Signalling pathways involved in endocrine resistance in breast cancer and associations with epithelial to mesenchymal transition (Review)

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Abstract. Both *de novo* and acquired endocrine resistance constitute a major therapeutic problem for treatment of hormone-positive breast cancer. Multiple explanatory mechanisms have been proposed through the study of cellular models which focus principally on receptor tyrosine kinase mediated signalling pathways utilizing SRC, PI3K, MAPK and SMADS. Many of the transducing molecules, particularly nuclear transcription factors such as SNAIL, TWIST, SNAIL2, ZEB, FOXC2, TCF/LEF and GOOSECOID are participants in proliferation as well as invasion and metastasis, involving a process of orchestrated cellular remodeling which is being likened to the process of epithelial to mesenchymal transition that takes place during embryonic development. We review the accumulating evidence that points towards the occurrence of this phenomenon as a consequence of the loss of endocrine control, with both processes being similarly characterized by depletion of cell adhesion proteins, E-cadherin, catenins and cytokeratins, increased association with the extracellular matrix through induction of metalloproteinases, fibronectin and collagen, and a switch to a mobile vimentin-based cytoskeletal structure with loss of apical basal polarity.

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Introduction

Endocrine therapy represents the most effective form of treatment for the majority of breast cancer patients whose tumours over-express the estrogen receptor (ER). In addition to ablative procedures (ovariectomy) and administration of anti-endocrine agents to inhibit ovarian function, treatment is reliant predominantly upon anti-hormonal agents termed selective estrogen modulators (SERMs). Until recent introduction of agents such as toremifene and raloxifene, tamoxifen has been the mainstay of treatment (1) inducing objective response or disease stabilization in over half of previously untreated metastatic breast cancer patients with ER[+] tumours (2). Further options include the use of pure anti-estrogens such as fulvestrant (Faslodex) which achieves its effects through receptor degradation, and application of aromatase inhibitors that reduce extra-gonadal peripheral estrogen synthesis from the adrenals and adipose tissue, including the breast. Both types of agents improve relapse-free survival and reduce incidence of contra-lateral breast cancers in women with early-stage cancer and increase overall survival in patients with advanced disease (3,4). Unfortunately, following initial response to SERMs and second line therapy with aromatase inhibitors, most patients subsequently develop resistance to both classes of drugs and become refractive to further attempts at endocrine manipulation. Added to the *de novo* resistance in patients whose tumours express levels of ER <10 fmol/mg protein, this presents a serious therapeutic problem, particularly in view of the increased aggressiveness of hormone insensitive breast cancers.

2. Estrogen receptor action

The classical mode of action of ER is related to the regulation of expression of genes with estrogen response elements (ERE) in their promoters through two distinct transcriptional activation domains; a hormone-independent activating function-1 (AF-1) located at the *N*-terminus of the receptor with its function regulated by phosphorylation, and the hormone-dependent AF-2, in the ligand-binding domain. In general, both domains have a synergistic effect in mediating positive gene regulation, although AF1 and AF2 can activate some gene promoters independently (5,6). Co-regulatory molecules modulate transcriptional activity of ER through interaction with the ER-ligand complex at the promoter; this activity is specifically enhanced

by binding to the AF-2 domain of co-activators such as nuclear-receptor co-activator 1 (NCOA1 or SRC1), NCOA2 (TIF2) and NCOA3 (AIB1, TRAM1, RAC3 or ACTR) (7,8). Co-activators enhance ER-driven transcription by several mechanisms that rely on the formation of large complexes including recruitment of histone-acetyltransferase at the promoter site. Binding of co-activators is inhibited by tamoxifen, causing blockade of transcription by AF-2 (9) which would explain its ability to function as a dual antagonist/agonist. Tamoxifen blocks the transcription of AF-2 dependent genes, while acting as an agonist in AF-1-dependent genes (10,11). Interaction with co-repressor proteins such as nuclear-receptor co-repressor 1 and 2, NCOR1 and NCOR2, repress ER-induced transcription by recruiting histone-deacetylase complexes (12-15). Microarray analysis of gene transcription showed that ~70% of estrogen-responsive genes, that are either transcriptional repressors or anti-proliferative genes, were down-regulated following treatment of MCF-7 cells with estradiol while genes involved in the induction of cell proliferation were up-regulated (16).

ER can also exert its function via a non-classical genomic action that affects gene expression at alternative regulatory DNA sequences such as AP-1 and SP-1. This type of interaction involves co-operation with other transcription factors which include the FOS-JUN complex (17-19). In addition, membrane ER interacts with and/or activates several kinases including the insulin-like growth factor-1 receptor (IGF-1R), SRC, phosphatidylinositol 3-kinase (PI3K), mitogen-activated protein kinase (MAPK), epidermal growth factor receptor (EGFR) and ERBB2 (20-26). Co-activators that can modify ER activity could also be phosphorylated by these cytoplasmic kinases (27-29) which is crucial for the activity of membrane functions of ER which probably have little significance in ER+ cells that express low levels of tyrosine kinase receptors such as members of the EGFR family (30).

Many interactions between the genomic and non-genomic pathways occur within the cellular context including ER induction of transforming growth factor α (TGFα) and amphiregulin expression (31,32). Both bind and activate EGFR leading to activation of MAPK and AKT signalling (33) that can also be activated via direct interaction with EGFR/ERBB2 heterodimers. In addition, specific G proteins are activated by ER binding to caveolin 1 at the cell membrane (34) which in turn leads to the activation of SRC that then activates matrix metalloproteinases which cleave transmembrane precursors of heparin binding-EGF (HB-EGF), an EGFR ligand (35). Estradiol-independent activation of ER through phosphorylation by several intracellular kinases has also been demonstrated (15,36). In response to several cytokines and growth factors such as ligands of EGFR or IGF-1R, pathways including MAPK/ERK, PI3K/AKT, p90rsk and p38 MAPK are activated, leading to ER phosphorylation at key positions in the AF-1 (serines 118 and 167 and threonine 311) and other domains (27,29,37,38).

3. Potential mechanisms of endocrine resistance

Better understanding of the pathways underlying intrinsic *de novo* and acquired resistance to endocrine therapy may point to novel strategies to overcome this problem and facilitate further improvements in breast cancer management.

In addition to possibilities common to the reaction of cells to xenobiotics, resistance might arise as a consequence of loss of expression or function of ERα, including auto-phosphorylation (39), modulation by activation of transmembrane tyrosine kinase receptors and interaction between downstream signal transduction pathways (40,41). Loss of ER expression is likely to be the most frequent mechanism for *de novo* resistance to tamoxifen but is insufficient to explain acquired resistance (42). A second ER (ERβ) that is interactive with ERα, but has different transcriptional activity, is generally considered to be an anti-proliferative factor (43-45) but its role in resistance still remains unclear and will not be discussed here. All reference in this review to ER should be assumed to mean ERα unless otherwise indicated. Fig. 1 lists some of the molecules that have been implicated in proliferation of resistant cell lines as well as those found to be over-expressed in ER- tumours. These are discussed below.

Co-activators and co-repressors. The SRC family of co-activators has a wide spectrum of effectors including nuclear receptors and transcription factors that include nuclear factor-κB (46) that has been implicated in endocrine resistance. Several members of this family, SRC-1/NCOA-1, SRC-2/GRIP1/TIF2/NCOA-2 and AIB1/SRC-3/RAC3/ACTRp/CIP share a common domain structure and functions (8). The role of SRC-1 in the agonistic action of tamoxifen in the uterus is a clinically significant example of the specificities of SRCS which occur only on non-consensus responsive sites (15).

The other interesting member of the SRC family is AIB1, which is over-expressed in >50% of breast tumours, with gene amplification in ~5-10% (48-50). The mouse AIB1 homologue SRC-3 shows tissue-specific expression and is essential for normal mammary gland development. The diversity of the co-activator family members is also demonstrated by gene knockout studies that show a distinct physiological role of SRC-3/AIB1 that is different from that of SRC-1 (51). In cultured MCF-7 cells, AIB1 is also highly up-regulated and reported to be necessary for their growth (52). It enhances tamoxifen agonistic activity (53) and may have a potential role in endocrine resistance. Over-expression and increased phosphorylation of A1B1 leads to constitutive ER-mediated transcription, which confers resistance *in vitro* and in xenograft models (39,54) and is associated with reduced patient responsiveness to tamoxifen (55). It is still unclear how phosphorylation activates ER co-activators. Recent studies suggest that it can enhance their nuclear sub-localization and interaction with ER (56) and may directly increase their own acetyl-transferase activity or stimulate their ability to recruit other transcriptional co-activators or integrators to the receptor complex (28).

Another co-activator, PELP1, confers tamoxifen resistance in its cytoplasmic localization (57). It functions as a scaffold protein that modulates ER activation of SRC and the ERK family kinases. It also promotes estrogen activation of PI3K31. Other co-activators found to be implicated in endocrine resistance are AP1 and SP1 (58-60).

