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Endocrine Resistance and Epithelial Mesenchymal Transition in Breast Cancer

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1. Introduction

Estrogen plays a major part in the regulation of cell proliferation and survival, controlling female physiology, reproduction and behaviour (Musgrove and Sutherland, 2009). It however assumes a more malevolent role in its association with breast cancer pathogenesis. Consequently, therapies have been designed to block the actions of estrogen mediated through its receptors (ERα and ERβ), or to simply reduce its levels in the body (Zilli et al., 2009). Since Beatson (1896) first introduced ovariectomy over a century ago as the first therapeutic modality to reduce the adverse effects of estrogen, endocrine therapy has developed into the cornerstone of breast cancer treatment for those 60-70% of patients whose tumours over-express ER and/or progesterone receptor (PR) (Massarweh and Schiff, 2007; Zilli et al., 2009). For three decades, selective estrogen receptor modulators (SERMS), predominantly tamoxifen, have proved to be effective agents for the suppression of breast cancer growth in both early and advanced disease (Normanno et al., 2005). Tamoxifen has significantly improved the quality of life and survival of many patients with metastatic disease, as well as displaying prophylactic benefit, particularly in women with ductal carcinoma-*in situ* (Fisher et al., 1999).

However, about half of ER+ patients with advanced disease and nearly all patients with metastatic disease fail to respond to first-line tamoxifen therapy. About 40% of patients receiving tamoxifen as adjuvant therapy experience tumour relapse and die from their disease, and a third of women treated with tamoxifen for 5 years develop recurrent disease within 15 years (Normanno et al., 2005). The introduction of pure estrogen antagonists such as fulvestrant, to overcome the apparent disadvantage of tamoxifen with its partial agonist properties, did not resolve the resistance problem (Osborne and Schiff, 2011). Second line therapy with other endocrine agents designed to inhibit peripheral extra-gonadal synthesis of estrogen in postmenopausal women produces some beneficial effects but for the most part serves merely to delay onset of endocrine resistance (Massarweh and Schiff, 2007). This refractiveness to continued administration of anti-estrogens and aromatase inhibitors poses a significant therapeutic problem that has been addressed by a large number of studies. Several theories have been proposed to explain this phenomenon, based on observations made with a variety of *in vitro* cellular models (Normanno et al., 2005). The consensus opinion seems to be that whereas *de novo* resistance is most likely due to low levels of ER expression, *acquired* resistance is predominantly the consequence of an attenuated response to other peptide growth factors that normally play a subsidiary role in cell proliferation.

These molecules exert their action through a variety of trans-membrane receptors that possess intrinsic tyrosine kinase activity. Fig 1 depicts the various potential influences that govern the behaviour of breast cancer cells.