The SRC substrate BCAR1 (also known as CAS) is a focal adhesion adaptor protein that activates proliferative, survival and invasion pathways and can induce tamoxifen resistance when over-expressed *in vitro* (61). Indeed, BCAR1-over-expressing breast cancers are less responsive to tamoxifen (62).
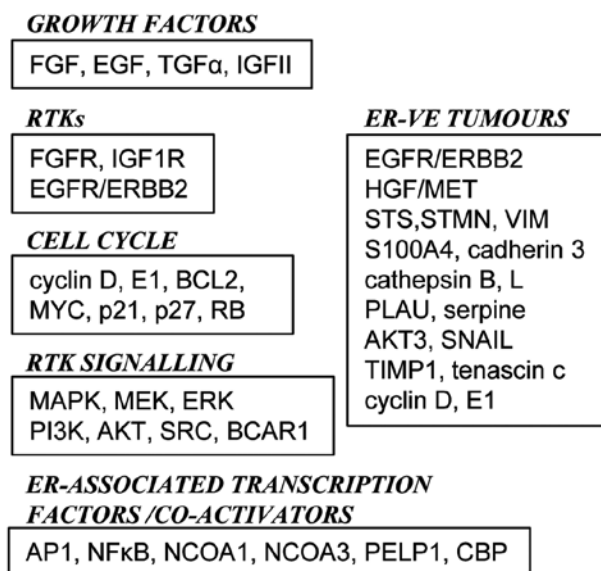
GROWTH FACTORS

| FGF, EGF, TGFα, IGFII |

RTKs

| FGFR, IGF1R
EGFR/ERBB2 |

ER-VE TUMOURS

| EGFR/ERBB2
HGF/MET
STS,STMN, VIM
S100A4, cadherin 3
cathepsin B, L
PLAU, serpine
AKT3, SNAIL
TIMP1, tenascin c
cyclin D, E1 |

CELL CYCLE

| cyclin D, E1, BCL2,
MYC, p21, p27, RB |

RTK SIGNALLING

| MAPK, MEK, ERK
PI3K, AKT, SRC, BCAR1 |

ER-ASSOCIATED TRANSCRIPTION FACTORS /CO-ACTIVATORS

| AP1, NFκB, NCOA1, NCOA3, PELP1, CBP |

Figure 1. Groups of molecules whose activity is related to tamoxifen resistance or found to be over-expressed in ER⁻ tumours. Note the considerable overlap with molecules reported to be involved in EMT.

BCAR1 binds and activates SRC with consequent phosphorylation of the SRC substrates EGFR and signal transducer and activator of transcription factor 5B, and effects on downstream signalling pathways (63). However, the ability of BCAR1 to confer anti-estrogen resistance may not require interaction with SRC. The putative guanine nucleotide-exchange factor BCAR3, which synergizes with BCAR1 to activate SRC, causes tamoxifen resistance *in vitro* (64). BCAR3 also activates RAC and p21- activated kinase 1 which is implicated in tamoxifen resistance through ER phosphorylation (65).

The recruitment of co-repressors normally involves binding of antagonists such as tamoxifen to the ER (7,14). Although *in vitro* tamoxifen resistance is thought to be associated with reduced co-repressor levels, no clinical data currently support this hypothesis (30). Some studies (66,67) suggest that, at least in cultured cells, modulation of the agonist/antagonist activity of tamoxifen can be affected by the differential expression or activity of co-activators and co-repressors. Progesterone and glucocorticoid receptor-mediated transcription that are provoked by their own selective receptor modulators can also be modified by co-activator/co-repressor ratio (68). This ratio was also reported to confer hyper or hyposensitivity to their ligands (69) providing a possible explanation for some clinical tamoxifen resistance and the tissue-specific actions of SERMs (15).

Tamoxifen metabolism. The metabolism of tamoxifen has also been implicated in intrinsic resistance to therapy. Plasma concentrations of tamoxifen metabolites can be affected by genetic polymorphisms in genes of tamoxifen-metabolizing enzymes. In tamoxifen-treated patients, a higher risk of disease relapse has been associated with women with selective cytochrome p450 2D6 (CYP2D6) genotypes. Indeed, CYP2D6 status may be used to identify patients who would benefit from tamoxifen therapy and those tamoxifen-treated patients who should not be co-administered potent CYP2D6 inhibitors (70).

Kinase activated growth pathways. It is increasingly recognised that growth factor stimulated pathways play a central role in acquired and intrinsic resistance to endocrine agents (71,72). Hiscox *et al* (73) have shown that tamoxifen resistance in MCF-7 cells is associated with elevated expression and activation of components of the EGFR signalling pathway (EGFR, ERBB2, TGFα, MAPK). The same authors noted that this change was accompanied by enhanced cell motility and matrix invasion. Cross-talk between the pathways adds to the already existing complexity of resistance. Some models have predicted that increased signalling through growth factor receptor pathways could promote the ER pathway, which would itself re-activate growth factor signalling leading to further transcriptional activation of estrogen-responsive genes and enhanced cross-talk. The effect of tamoxifen in inhibiting cell growth might itself be inhibited through the actions of increased growth factor signalling which would contribute to endocrine resistance. This mechanism has important clinical implications as many tyrosine kinase inhibitors used in ERBB2-targeted therapy such as trastuzumab and the EGFR tyrosine kinase inhibitor, gefitinib, might be useful agents to prevent tamoxifen resistance (73). Erlotinib (Tarceva) and imatinib (Gleevec) that also preferentially target EGFR have been therapeutically effective in treating several malignancies including small cell lung and pancreatic cancers. ER interacting with several kinases, including that of IGF-1R and the p85 regulatory subunit of PI3K via adaptor proteins, SRC and SHC, result in different cell survival and proliferative signals via the AKT and MAPK pathways.

EGFR/ERBB2 activated pathways. Some studies suggest that ER expression and/or function could also be reduced by the activation of growth factor signalling. For example, MCF-7 cells treated with EGF, IGF-I, TGFβ or phorbol myristate acetate had lowered ER mRNA and protein (74-77) and increased signalling through EGFR, PI3K/AKT, PKA and PKC.

The transcriptional activity of ER has been shown to be repressed by heregulin which can activate both EGFR and ERBB2 through formation of heterodimers with either ERBB-3 or ERBB-4 (78,79). Transfection of constitutively active ERBB2, RAF1 or MEK results in significant reduction in expression of ER mRNA and protein and estrogen-regulated genes, resulting in development of estrogen independence (80-83). Cumulatively, these data suggest that growth factor signalling contributes to transcriptional repression of ER expression in breast cancer cells, resulting in endocrine resistance. A 5-10-fold increase in mRNA and protein expression of ERBB2 and EGFR has been noted in tamoxifen resistant MCF-7 cells (84). ERBB2 is frequently over-expressed in 20-30% of breast cancers and is associated with a more aggressive phenotype (85). Furthermore, this increased expression also appears to activate EGFR/ERBB2 heterodimers and to cause increased phosphorylation of MAPK, AKT and nuclear ER on serine residues 118 and 167 (86-89). EGFR/ERBB2 signalling was reported to be involved in cellular models with acquired tamoxifen resistance (72) and its inhibition by gefitinib or trastuzumab resulted in

growth suppression of these cells (90). Furthermore, the addition of EGF-like peptides increased phosphorylation levels of ER and ER-induced transcription which was blocked by treatment with gefitinib. Increased synthesis of TGFα and amphiregulin that maintains the EGFR/ERBB2 autocrine loop operating in tamoxifen-resistant cells was a result of this enhanced transcriptional activity of ER (87). Adapted resistance to fulvestrant in MCF-7 cells was also correlated with increased EGFR signalling (91). However, fulvestrant resistance to aromatase inhibitors can also be mediated by up-regulation of the ERBB2/HER2 pathway (42). Patients presenting with acquired resistance to tamoxifen showed increased levels of ERBB2 and increased activation of p38-MAPK (92) which was only observed in MCF-7 xenografts subjected to combined estrogen deprivation and tamoxifen, but not with estrogen deprivation alone. In addition, a phase-II clinical trial on tamoxifen-resistant advanced breast cancer patients treated with gefitinib showed beneficial effects of anti-EGFR agents (93). Increased levels of EGFR and MAPK activity was also associated with the acquired endocrine resistance of long-term tamoxifen treated MCF-7 cells (94).

The X-linked tumour suppressor forkhead box P3 and the zinc finger transcription factor GATA4 can repress ERBB2 expression, even in a cell line with ~10-fold amplification of ERBB2, and their expression is negatively correlated with ERBB2 expression in breast cancer (95,96). In addition, ER-mediated repression of ERBB2 was reported to be dependent on competition between the paired-domain transcription factor PAX2 and the ER co-activator NCOA3 for binding and regulation of ERBB2 transcription and, in turn, tamoxifen responsiveness (97). However, increased PAX2 expression and consequent repression of ERBB2 was associated with increased survival following tamoxifen treatment, and loss of PAX2 expression in the presence of increased NCOA3 expression predicted a poor outcome (97), indicating that this mechanism is of direct clinical relevance.

High levels of AIB1 can greatly reduce the antagonist effects of tamoxifen in tumours that over-express growth factor receptors such as ERBB2. A1B1 is phosphorylated and activated by several signalling kinases, including p42/44 MAPK that can be activated by ERBB2 itself hence forming a link between ERBB2 and A1B1 in breast cancer progression (28). This suggests that relatively high levels of co-activators such as A1B1 can cause a lower response to tamoxifen therapy in tumours expressing them caused by the increased estrogen agonistic activity of the tamoxifen-bound ER (98). It was also reported that following treatment of the ERBB2-over-expressing MCF-7/HER2-18 cells with tamoxifen, co-activators such as AIB1 were recruited to the ER-tamoxifen complex more than co-repressors. In these cells, tamoxifen tends to act as an agonist in the presence of low levels of estrogen. EGFR/ERBB2 signalling is also activated promoting the activation of both MAPK and AKT signal transduction pathways (26) which would phosphorylate and functionally activate both ER and AIB1. These phenomena can be blocked by treatment with gefitinib and often result in inhibition of cell growth, suggesting the possible involvement of EGFR/ERBB2 signalling in the growth-promoting activity of tamoxifen in these cells. Interestingly, gefitinib had little effect on estrogen-induced growth which is consistent with clinical observations indicating that co-expressing ERBB2 and AIB1 results in a poor outcome if associated with tamoxifen treatment (55). Furthermore, patients that express either ERBB2 or EGFR are relatively resistant to tamoxifen but are sensitive to aromatase inhibitors (99,100). A shorter disease-free survival in patients presenting with high levels of A1B1 was observed after tamoxifen administration as an adjuvant treatment (55). On the other hand, untreated patients presenting with high levels of A1B1 had a better outcome (30).

IGF-1R signalling is also involved in tamoxifen-resistant cells, with IGF-II induced increase of IGF-1R phosphorylation and subsequent EGFR activation (101,102). Tamoxifen treatment of MCF7 cells reduces both total and activated IGF-IR. A specific IGF-1R inhibitor, as well as an IGF-II neutralising antibody, can not only reduce ER phosphorylation and tumour proliferation but also affect EGFR signalling events; but EGF mediated actions are not blocked. Although unclear in breast cells, in some tumours IGF-1R can induce metalloproteinase-dependent release of heparin-EGF and amphiregulin to transactivate EGFR.

De-regulation of ERBB2, EGFR and IGF-1R pathways could occur as a result of genetic or epigenetic modifications, such as amplification of ERBB2, activating mutations in PIK3CA, which encodes a catalytic subunit of type I PI3Ks, and loss of heterozygosity or methylation of PTEN, a tumour suppressor that inhibits the PI3K pathway (63). In other cases, this de-regulation reflects aberrations in upstream regulators, such as the activation of AKT in association with the loss of PTEN expression or over-expression of ERBB2 (63,103) and activation of IGF-1R and ERBB3 following the loss of PTEN (104).

PI3K cell survival pathway. Non-genomic activity of ER may be influenced by the PI3K pathway that is activated by growth factor induced tyrosine kinase receptor phosphorylation. ER displays ligand-dependent binding to the p85a regulatory subunit of PI3K, resulting in the activation of AKT which increases cellular proliferation and decreases apoptotic responses (105,106) and which is the target of several other receptor stimulated pathways through IGF-1R, EGFR and ERBB2 (107). PI3K also causes ER phosphorylation at serine-167 by AKT activation resulting in ligand-independent activation (29). In one study, it was shown that addition of the PI3K pathway inhibitor LY294002 enhanced the pro-apoptotic effects of tamoxifen, primarily in the cell line with the maximum endogenous levels of AKT activity (106). MCF-7 cells transfected with AKT show a reduction in tamoxifen induced inhibition of cell growth (29), supporting the notion that high expression of AKT is associated with tamoxifen resistance.

Stress-activated protein kinase/c-JUNNH2 terminal kinase pathway. ER can interact with the stress-activated protein kinase/c-JUNNH2 terminal kinase pathway either by binding with the AP-1 transcription complex, by dimerization of JUN and FOS family members that bind to AP-1 response elements on DNA (108), or by direct p38 MAPK activation. AP-1 transcriptional activity is enhanced by increased abundance of any of its components or by the JUN NH2-terminal kinases (JNKs) or stress-activated protein kinases that are induced by cellular insults such as oxidative stress (109). One study reported that increased AP-1 DNA binding is associated with the development of tamoxifen resistance in MCF-7 (110). This

was also reported in a study of 30 primary human breast tumours with acquired tamoxifen resistance, compared with 27 untreated controls (58). The agonistic effects of tamoxifen at AP-1 sites can also be induced by tamoxifen induction of intracellular oxidative stress that leads to the activation of JNK and increased AP-1 activity (59,111). New and Han (112) showed that activation of the p38 MAPK pathway, that targets further protein kinases and transcription factors, occurred in response to various extracellular stimuli including cytokines, growth factors, chemical and physical stress. Other studies showed that 4-hydroxytamoxifen resistant cell lines displayed a non-active p38 signalling that was directly related to apoptosis inhibition (113,114).

Keratinocyte growth factor pathways. It has been suggested that keratinocyte growth factors (KGFs) are important regulatory factors acting as estromedins for the stimulation of breast cancer cell growth. KGF also stimulates aromatase activity, promoting conversion of androgens to estrogens in primary cultured human breast cells. KGF might increase endocrine resistance via reduction in ER, PR and PTPγ as well. The functional analysis of KGF-13 suggests possible applications of short receptor-binding peptides derived from intact KGF as therapeutic agents (115).

NOTCH pathway. The NOTCH pathway is implicated in both cell fate in the normal human mammary gland (116) and regulation of cancer stem cells (CSCs) in both ductal carcinoma *in situ* (117) and invasive carcinoma of the breast (118,119). Luminal type breast cancers express low levels of NOTCH and ERBB2 but high levels of ER compared to basal types, which show the opposite pattern (120). Estrogen signalling down-regulates the NOTCH pathway; estradiol-induced reduction of NOTCH 1 and 4 in both T47D and MCF7 cells which could be abrogated by either tamoxifen or fulvestrant (120). In a mouse xenotransplantation assay using BT474 cells, tumours were treated with tamoxifen alone or in combination with a γ secretase inhibitor (121). Combination therapy was significantly superior to the use of tamoxifen alone and the authors concluded that tamoxifen antagonism of the estrogen stimulus leads to the re-activation of the NOTCH signalling pathway promoting proliferation and survival.

Cell cycle regulators. Data from experimental model systems indicate that anti-estrogens are both cytostatic and cytotoxic. Neoadjuvant endocrine therapy leads to decreased cell proliferation (122), and in cell culture anti-estrogen treatment leads to a G1 specific cell cycle arrest and a consequent reduction in growth rate (123). The molecules involved in the anti-estrogenic effects on cell cycle progression have central roles in the control of G1 phase progression downstream of other growth factors, as well as estrogen. Aberrant expression of several such estrogen and anti-estrogen targets confers resistance *in vitro* and is associated with reduced tamoxifen responsiveness in patients. Over-expression of MYC, cyclin E1, D1 or its splice variant D1b, or the inactivation of the Rb tumour suppressor, an important substrate for cyclin-dependent kinases (CDKs) that are active in G1 phase, and the decreased expression of the CDK inhibitors p21 or p27, results in decreased anti-estrogen sensitivity *in vitro* (124-132). The over-expression of MYC and consequent tamoxifen resistance is accompanied by transcriptional repression of CDKN1A

(133), relieving the inhibitory effect of p21 on cyclin E1-CDK2 complexes. Cyclin D1 over-expression leads to an increased abundance of cyclin D1-CDK4 complexes which indirectly activate cyclin E1-CDK2 by sequestering p21 and p27 (126,134), and to the activation of cyclin E2-CDK2 by increased transcription of CCNE2 which encodes cyclin E2 (135). In addition to its cell cycle regulatory role, cyclin D1 interacts with several transcription factors, including ER and STAT3 (136). Moreover, tamoxifen induces cyclin D1 binding to ER at the expense of cyclin D1-STAT3 binding, activating both STAT3 and ER. This is an additional mechanism by which cyclin D1 over-expression can affect tumour response to tamoxifen (137). Therefore, MYC and cyclin D1 over-expression can potentially affect anti-estrogen sensitivity at several levels and this is associated with tamoxifen resistance in patients (138,139). There is also more limited evidence for a relationship between over-expression of cyclin E1, Rb inacti-vation and reduced expression of p27 and clinical response (130,138-140). Over-expression of MYC, cyclin D1 and cyclin E1 in breast cancers is at least two to three times more common than amplification of the corresponding genes (138). Rb inactivation is also more common than its deletion or mutation (130) possibly due to activation of upstream mitogenic signalling pathways and de-regulation of transcriptional regulators including members of the E2f family. The gene encoding p27, CDKN1B, is rarely mutated or deleted in breast cancer, but its expression is frequently reduced by oncogenic activation of mitogenic signalling (for example by ERBB2 over-expression or SRC activation) with increased degradation (140).

The microRNAs (miRNAs) miR-221 and miR-222 reduce p27 expression and confer resistance to tamoxifen, although the precise mechanism is unclear, as these miRNAs also reduce ER expression and are over-expressed in ERBB2-over-expressing breast cancer (141,142). There is conflicting data on the relationship between p21 expression and outcome in breast cancer (138). The role of p21 in tamoxifen response has not been studied extensively, although the ERBB2 repressor forkhead box P3 is essential for p21 expression (143) and there is some evidence for p21 de-regulation in cancers with ERBB2 over-expression or AKT activation (139). The latter results in the mis-localization of p21 to the cytoplasm, a phenomenon associated with poor response to tamoxifen (144).

Targeting CDK10 also causes resistance to tamoxifen and other endocrine agents *in vitro*. Iorns *et al* (145) observed that CDK10 silencing increased ETS2-driven transcription of c-RAF. This led to MAPK pathway activation and loss of tumour cell dependency on estrogen signalling suggesting that CDK10 could be an important determinant of endocrine resistance. Furthermore, patients with ER⁺ tumours that expressed low levels of CDK10 were associated with poor clinical response to tamoxifen.

Apoptosis. Treatment with high concentrations of anti-estrogens, estrogen withdrawal or aromatase inhibitor treatment of cells transfected with aromatase leads to the activation of the cellular stress response and apoptosis in breast cancer cells (146,147). The mechanisms are not well defined, but several molecular consequences that promote apoptosis have been documented, including regulation of BCL-2 family members and increases

in the apoptotic second messenger ceramide. Cross-talk between the apoptotic effects of anti-estrogens and the tumour necrosis factor (TNF) pathway, as well as anti-estrogen effects on survival signalling through the PI3K-AKT, NF-κB and interferon pathways is also likely to contribute to anti-estrogen-mediated apoptosis (148). Other observations indicate that autophagy is a mechanism of cell survival in breast cancer cells that are resistant to apoptotic concentrations of tamoxifen (149).

It has been difficult to establish the role of apoptosis in the clinical setting. Neoadjuvant studies have yielded conflicting data and have been limited by small patient numbers and the methodological difficulties of measuring apoptosis *in vivo* (150). As tumour growth reflects the balance between cell proliferation and cell death, disruption of this balance is expected to affect clinical response. There is accumulating evidence for the increased expression of anti-apoptotic molecules, for example BCL-2 and BCLXL, and decreased expression of pro-apoptotic molecules such as BAK, BIK and caspase 9, in attenuated responses to tamoxifen (148). Although many of these responses are probably consequences of the activation of survival signalling through the PI3K-AKT pathway, as a result of over-expression of receptor tyrosine kinases and increased 'non-genomic' signalling from cytoplasmic ER, other pathways have been reported. For example, increased DNA-binding and transcriptional activity of NF-κB are features of tamoxifen-resistant cells, and tamoxifen sensitivity can be restored by parthenolide, a specific NF-κB inhibitor (60,148). Tamoxifen insensitivity *in vitro* is also associated with the down-regulation of IRF1, an interferon-responsive putative tumour suppressor that binds NF-κB and is essential for apoptosis. Furthermore, over-expression of a splice variant of human X-box-binding protein 1, a transcription factor that controls the unfolded protein response, is also associated with tamoxifen resistance *in vitro* and poor survival in patients with breast cancer treated with tamoxifen (151-153). Correlation of NF-κB, XBP1 and IRF1 expression in patients with breast cancer (154) suggests that these molecules may function in a common pathway (155). Fig. 2 illustrates the involvement of some of the signalling pathways that have been described in the preceding sections.