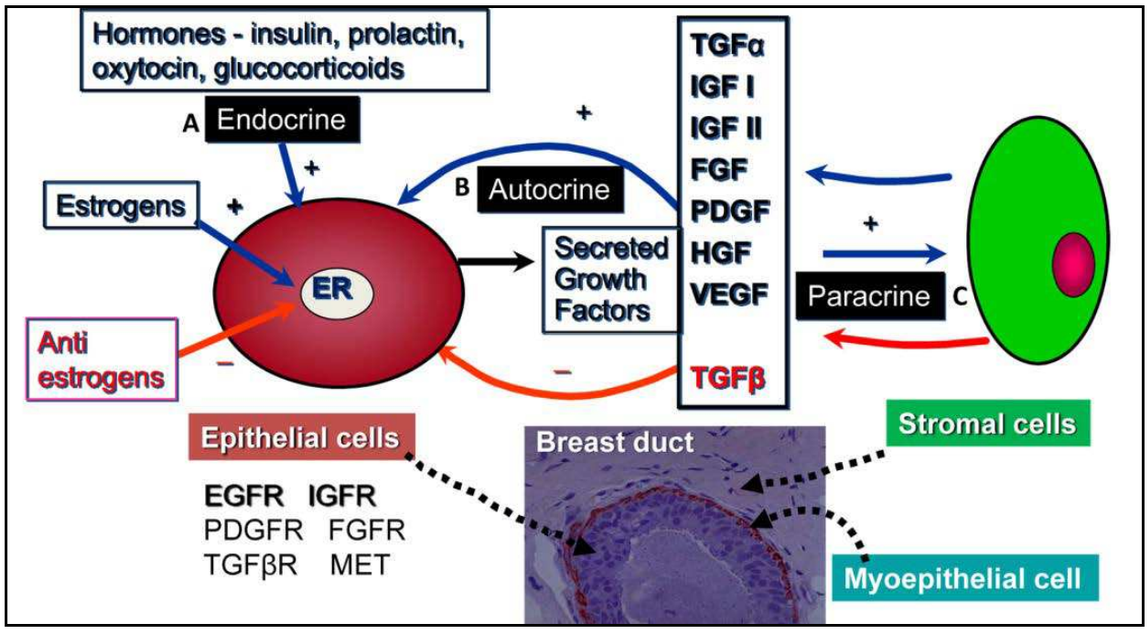


Fig. 1. Factors affecting growth and proliferation of breast cancer cells. Breast epithelial cells are subject to various influences that can either promote or inhibit cellular activity. (A) Endocrine stimulation by a variety of hormones, most significantly estrogen, promotes long term effects. (B) Autocrine stimulation involves, under various conditions, the production and secretion of a number of peptides that act back on the producer cell to modify its activity through membrane bound receptors that frequently possess intrinsic tyrosine kinase activity which initiates a signalling cascade that terminates in the action of transcriptional regulators to modify gene expression. (C) Paracrine stimulation is effected by the action of mediators which include the listed peptide growth factors as well as others originating from myoepithelia (in the normal breast) and stromal elements that include fibroblasts and macrophages in tumours. All of these pathways have been found to operate both *in vitro* (in tumour-derived cell lines) and *in vivo*, but their relative contributions vary considerably in both cases and may be influenced not only by biological heterogeneity but also by therapeutic interventions.

It is also a general experience that endocrine resistance is associated with increased aggressiveness and frequent metastasis (Hiscox et al., 2007), characteristics that more often typify ER-ve tumours. Identification of ligands, receptors and downstream signaling molecules with increased activity in the resistant phenotype, both in cell culture and in tumour biopsies, has highlighted a bewildering collection of molecules that may play a direct causative role, be a consequence or simply innocent bystanders in the progressive cellular change towards endocrine independence. For the purposes of therapeutic discrimination, attempts have been made to reduce this plethora, generated principally by microarray analyses (eg Charafe-Jauffret et al., 2006 ; Luqmani et al., 2009; Al Saleh, 2010) to

a manageable number, and given the designation of 'gene signature' by virtue of selectively circumscribing a particular sub-group of patients.

In a separate scenario, new insights have been gained into our understanding of cell differentiation from studies that have demonstrated that epithelial cells have the potential to trans-differentiate into mesenchymal cells (epithelial to mesenchymal transition: EMT) and vice versa (mesenchymal to epithelial transition: MET). Many recent reports have indicated that this process, which was previously observed during transition between developmental stages, is synonymous with the process of tumour metastasis. Both processes share similar pathways of activation. Our recent data (Luqmani et al., 2009; Al Saleh, 2010; Al Saleh et al., 2011a) suggests that there may also be causal links between the development of endocrine resistance and the onset of EMT. In this report we summarise the molecular pathways of ER activity, the mechanisms proposed to account for resistance and finally review the evidence for the above hypothesis.

2. Mechanisms of estrogen receptor induced cell proliferation

ERα and ERβ are transcribed from distinct genes located on separate chromosomes (6 and 14, respectively) (Green et al., 1986; Kuiper et al., 1996). These receptors differ in their tissue distribution, with ERα being highly expressed in the pituitary gland, ovaries (thecal and interstitial cells), uterus, liver, kidneys, adrenals and the mammary glands while ERβ is found mainly in the prostate, bone, ovaries (granulosa cells), lungs and in various parts of the central and peripheral nervous system (Emmen et al., 2005; Kuiper et al., 1997). Nevertheless, ERα and ERβ do overlap in their expression in some tissues (Zilli et al., 2009). More importantly, the two receptors have different roles in breast development. Only ERα appears to be essential for ductal growth although both receptors are present in the breast. ERα-knockout mice show very little growth of mammary ducts, while ERβ-knockout mice develop a normal mammary gland with regular ductal branching (Förster et al., 2002; Lubahn et al., 1993). This suggests that ERβ might be exerting pro-differentiative and anti-proliferative functions. In addition, increased ERα/ERβ ratio in breast cancer as compared with benign tumours and normal tissues suggest that ERα is most closely associated with breast cancer pathogenesis, while ERβ can protect against the mitogenic activity of estrogens in pre-malignant lesions (Roger et al., 2001; Shaw et al., 2002). It has even been suggested that the estrogen-induced proliferation of ER+ breast cancer cells can be inhibited by ERβ over-expression (Ström et al., 2004; Williams et al., 2008). Thus ERα remains the main focus of attention in studies on breast cancer. Unless otherwise specified, 'ER' in this review will refer to ERα.