4. Epithelial to mesenchymal transition

At the leading edge of invasiveness and metastasis, solid tumours that are epithelial in origin, start to lose their characteristic cell-cell adhesive structures, change their polarities and undergo extensive re-organization of their cytoskeletal systems. They switch expression from keratin-to-vimentin type intermediate filaments, become isolated, motile, and resistant to anoikis with enhanced survival. They gain resistance to cytotoxic drugs and acquire the ability to invade neighbouring tissue and penetrate into the vasculature to metastasize. This has been reported with non-small cell lung carcinomas, pancreatic, colorectal, and hepatocellular cancers (156). This type of behaviour is identified with cells undergoing epithelial-to-mesenchymal transition (EMT) (157), a phenomenon that was originally defined as the cellular remodelling that occurs during heart morphogenesis and was first observed and recorded by Greenburg and Hay (158). It is seen as a series of changes

from characteristic cobblestone-like epithelial morphology to a spindle, fibroblast-like shape with migratory protrusions. Carcinomas of epithelial origin may acquire a mesenchymal-like state in order to facilitate their migration and invasion. This is illustrated in Fig. 3 showing that the morphological and behavioural changes are accompanied by a distinct change in the gene expression profile. When the migrating metastatic cells reach their secondary site of carcinogenesis, presumably in order to survive in the local milieu, the cancer cells revert to their epithelial state to form organized tumourigenic nodules, a reverse process referred to as epithelial to mesenchymal transition (MET). During EMT and MET, a bimodal communication exists between the host fibroblasts, extracellular matrix/basement membranes, and the immune cells.

Although evidence supporting the occurrence of EMT is slowly growing from *in vitro* data such as the pathway blockade by agents like NFκβ and SMADs (159,160), it seems to have gone largely unnoticed during routine histological examination of tissue biopsies. This has led to much scepticism among some pathologists about whether this process actually occurs *in vivo* (161-163) or is just an *in vitro* phenomenon resulting from manipulation of cells in an artificial environment. Nevertheless, the accumulating molecular evidence is presenting a strong argument for closer cytological as well as molecular examination of the process of metastasis.

Genes that are activated in EMT during carcinoma progression and metastasis are also active in early embryogenesis, tissue morphogenesis, and wound healing, suggesting that EMT involves a re-activation of these developmental events during tumour progression. This process of de-differentiation is carried out through a series of transcriptional re-programming steps involving the participation of a number of transcription factors such as ZEB1/TCF8, SNAIL, ZEB2, SNAIL2, E12/E47, FOXC2, GOOSECOID and TWIST (164). This is followed by the activation of abnormal survival signals via receptors such as platelet derived growth factor receptor (PDGFR), fibroblast growth factor receptor (FGFR), cMET, transforming growth factor β receptor (TGFβR), IGF-IR, human growth factor receptor (HGFR) and EGFR and regulatory kinases such as PI3K, AKT and mTOR.

The evidence for EMT-associated tumour motility is supported by network signalling pathways mediated by fluctuating levels of TGFβ, EGF, PDGF, ERK/MAPK, β-catenin, SMADS, RAS, c-FOS, integrins β4 and α5, and most importantly the dissolution of cell-cell junctions mediated by SNAIL, SNAIL2 and E2a transcription factors (165).

The actual triggers of EMT in breast cancer are not yet known. MCF10A cells, an immortalised line derived from spontaneous transformation of normal epithelial cells obtained from reduction mamoplasty, have been seen to undergo EMT in response to multiple factors including a combined expression of H-RAS and ERBB2 in 3D marginal culture, undergoing monolayer wounding enhanced by EGF and IGF-IR over-expression, introduction of constitutively active p65 subunit of NF-κB, chronic exposure to TNF-α and expression of Rb suppressor associated protein 46 (RbAp46/RbBP7) (166,167).

Molecular profiling of 'triple-negative' (ER⁻, PR⁻, ERBB2⁻) metaplastic breast tumours indicates an enrichment of stem cell-like and EMT markers (168). Data from c-MYC initiated murine tumours indicate that whilst EMT may not be an
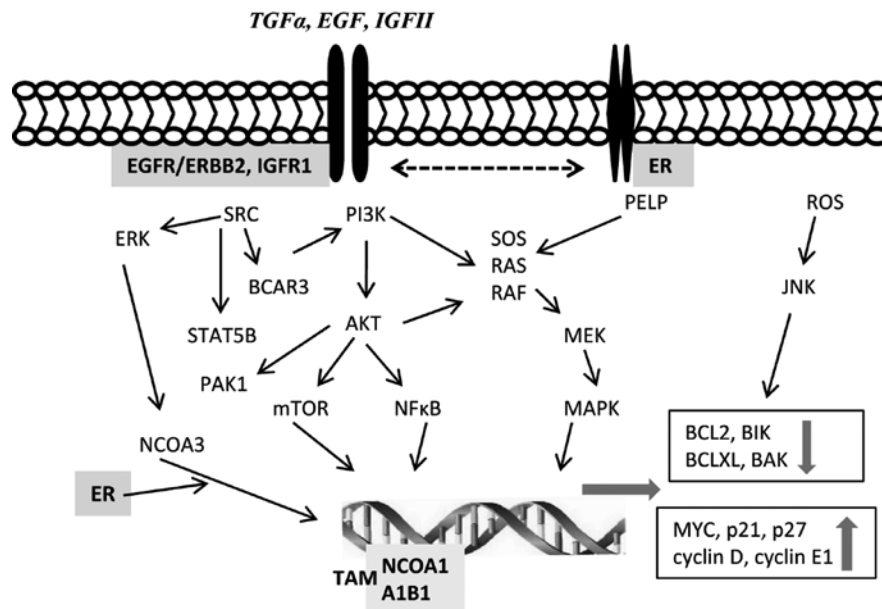
Figure 2. Pathways postulated to be involved in growth of endocrine-resistant cells as discussed and referenced in the text. Cross-talk between membrane bound ER and receptor tyrosine kinases is thought to elicit downstream transcriptional complexes at target gene promoter sites including those with A1B1 and NCoA1 which result in ligand independent ER and or tamoxifen agonist activity leading to increased cell cycle regulators and decrease in apoptotic mediators.
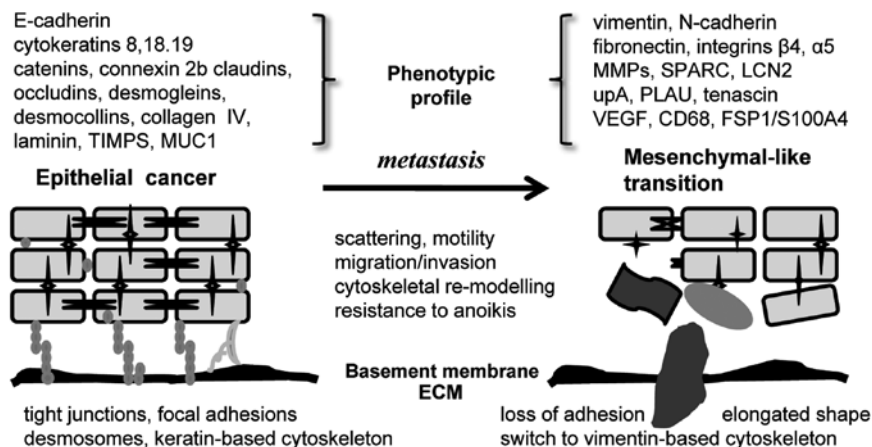

Figure 3. Epithelial to mesenchymal transition. Morphological changes initiated through a series of cell signalling events lead to loss of epithelial apical basal polarity, detachment from the tumour and a remodelling of the cellular architecture allowing individual cells to gain motility and adopt a molecular phenotype resembling that of mesenchymal cells. Examples of the most commonly reported genes found to be over-expressed in the two types of cells is shown above each structure.

absolute necessity for tumour progression/metastasis, there is clear morphological evidence for involvement of EMT in the process (169). By following 'genetically tagged' stromal and mammary epithelial cells, these authors concluded that stromal fibroblasts associated with MYC-induced tumours were of epithelial origin. Many of these cells had switched from cytokeratin and E-cadherin expression to vimentin and fibronectin. It is also reported that EMT markers are present in the tumour-host interface in colorectal cancers (170), but not in the bulk tumour providing strong evidence of EMT involvement in regulating invasiveness and tumour aggressiveness.

Cell adhesion molecules. A defining feature of EMT is the loss of the homotypic cell adhesion molecule E-cadherin and the occludins (171,172) which together with other proteins such as the claudins and tight junction proteins are an integral component of epithelial cell adherens junctions that form the cohesive architecture of normal epithelia. Indeed, the loss of E-cadherin function is believed to play a pivotal role in the transition of breast tumours from a benign to an invasive state. Reduction of E-cadherin expression correlates with poor differentiation, invasiveness, aggressive metastatic behaviour, and an unfavourable prognosis (173-175). The importance of E-cadherin in the maintenance of normal tissue architecture and

epithelial cell behaviour is reflected in the distinct mechanisms that converge to compromise its expression and function in breast cancer (171). Although inactivating mutations and the subsequent loss of heterozygosity at the E-cadherin CDH1 gene locus are important mechanisms for permanently silencing E-cadherin expression in many lobular breast cancers (175), EMT and metastatic progression are mostly associated with a reversible down-regulation of E-cadherin expression at the transcriptional level (176). This arises either through hypermethylation of the CDH1 promoter or transcriptional repression. Indeed, ductal breast cancers show heterogeneous loss of E-cadherin expression consistent with the notion that the tissue microenvironment at the invasive front may transiently down-regulate E-cadherin transcription (175). Consistent with the function of E-cadherin as a breast cancer invasion suppressor, experimental knockdown of E-cadherin is sufficient to confer metastatic ability (174). Conversely, exogenous E-cadherin inhibits the migration and extracellular matrix (ECM) invasion of metastatic breast cancer cells, although E-cadherin expression by itself is insufficient to completely reverse the mesenchymal phenotype (177-179). The consequences of E-cadherin loss for EMT are far reaching and reflect its dual roles in cell adhesion and signalling. First, E-cadherin loss causes the disassembly of inter-cellular adhesion complexes, thus loosening contacts between neighbouring epithelial cells and disrupting the overall tissue architecture. Second, the physical absence of E-cadherin serves to dissipate E-cadherin-mediated tethering of β-catenin to the cell membrane, thus permitting activation of WNT signalling (174). Furthermore, E-cadherin loss sets into motion multiple downstream transcriptional pathways leading to EMT and the induction of its own transcriptional repressors, SNAIL TWIST and ZEB1 (EF1), in a feed-forward loop that sustains E-cadherin repression and potentiates EMT (174).

Up-regulation of SNAIL, associated with reduced E-cadherin gene expression (180), is a consistent feature of high grade ductal carcinomas (181) and implicated in tumour recurrence (182). TWIST expression has been observed in breast tumour metastases and correlated with nodal involvement (178,183). SNAIL2 likewise has been associated with disease aggressiveness in metastatic breast carcinoma (184). Loss of E-cadherin was also reported to be associated with the loss of cytokeratins CK8, 18 and 19 (179). Down-regulation of E-cadherin during EMT is often associated with a process known as 'cadherin switching' (185) which involves the induction of non-epithelial cadherins (e.g., N-cadherin and/or cadherin-11) (171,179,186) typically found in mesenchymal cells. SNAIL, ZEB2/SIP1 and SNAIL2, can induce N-cadherin (and cadherin-11) expression during EMT, implicating it in the transcriptional re-programming of de-differentiating epithelial cells (179,187,188). N-cadherin is highly expressed in invasive and metastatic human breast cancer cell lines and tumours and correlates with aggressive clinical behaviour. However, N-cadherin ectopic expression also promotes motility, invasion and metastasis in E-cadherin-positive breast cancer cells (189) possibly in synergy with FGF2 (190) without impacting on their epithelial phenotype, suggesting that the malignant functions of N-cadherin are dominant over E-cadherin and pointing to EMT-independent routes to metastasis. Interestingly, the association of cadherin switching,

in ER-targeting shRNA transfected MCF-7 cells, with increased motility reflected by membrane ruffling and F-actin rearrangement suggests that the loss of cellular adhesion molecules following acquisition of endocrine resistance is a major factor influencing the cells to move and invade into the surrounding tissues (191). The general role of cadherins has been extensively reviewed by Berx and van Roy (192).

Besides E-cadherin loss, the integrity of adherens junctions may be compromised through down-regulation of the catenins, which are mainly localized within the normal epithelia as complexes contributing to inter-cellular adhesion by means of molecular bridges linking E-cadherin to the actin cytoskeleton. There is evidence for involvement of β-catenin-mediated gene transcription and establishment of EMT (193). EGFR modulation of β-catenin (tyrosine) phosphorylation was a feature of tamoxifen resistant breast cancer cells, accompanied by loss of association between β-catenin and E-cadherin, increased β-catenin and elevated transcription of its target genes implicated in tumour progression and EMT (194). Components of tight junctions and desmosomes such as claudins, occludins, desmogleins and desmocollins, as well as epithelial cell polarity genes, are co-ordinately down-regulated during different EMT programs, contributing to the disbanding of inter-cellular contacts and the loss of apicobasal polarity (188,195).

Cytoskeletal proteins. Vimentin is a component of type III intermediate filaments and the archetypal mesenchymal marker most commonly used to categorize EMT (196) but it should be used in conjunction with other markers, as it has been noted that epithelial cell lines can also begin to express vimentin as part of the adaptation to *in vitro* culture conditions (197). Nevertheless, elevated vimentin expression correlates well with increased cell migration, invasion and EMT induction in several breast cancer cell lines (198,199). It is co-ordinately regulated together with other mesenchymal markers, such as the ECM molecule tenascin C (200,201), whose expression in human breast carcinomas correlates positively with over-expression of ERBB2 and down-regulation of ER. Although the mechanisms underlying E-cadherin down-regulation are fairly well documented, the molecular events triggering vimentin expression during EMT are less well delineated. That vimentin expression is a late event in EMT points to a temporal sequence of genetic events in which loss of epithelial features directly precedes and leads to up-regulation of mesenchymal genes (201). Direct activation of vimentin expression in human breast tumour cells (198) by β-catenin/T-cell factor/lymphocyte enhancer factor-1, is consistent with the activation of β-catenin as a downstream event ensuing from loss of E-cadherin. The indirect promotion of vimentin expression by ZEB2/SIP1 during EMT in a β-catenin-independent manner (202), suggests the existence of as yet unknown transactivators driving EMT associated vimentin expression.

The appearance of another mesenchymal marker, FSP1/S100A4, is considered an important early event in the pathway leading to EMT, and its altered expression levels correlate with breast cancer progression. As a mesenchymal/EMT marker in a murine model of breast cancer FSP1/S100A4 has provided valuable *in vivo* evidence implicating EMT in the onset of metastasis (171) facilitated through the activation of matrix metalloproteinases (MMPs) such as MMP9 or SPARC (179).

Matrix metalloproteinases. A family of more than 28 members, matrix metalloproteinases are up-regulated in nearly every tumour type and are intimately involved in cancer progression through cleavage and release of bioactive molecules that inhibit apoptosis and stimulate invasion, as well as through degradation of extracellular matrix (ECM) components that promote tumour cell growth. Cells treated with MMP-3 showed increased expression of the activated splice variant RAC1b, stimulating elevation of the levels of cellular reactive oxygen species, which in turn were responsible for increased expression of SNAIL and induction of EMT (203).

Lipocalin2 (Lcn2) is a member of a family of small extra-cellular proteins that play important roles in cell regulation, proliferation, and differentiation and is an MMP-9 associated protein. Yang *et al* (204) observed that LCN2 was over-expressed in human breast cancer cells and found to up-regulate mesenchymal markers, including vimentin and fibronectin, down-regulate E-cadherin, and significantly increase cell motility and invasiveness in previously non-invasive MCF-7 cells. They found that siRNA-mediated LCN2 silencing in aggressive breast cancer cells inhibited cell migration and development of the mesenchymal phenotype. Reduced expression of ERα and increased expression of the key EMT transcription factor SNAIL2, were observed with LCN2 expression. Over-expression of ERα in LCN-2 expressing cells reversed the EMT and reduced SNAIL2 expression, suggesting that ERα negatively regulates LCN2-induced EMT.

Transcription factors. Kruppel-like factor 8 (KLF8) was initially identified as a transcriptional repressor of kruppel-like C2H2 zinc-finger transcription factor family proteins. It was reported as a potent inducer of EMT and epithelial cell invasion, and a novel repressor of E-cadherin in epithelial cells (205). Its up-regulation is associated with the loss of E-cadherin expression in breast carcinoma cells and their consequent invasiveness. Their data showed that KLF8 was up-regulated in 50% of E-cadherin-ve metastatic breast tumours, promoting cell motility, possibly by a mechanism secondary to its repression of E-cadherin. Alternatively, its effect may be due to direct regulation of other proteins that are critical to cell migration.

The ZEB family of zinc finger transcription factors have established functions in normal embryonic development. Recently, their association with EMT has led to investigations on their potential role in malignancy. ZEB1 up-regulation correlates closely with epithelial de-differentiation in both invasive ductal and undifferentiated lobular breast tumours. E-cadherin and other epithelial differentiation markers could be re-expressed by the induced down-regulation of δEF1/ZEB1 (downstream of SNAIL expression) (206) in breast cancer cell lines (207-209). Intriguingly, ZEB1 is also highly expressed in tumour-associated stromal cells, and there is speculation as to whether these mesenchymal cells are derived from tumour cells undergoing ZEB1-directed EMT (195). ZEB1 and ZEB2 bind to the E-box elements in the E-cadherin promoter causing transcriptional repression and EMT activation (197,198).

Several interconnected signal transduction pathways and a number of extracellular signalling molecules have been implicated in the regulation of EMT-like processes during breast cancer progression (210,211). Thus, EMT can be induced in breast cancer cell lines *in vitro* by extraneously added cytokines [e.g., tumour necrosis factor-α (TNF-α)] and growth factors (e.g., TGF-β, PDGF, HGF and FGF) through their respective cell surface receptors, leading to the activation of intracellular effectors such as RAS and other small GTPases, SRC, NF-κB, SMADs, β-catenin and integrins. EMT can also be induced by alterations in components of the ECM and in response to ECM remodelling by secreted MMPs (210,211). All these instances highlight the importance of the tumour microenvironment in initiating EMT.

Intracellularly, the pathways include the basic helix-loop-helix transcription factor TWIST, SNAIL1 and SNAIL2, the zinc-finger homeobox repressors ZEB1 and ZEB2/SIP1, GOOSECOID, and FOXC2. The activities of these EMT inducing transcription factors mostly converge in the transcriptional repression of E-cadherin, culminating in the suppression of the epithelial phenotype with concomitant upregulation of mesenchymal traits (210,211).