In what is now referred to as the nuclear or genomic action of ER, binding of estrogen induces activation of the receptor by initiating its dissociation from cognate heat shock proteins, and leads to conformational changes, dimerisation and autophosphorylation (Osborne & Schiff, 2005). The activated ER binds to estrogen response elements (EREs) located in the promoter regions upstream of estrogen-regulated genes. Frasor et al., (2003) observed from microarray analysis of gene expression in MCF-7 cells that about 70% of such estrogen-regulated genes were actually down-regulated following treatment with estradiol. Many of these genes are transcriptional repressors, or genes with anti-proliferative or pro-apoptotic function. On the other hand, there is increased expression of genes inducing cell proliferation and survival. Up-regulation of gene expression is mediated through two domains; activating function-1 (AF-1) and activating function-2 (AF-2). AF-1 is a hormone

independent domain located at the N-terminus of the receptor with its function regulated by phosphorylation. AF-2 is the site where ligand-binding actually occurs and is therefore hormone dependant. Almost all gene promoters are activated through both AF-1 and AF-2, though some are activated independently by AF-1 or AF-2 (Gronemeyer 1991; Osborne et al., 2001). Subsequent to formation of the ER-ligand complex, binding of co-regulatory molecules such as nuclear-receptor co-activator 1 (NCOA1 or SRC1), NCOA2 (TIF2) and NCOA3 (AIB1, TRAM1, RAC3 or ACTR) (Leo & Chen 2000; McKenna et al., 1999) enhance the transcriptional activity of ER accompanied by increased activity of histone-acetyltransferase (HAT) at the promoter site. Other co-regulatory molecules can also partly suppress the transcriptional activity of ER by recruitment of histone-deacetylase complexes such as nuclear-receptor co-repressor 1 (NCOR1) and NCOR2 that influence ER-induced transcription (Chen & Evans, 1995; Horlein et al., 1995). Several of these groups of molecules have been reported to have prominent roles in cancer. AIB1 (SRC-3) is over-expressed in almost two thirds of all breast cancers and associated with a shorter disease-free survival in patients receiving tamoxifen as adjuvant treatment (Osborne et al., 2003). In untreated patients, high levels of AIB1 were associated with improved outcome, consistent with studies that suggest the possibility of an association between an enhanced agonistic effect of tamoxifen and the high levels of co-activators. However, ER can also co-operate with FOS/JUN and bind with other transcription factors such as AP-1 (activator protein-1) and SP-1 (specificity protein-1) at their specific sites on DNA (Kushner et al., 2000; Ray et al., 1997; Safe 2001) commonly designated as serum response elements (SRE).

In addition to its classical mode of action through a nuclear-located receptor, estrogen has also been reported to interact with membrane associated receptors, leading to a more rapid reaction than would be expected from a transcriptionally mediated response, such as initiation of cAMP production (Rosner et al., 1999; Zivadinovic et al., 2005) and activation of intrinsic kinases present in other plasma membrane receptors such as insulin-like growth factor-1 receptor (IGF-1R), epidermal growth factor receptor (EGFR) and ERBB2 (Bunone et al., 1996; Campbell et al., 2001; Font de Mora & Brown 2000) as well as receptors for fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF). It has been suggested that interaction of SERMs including tamoxifen with such membrane associated receptors may be responsible for their agonist behaviour. There is however much controversy over this issue, with other studies discounting the involvement of such postulated receptors as G protein-coupled receptor as targets of estrogen action (Otto et al., 2008). However this may be, any non-genomic interactions of estrogen would depend on the levels of the above-mentioned kinases, and they will likely be modest in ER+ breast cancer cells that express low levels of tyrosine kinase receptors such as EGFR and ERBB2 (Normanno et al., 2005).