Many studies have demonstrated the upregulation of EMT-inducing transcription factors during breast cancer progression and accorded their prognostic significance (172,178,210). High levels of TWIST expression are associated with invasive lobular carcinomas (178), which originate from the epithelial cells of the milk-secreting lobular alveoli. EGF was reported to reduce E-cadherin expression and increase mesenchymal markers such as TWIST. Forced expression of EGFR in deficient cells reactivates TWIST expression but is suppressed by EGFR and Janus-activated kinase (JAK)/signal transducer and activator of transcription 3 (STAT3) inhibitors. TWIST expression is not significantly inhibited by kinases and signal transducers that target PI3K and MEK/ERK. Furthermore, TWIST promoter is significantly activated by constitutively active STAT3 but is suppressed by the JAK/STAT3 inhibitor and dominant-negative STAT3. A 26-bp promoter region that contains putative STAT3 elements was also found to be required for the EGF-responsiveness of the TWIST promoter. Chromatin immunoprecipitation assays showed that TWIST promoter binding to the nuclear STAT3 is induced by EGF. An immunohistochemical study of 130 primary breast carcinomas showed parallel associations between non-nuclear EGFR and TWIST and between phosphorylated STAT3 and TWIST (212,213).

GOOSECOID is another EMT-inducing transcription factor whose expression is significantly associated with ductal type breast tumours (214). It is known to initiate cell migration from the vertebrate Spemann organiser and its ectopic expression in breast cancer cells can lead to invasion associated events; down-regulation of E-cadherin, α-catenin and γ-catenin proteins, concordant with the apparent loss of adherens junctions, up-regulation of N-cadherin and vimentin, scattered distribution in culture, spindle-like morphology and increased cellular motility (214,215). TWIST and GOOSECOID induce FOXC2, a transcription factor of the FOX family of forkhead helix-turn-helix DNA binding proteins involved in EMT and organ development in many tissues (216). FOXC2 expression is associated with highly aggressive basal-like breast cancers (217) and interestingly, could induce mesenchymal gene transcription independently of E-cadherin repression.

Over-expression of SNAIL correlates with advanced tumour grade, lymph node metastasis, and poor clinical outcome in

infiltrating ductal breast carcinomas (181). Significantly, in one of the first demonstrations of tumour related EMT *in vivo*, spontaneous up-regulation of SNAIL was associated with the recurrence of tumours with EMT characteristics in a reversible ERBB2-induced mouse breast cancer model (182). Loss of ER reduces MTA3 mediated inhibition of SNAIL hence promoting EMT (191). However, like SNAIL, the expression of SNAIL2 has been associated with poor clinical outcome in breast tumours (183). Indeed SNAIL2 has been implicated in the formation of tubular structures believed to represent a collective mode of migration at the invasive front of ductal breast carcinomas (218).

The re-expression of embryonic transcription factors during breast cancer progression may arise as a result of accumulating genetic instabilities or through reciprocal interactions with the local tissue microenvironment. In support of the former, loss of SINGLEMINDED-2s in the mouse mammary gland induces SNAIL2-mediated EMT, indicating how gene inactivation may circumvent EMT dormancy in the adult mammary gland (219). In support of the latter, there is evidence for the heterogeneous expression and nuclear translocation of SNAIL (220) and SNAIL2 (221) following exposure to hypoxia at the invasive front, highlighting the importance of the microenvironment in providing extracellular signals for initiation of EMT at metastasis.

Tumour progression and metastasis is promoted by the stabilization of the hypoxia-inducible factor-1α (HIF-1α) transcription complex caused by intra-tumoural hypoxia and ultimately causing treatment failure and mortality in several human cancers. TWIST was shown to be associated with HIF-1α in inducing EMT and tumour metastasis by hypoxia or over-expression of HIF-1α (222). TWIST expression was found to be regulated by HIF-1 binding to the hypoxia-response element (HRE) in the TWIST proximal promoter. Furthermore, TWIST deficient mice showed similar phenotypes to HIF-1α null mice. However, EMT reversal was achieved by siRNA-mediated repression of TWIST in HIF-1α-over-expressing or hypoxic cells. Patients co-expressing HIF-1α, TWIST and SNAIL in primary head and neck tumours had the worst prognosis, caused by increased tumour metastasis providing evidence of a key signalling pathway involving HIF-1α and TWIST that promotes EMT and tumour metastasis in response to intra-tumoural hypoxia (222).

Tyrosine kinase receptors. AXL is a member of the TAM (Tyro-Axl-Mer) receptor tyrosine kinases (RTK) that are known to have diverse effects on regulating cellular responses that include cell proliferation, cell survival, migration, autophagy, angiogenesis, natural killer cell differentiation and platelet aggregation (223). AXL shares the vitamin K-dependent ligand growth arrest-specific 6. On the basis of its expression in many embryonic tissues it is thought to be involved in mesenchymal and neural development, with a restricted expression in smooth muscle cells in adult tissues (224). AXL was reported to be associated with EMT and its activation is linked to many signal transduction pathways, including AKT, MAPK, NF-κB, STAT, and others (225). AXL was originally identified as a transforming gene from a patient with chronic myelogenous leukemia, and since then it has been correlated with various high-grade cancers associated with poor prognosis

(223). It was shown that the presence of AXL is important for the growth of breast carcinoma and glioma cell xenografts (226,227). It was also reported that the sole expression of AXL in breast cancer predicts poor overall patient survival. Furthermore, maintaining breast cancer invasiveness, growth in foreign microenviroments, and metastatic spread requires elevated AXL levels. All of these studies indicate that AXL is a unique EMT effector that is essential for breast cancer progression (186). Endocrine-resistant breast cancer cells show highly elevated expression of AXL (191).

Tumour suppressor genes. The Rb protein is mutated or expressed at low levels in breast carcinomas suggesting that a relationship may exist between loss or reduction of Rb function and a less-differentiated state, increased proliferation, and high metastatic potential. siRNA mediated knockdown of Rb in MCF-7 cells (228) disrupted inter-cellular adhesion and induced a mesenchymal-like phenotype. In addition, Rb depletion resulted in reduced expression of E-cadherin.

p21$^{CIP1/WAF1}$ is a downstream effector of several tumour suppressors and also behaves as a cyclin-dependent kinase inhibitor of cell proliferation. It is also associated with EMT. Although its involvement in regulating features of tumour stem cells *in vivo* is unclear, deletion of p21CIP1, which enhanced the rate of tumourigenesis induced by mammary-targeted H-RAS or c-MYC, enhanced immunohistochemical features of EMT. Silencing of p21^{CIP1} also enhanced features of EMT in transformed immortal human MEC lines. p21^{CIP1} attenuated oncogene-induced BT-IC and mammosphere formation, suggesting a link between loss of p21^{CIP1} and acquisition of breast cancer EMT and stem cell properties *in vivo* (229).

The homeobox genes. HOX genes, members of the homeobox gene family, are also believed to be associated with tumour progression. These are evolutionarily conserved genes encoding master transcription factors that play fundamental roles in regulating embryonic development and maintaining homeostasis through strictly regulated expression in various tissues and organs during adulthood. Several studies have described the association of HOX genes in the pathogenesis of multiple cancers; HOXA7 and HOXD13 in lung (230), HOXC4 and HOXC8 in prostate (231), HOXB7 in ovarian (232) and HOXA10 in endometrial cancer (233). It was reported that 60% of breast cancers had no HOXA5 expression (234). Furthermore, HOXA5 expression in MCF-7 cells caused p53-dependent apoptosis, whereas in HS578T cells expressing mutant p53, cell death was due to activation of the caspase pathway (235). HOXD10 was also reported to be extensively reduced as malignancy increases in epithelial cells. Restoring the expression of HOXD10 in MDA-MB-231 which is a highly invasive breast cancer cell line, could significantly reduce migration (236). In addition, increased MCF10A cell motility and invasion *in vitro* was associated with HOXB13 over-expression, while its ratio versus interleukin-17β receptor was predictive of tumour recurrence during adjuvant tamoxifen monotherapy (237). Microarray analysis of purified epithelial cells showed that HOXB7, which is involved in tissue remodeling of the normal mammary gland (238) was expressed in higher levels from normal epithelial cells to primary metastatic breast tumours to bone
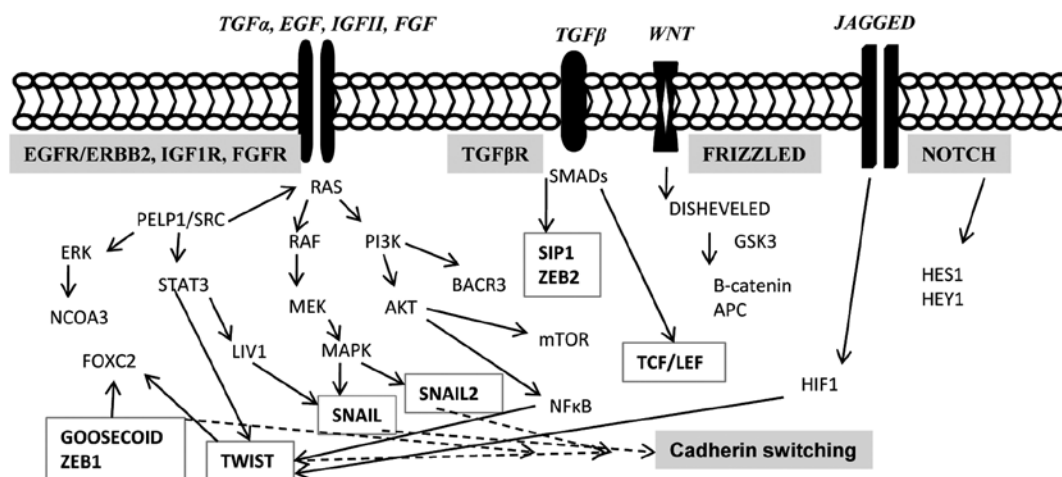
Figure 4. Signal transduction pathways implicated in EMT, showing some of the inter-relationships that are described and referenced in the text. Ligand stimulation of a variety of membrane receptors leads through intermediary molecules to activation of a set of nuclear transcription factors that regulate expression of epithelial/mesenchymal associated genes shown in Fig. 3. Note that many of these molecules are also involved in endocrine resistance. The so-called 'Master EMT regulators' are boxed.

metastatic lesions and is associated with development of breast cancer (239,240). HOXB7 was located to a novel amplicon at 17q21.3, by the use of cDNA-based comparative genomic hybridization. This amplification was associated with poor prognosis in a panel of 186 breast cancer cases (241). Furthermore, HOXB7 over-expression regulated the expression of several growth and angiogenic factors, including basic FGF, vascular endothelial growth factor (VEGF), inter-leukin 8, ANG1, ANG2, and MMP9 in SKBR3 breast cancer cells, leading to formation of vascularized tumours when grown as xenografts in nude mice (239,240). HOXB9 has a similar effect leading to increased cell motility and acquisition of mesenchymal phenotypes (242). Another homeoprotein, SIX1, has been shown to stimulate mammary tumour progression and metastasis in mouse models, through induction of EMT (243,244).

EMT and tumour microenvironment. Although the tissue microenvironment is initially hostile to the development of a tumour, as cancers evolve, they circumvent the cytotoxic signals emanating from the surrounding tissue and provoke an inflammatory response that recruits tumour-infiltrating immune cells to the host-tumour interface. The cross-talk and reciprocal interactions between the tumour, its surrounding stroma, and infiltrating immune cells in the form of secreted MMPs, ECM components, growth factors, and cytokines enable localized tissue remodelling to promote tumour cell motility, EMT and the initiation of metastasis. Consistent with this model, co-culture of breast tumour cells with macrophages greatly enhances their migration and invasion by inducing EMT through NF-κB-mediated SNAIL stabilization (245). Furthermore, interleukin-6 induces EMT and promotes the invasiveness of MCF-7 cells, with clear ramifications for breast cancer patients with elevated serum levels of this cyto-kine (246).

Fig. 4 summarises various aspects that have been discussed above to illustrate how the diverse signalling pathways, some of which are implicated in endocrine resistance, lead to accumulation of several key mediators of the EMT.

5. EMT, CSCs and endocrine resistance

In their model of tamoxifen resistance, Hiscox *et al* (194,247) showed that β-catenin, regulated by an autocrine action of EGFR, could act as both a mediator of E-cadherin dysfunction and a regulator of gene expression and promote development and progression of EMT. Development of endocrine resistance was accompanied by a change in β-catenin phosphorylation, loss of function of adherens junctions and increased transcription of β-catenin target genes. Interestingly, increased EGFR expression has been demonstrated within the EMT micro-environment, and its activation via autocrine production of EGFR ligands such as TGFα and amphiregulin has been reported to facilitate the EMT process (248).

Modulation of EGFR activity using gefitinib established it as a key player in the development of an aggressive, EMT-like phenotype in acquired tamoxifen-resistant breast cancer cells (71).

It has been suggested that the acquisition of enhanced EGFR/ERBB2 pathway signalling in ER+ breast cancer with tamoxifen resistance potentially results from selection of a more stem cell-like phenotype. Expression of EGFR is seen in stem cells of the normal mammary gland in mice and humans (249,250) whilst ER is predominantly expressed in the more differentiated luminal cells (251-253). The EGFR pathway was activated in CSCs of ductal carcinoma *in situ* (DCIS) of the breast. There is emerging evidence for a role of the ERBB2 pathway in the function of CSCs. Expression of ERBB2 and presence of ALDH1+ CSCs was positively correlated in one series of 491 breast cancer patients (254). The CSC populations of four ERBB2+ breast cancer cell lines have been demonstrated to express more ERBB2 mRNA and protein compared with the non-CSC population, regulated at the level of transcription. Furthermore, trastuzumab reduced mammosphere-forming

capability and tumourigenicity on serial xenotransplantation (255). In a clinical study in ERBB2 over-expressing large primary breast cancers, lapatinib reduced the CD44$^+$/CD24 CSC fraction and mammosphere forming efficiency of the residual tumour, although not statistically significant (256). Notably treatment with chemotherapy alone increased the proportion of CSCs in the residual breast cancer (256,257). So it seems plausible that up-regulation of the EGFR/ERBB2 pathway in endocrine-resistant breast cancer could indirectly reflect an enrichment of a CSC phenotype (258). The γ secretase inhibitor DAPT or a NOTCH 4 neutralizing antibody significantly reduced mammosphere formation in primary human DCIS.

Antagonism of the NOTCH pathway causes amelioration of the effect of trastuzumab that reduces mammosphere formation in ERBB2 over-expressing cell lines (255). A 2-6-fold increase in NOTCH 1 activity induced nuclear accumulation of NOTCH 1 intracellular domain, and increased expression of NOTCH down-stream targets including HES and HEY 1 was seen in MCF-7, BT474 and SKBr3 cell lines after treatment with trastuzumab or lapatinib (259). Inhibition of the NOTCH pathway led to re-sensitisation to trastuzumab and the combination of NOTCH antagonism and trastuzumab inhibited growth in both trastuzumab sensitive and resistant cell lines (259).

Borley *et al* (260) reported that the antiestrogens tamoxifen and fulvestrant can promote an invasive phenotype in E-cadherin deficient ER$^+$ breast cancer cells through activation of SRC. However, estrogen deprivation mimicking the action of aromatase inhibitors did not promote invasiveness in the same cells. This means that acquired anti-estrogen resistance induced breast cancer cell invasion in the absence of E-cadherin but not necessarily EMT behaviour. An immunohistochemical profiling of SRC expression and signalling through EGFR showed simultaneous activation of GRB2/RAS/MAPK pathway, phospholipid metabolism involving PLD, PLCγ, and P13K and activation of the cytosolic SRC family kinases, ultimately promoting EMT-like events. SRC kinase was shown to modulate EMT-like characteristics in acquired endocrine resistance. src activation assayed in breast cancer biopsies showed a correlation with presence of distant metastasis, and shortened survival with tamoxifen therapy in ER$^+$ patients. Significantly, targeted inhibition of SRC kinase activity in tamoxifen-resistant breast cancer cells is accompanied by an efficient reduction in their invasive and migratory behaviour and even causing its reversion (MET) (247). *In vitro* studies suggest a role for c-MET signalling as a promoter of tumour progression and metastasis *in vivo*. HGF/scatter factor mediated activation of c-MET RTK induced 'cell scattering', a process fundamental to EMT. Analysis of ER$^-$ Faslodex-resistant MCF-7 cells showed significantly elevated levels of the HGF/scatter factor receptor gene, c-MET. In addition, high levels of c-MET in breast tumours correlated with a significantly reduced survival rate (261).

Mani *et al* (262) demonstrated for the first time that in addition to endowing cells with migratory and invasive potential, passage through EMT confers properties of breast CSCs. In breast tumours, the CD44$^+$/CD24$^-$/low antigenic phenotype defines a subpopulation of breast cancer cells enriched with cells with stem cell-like qualities, namely the ability to self-renew and reconstitute differentiated tumours (263). Thus, immortalized human mammary epithelial cells exposed to a range of different EMT-inducing stimuli (ectopic expression of SNAIL or TWIST or TGF-β treatment), acquire the ability to self-renew and express the CD44$^+$/CD24$^-$/low antigenic phenotype. Conversely, CSC isolated from human breast cancer tissues express high levels of mRNAs encoding mesenchymal markers (262). Similarly, Morel *et al* (264) have shown that aberrant activation of the RAS/MAPK pathway generates a population of CD44$^+$/CD24$^-$/low cells displaying combined EMT and stem cell attributes. Moreover, hypoxia-induced SNAIL2 expression is associated with the acquisition of a basal-like breast cancer phenotype and high levels of the stem cell regulatory genes CD133 and BMI1 (221). More recently, *in vivo* EMT induction by infiltrating CD8 T-cells has been shown to generate mesenchymal tumour cells with CSC properties (265), highlighting once again the paradoxical role of inflammation in exacerbating breast cancer progression. Furthermore, genome-wide transcriptional profiling has shown that metaplastic breast cancers and claudin-low tumours frequently exhibit EMT and stem cell-like features, likely contributing to their poor outcomes (168).

Collectively, these findings demonstrate that induction of EMT in differentiated breast epithelial tumour cells is sufficient to generate a sub-population of cancer cells with stem cell characteristics and the propensity to metastasize. Thus, EMT is not only important for cells to escape from the immediate vicinity of the tumour, but may also sustain primary tumour growth as well as promote the initiation and establishment of secondary tumours. The molecular pathways linking stem cell-like characteristics with EMT remain largely undefined. In a recently described mouse model of breast cancer metastasis to the lung, inhibition of WNT signalling through LRP6 was found to reduce stem cell-like properties and cause EMT reversal, restoration of the epithelial phenotype, and suppression of SNAIL2 and TWIST expression (266). Several components of the WNT signalling pathway have thus been implicated in mediating breast cancer metastasis from the orthotopic site to the lungs, suggesting that pharmacologic agents targeting the WNT pathway may be useful in controlling EMT-mediated breast cancer recurrence and metastasis (171).

Recent work by Weinberg's group (262) has linked the mesenchymal cell phenotype to stem cells in normal tissue and to CSCs. Immortalised human mammary epithelial cells (HMECs) induced to undergo EMT exhibited stem cell markers and had increased capacity to form mammospheres enriched in stem cells. Similarly stem cells isolated from normal and cancerous human and mouse mammary glands demonstrated markers of mesenchymal phenotype normally apparent in EMT. This included up-regulation of SNAIL and SNAIL2 and also the TGFβ signalling pathway which has been previously implicated in stem cell function (253).

Interestingly, recent analysis of a panel of breast cancer cell lines of luminal, intermediate and basal phenotypes has shown a significantly increased fraction of CSCs (defined by CD44$^+$/CD24lo/ESA$^+$ expression) in basal type breast cancers compared to hormone-sensitive luminal cancers (291). Furthermore, a positive correlation was shown between CSC number and cell line tumourigenicity in *in vivo* models (267).

As ER negatively regulates the expression of the key transcription factors regulating EMT such as SNAIL and SNAIL2 (268,269) a functionally redundant ER in endocrine-resistant breast cancer might therefore promote a more mesenchymal stem-cell-like phenotype. In an MCF-7 model, tamoxifen resistant cells showed enhanced mammosphere forming capacity compared to tamoxifen sensitive cells, suggesting an increased CSC fraction (121).

Interestingly, EMT was implicated in the progression to distant metastatic disease and therapeutic resistance. It was shown that following adriamycin treatment of MCF-7 cells, TWIST 1 expression is induced and only cells undergoing adriamycin-induced EMT display enhanced invasion and multidrug resistance. TWIST 1 depletion by RNA interference blocked mesenchymal transformation, partially reversed multi-drug resistance, and abolished invasion induced by adriamycin (270). Furthermore, TWIST1 RNA interference may show efficacy in adriamycin-based chemotherapies for breast cancer (271). Resistance to ERBB2 targeted therapy is a clinical problem that may undermine the success of ERBB2 targeted therapies such as trastuzumab and lapatinib. The ERBB2 pathway may play an important role in the maintenance of breast CSCs, induction of which, during EMT, may be the basis of resistance in ERBB2 targeted therapy (272). More generally, breast cancer cells may acquire resistance to conventional and targeted therapies upon conversion to a mesenchymal-like phenotype. A recent screen using transformed human breast cells that were experimentally transformed into mesenchymal cells showed that these cells which exhibit CSC properties, are more resistant to conventional chemotherapeutic drugs such as paclitaxel or doxorubicin (273).

Our group has established an endocrine-resistant cell line (termed pII) by transfection of MCF-7 cells with an shRNA targeting the ER (274,275). These cells have constitutively down-regulated ER, exhibit a higher proliferative rate, increased aggressive behaviour reflected by accelerated motility and ability to penetrate into simulated extracellular matrix components, co-incident with a change from an epithelial morphology to a more mesenchymal one (191,276). Rearrangement in these cells of the α-actin cytoskeleton reflects increased appearance of lamellipodia and microspikes, features known to drive cellular motility (277). The morphological changes are accompanied by loss of E-cadherin and catenin and other epithelial markers such as keratins 18 and 19 and the gain of expression of mesenchymal markers such as N-cadherin, tenascin C and fibronectin, PLAU, VEGF, CD68 and vimentin, characteristics shared with the MDAMB231 ER⁻ cell line and hallmark features of cells undergoing EMT (171). Genome-wide micro-array analysis confirmed a shift towards higher expression of genes involved in cell motility and interaction with the ECM as well as those characterising basal-like metaplastic and claudin-low tumour types reported to increase cell survival, stem cell-like properties, migratory and invasive capacity, thus promoting metastasis. Transcriptional repressors of E-cadherin that include ZEB1, ZEB2/SIP1 and SNAIL2 (174) were also elevated. Another significant group that is elevated in these cells are included in the '24 gene signature' of genes proposed as predictive of invasiveness (278): integrin, TIMP-2 and TIMP-3, MT1-MMP, PAI-1, Osteonectin/SPARC, thrombo-spondin-1, collagen (VI) α1 and collagen (I) α2. A further

interesting feature is the ability of various tyrosine kinase inhibitors to block proliferation, motility and invasion in these cells (191) reflecting an important role of peptide growth factor receptors, particularly EGFR. So far, all the experimental data are consistent with the view that in these cells that have acquired endocrine independence, there is an associated change that closely mimicks EMT.

6. Reversal of EMT

The process of EMT associated with tumour progression and invasion presents a dramatic phenotypic change in which well differentiated epithelial cells possessing extensive junctional networks and a distinct pattern of gene expression, apparently re-organise and acquire molecular and morphological characteristics of cells of a completely different embryonic lineage with its own unique features. As detailed in the preceding sections, this is a seemingly complex multidimensional process involving an extraordinarily large number of contributory molecules of diverse function from structural components to receptors to signalling molecules and transcription factors. Which of these are simply *in vitro* phenomena and which have physiological significance remains to be confirmed. However that may be, it is clear that EMT presents a challenge to existing notions of cell lineage and differentiation. Reversal of this transition would have not only obvious therapeutic implications but also shed light on cellular mechanisms controlling cell specificity and function. In this respect, it is interesting that whereas tumour cells can undergo EMT in response to the inductive signals produced by an inflammatory tumour microenvironment, their exposure to the non-inflam-matory microenvironment of a distant metastatic site may trigger at least partial EMT reversal and MET, a necessary process for tumour re-population. Metastatic lesions usually exhibit typical epithelial characteristics even when derived from poorly differentiated primary tumours (211).

TGF-β signalling pathway. As a key mediator of fibrosis and a facilitator of metastasis, the TGF-β signalling pathway may be a good target for EMT reversal (172,279). TGF-β induces EMT by both SMAD-dependent and independent signalling events (164,265,280,281). SMAD4 is frequently mutated in pancreatic and colorectal cancers and in a subset of juvenile polyposis syndrome patients who have inherited mutated alleles of SMAD4 (282).

In advanced disease, TGF-β can stimulate invasion and metastasis of tumour that has become TGF-β insensitive. Invasion and metastasis of tumour cells can be inhibited by ectopic expression of dominant negative TGF-β receptors (283). Colon cancer patients with inactivating mutations in TGF-β type II receptor have a lower metastatic potential and increased survival (284,285). TGF-β1 ligand activates a heteromeric receptor of two transmembrane serine/threonine kinases, type I and II receptors (TβRI and TβRII) (TβRII transphosphorylates TβRI, activating its kinase function) to exert its signalling effects. Activated TβRI phosphorylates the intracellular proteins SMAD 2 and 3 which then associate with SMAD 4, translocating to the nucleus where the complex interacts with other transcriptional co-activators and co-repressors to regulate expression of numerous genes (174). This type of signalling

that depends on SMAD up-regulates the expression of many transcription factors such as SNAIL, SNAIL2, TWIST, and members of the ZFH family, ZEB1 and ZEB2 (178,179,188). Many other signalling proteins have also been implicated in the induction of EMT by TGF-β1. These include RAS/MAPK (286), integrin β-1 (181), integrin-linked kinase (214), p38 mitogen-activated protein kinase (p38 MAPK) (217), RHOA kinase (ROCK) (182), PI3K (183), JAGGED1/NOTCH (218), SARA (219), NF-κB (220), PAR 6 (175,221) and ERK (245). The inhibition of TβRI kinase and ROCK resulted in full reversal of the metastatic phenotype and gene expression patterns associated with EMT. This is caused by the mesenchymal gene expression blockade caused by TβRI kinase which is mediated by down-regulating ZEB1 and ZEB2, while the epithelial structure is maintained by the ROCK inhibitor (281).

Another mechanism of EMT reversal involves expression of GATA3, a transcription factor regulating T lymphocyte differentiation and maturation. It is absent from metastatic lesions but expressed in early stage well differentiated breast cancers. A cuboidal-like epithelial phenotype and reduced cell invasive activity associated with increased E-cadherin expression and decreased N-cadherin, vimentin, and MMP-9 was mediated by ectopic expression of GATA3 in MDA-MB-231 cells that grew as non-metastatic smaller primary tumours. Control cells grew as larger metastatic tumours in xenografted mice. E-cadherin expression was induced by the binding of GATA-like motifs located in the E-cadherin promoter. MCF-7 cell fibroblastic transformation and increased invasion and metastasis resulted from siRNA induced GATA3 knockdown. Several studies on human breast cancers showed that GATA3 expression was correlated with elevated E-cadherin levels, ER expression, and longer disease-free survival indicating that GATA3 may drive invasive breast cancer cells to undergo EMT reversal which would ultimately lead to the suppression of cancer metastasis and invasion (213).

uPA. Urokinase-type plasminogen activator (uPA) and plasminogen activator inhibitor type I (PAI-1) system also have an important role in tumour invasion and metastasis (287-289) interacting with the matrix metalloproteinases system. Both molecules are well established as prognostic indicators of equal or greater utility than ER (290) and are also useful predictors of distant metastases in a subset of early, node-negative breast cancer patients (291). Hypoxia can promote EMT by inducing uPAR-dependent cell-signalling in cancer cells. MDA-MB 468 cells cultured in 1% O_2 exhibited increased uPAR expression, disruption of inter-cellular junctions, increased vimentin expression and loss of E-cadherin, indicating EMT (292). This effect could be reversed by shifting the cells to 21% O_2. EMT could also be reversed by silencing expression of endogenously-produced uPA, or by targeting uPAR-activated cell-signalling factors such as PI3K, SRC family kinases and extracellular signal-regulated kinase. MDA-MB 231 cells express high levels of uPA and uPAR and have a characteristic mesenchymal morphology under normoxic conditions (21% O_2). Silencing of uPA in these cells leads to decreased expression of vimentin and SNAIL, and induces a morphology characteristic of epithelial cells. Thus uPAR-initiated cell-signaling represents a potential target for EMT reversal in cancer cells. EMT reversal

has also been observed during re-programmed gene expression in the mouse embryo induced by mutations in the TCF8 gene which leads to developmental defects caused by a reduction in progenitor cell proliferation and cell migration (202,281).

7. Concluding remarks

There is considerable accumulated evidence which shows that the acquisition of resistance of breast cancers to endocrine therapies is accompanied by the gain of aggressive characteristics both *in vivo* and *in vitro*. EMT-like processes accompany acquisition of endocrine resistance by promoting a migratory phenotype that shows evidence of a switch from a network of cytokeratin intermediate filaments to one based upon vimentin. Underlying these events are complex signalling cascades which may be activated by numerous cytokines/growth factors. So, the ability of anti-growth factors and kinase inhibitors to antagonise such events may ultimately be of significance in the treatment of cancer patients as a means to prevent or reduce tumour dissemination. The link between development of endocrine resistance and EMT offers new therapeutic targets and new insights into the mechanisms underlying both processes in breast cancer progression. It remains to be seen whether the pleithora of phenotypic changes are indeed reversible, as suggested by the apparent re-differentitation back into epithelial character of blood-borne metastasizing cells that re-enter distant tissues to form secondary neoplastic deposits. Our current studies are addressing the issue of whether the EMT that we observe to be a direct consequence of loss of ER function in cultured cells, can be reversed simply by permitting the re-expression of ER. The nuclear transcription factors that trigger the loss of E-cadherin and other epithelial components could be key molecular targets whose blockade could prevent EMT induction and possibly block endocrine resistance.

Acknowledgements

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