Ligand independent activation of ER can occur via the downstream signaling cascades transmitted through membrane receptor tyrosine kinases such as EGFR, ERBB2, and IGF1R In particular. MAPK/ERK, PI3K/AKT, p90RSK and p38 MAPK pathways can specifically activate ER at key positions (serine 118 and 167 and threonine 311) in the AF-1 domain and in other domains (Bunone et al., 1996; Campbell et al., 2001; Joel et al., 1998; Kato et al., 1995). Expression of ligands and receptors such as transforming growth factor-α (TGFα), IGF1 and IGF1R can be increased by estrogen and those can then initiate signalling while expression of other receptors such as EGFR and ERBB2 is decreased by estrogen signaling (Kushner et al., 2000; Massarweh et al., 2008; Umayahara et al., 1994; Vyhlidal et al., 2000; Yarden et al., 2001). In addition, activation of the PI3K/AKT and the p42/44 MAPK

pathways by these receptors down-regulates the expression of ER and PR causing reduction in estrogen dependency while activating the transcriptional function of ER, which suggests a contribution of this cross talk to the relative resistance to endocrine therapies in tumours with amplified ERBB2 expression (Bayliss et al., 2007; Brinkman and El-Ashry, 2009; Creighton et al., 2010; Lopez-Tarruella and Schiff, 2007).

The two types of ER actions, genomic and non-genomic, are not mutually exclusive and do overlap. For example, ER induces the expression of transcripts for both TGFα and amphiregulin (Normanno et al., 1993; Saeki et al., 1991) which can both bind and activate EGFR resulting in activation of MAPK and AKT signaling which are also activated by direct interaction with ER (Salomon et al., 1995). ER binding to membrane caveolin-1 leads to the activation of specific G proteins resulting in the activation of SRC and in turn of matrix metalloproteinases that cleave transmembrane precursors of the EGFR ligand, heparin binding-EGF (HB-EGF) (Levin, 2003; Razandi et al., 2003). Fig 2 illustrates the major identified downstream events involving ER activation.

3. Mechanisms of endocrine resistance

It should be noted that most tumours are heterogeneously composed and a biphasic response to treatment could reflect the survival and eventual clonal outgrowth of an intrinsically resistant minor sub-population.

3.1 Alterations in ER expression or function

Since all endocrine therapies target ER, the expression of the latter is the main predictor of the outcome of such therapies. The *de novo* resistance is clearly caused by the lack of ER expression which can be due to histone deacetylation (Parl, 2003) or associated with aberrant methylation of ER CpG islands that deactivates chromatin (Ottaviano et al., 1994; Weigel & deConinck, 1993). Interestingly, ER expression can be restored in ER-ve breast cancer following co-treatment with DNMT1 and HDAC inhibitors (Robertson et al., 2000; Rountree et al., 2000; Yang et al., 2001).

It was initially thought that acquired resistance might be due to missing or non-functional variants of ER. However, only 17–28% of patients with acquired endocrine resistance lack ER expression (Gutierrez et al., 2005; Johnston et al., 1995). Furthermore, approximately 20% of tamoxifen-resistant patients will eventually respond to second-line treatment with aromatase inhibitors or fulvestrant (Howell et al., 2005; Osborne et al., 2002). Although a number of exon-deleted receptor isoforms have been described, their frequency is insufficient to account for resistance.

Furthermore, these mutations have been detected in ER-ve tumours (Herynk & Fuqua 2004). A mutation that results in a hypersensitive receptor that shows enhanced binding of co-activators in the presence of low estrogen levels (a single amino acid substitution changing lysine 303 to arginine) was found in 20 of 59 hyperplastic breast lesions (Fuqua et al., 2000). However, the role and frequency of such mutations in primary breast carcinomas and their relation to endocrine resistance needs to be explored in a larger number of patients.

Patients carrying inactive alleles of cytochrome P450 2D6 (CYP2D6) (approximately 8% of Caucasian women) fail to convert tamoxifen to its active metabolite, endoxifen (4-hydroxy-N-desmethyl-tamoxifen), and are consequently less responsive to tamoxifen, which is considered to be a significant factor in resistance to therapy (Hoskins et al, 2009). The baseline levels of endoxifen are elevated in patients carrying the wild-type CYP2D6 and who

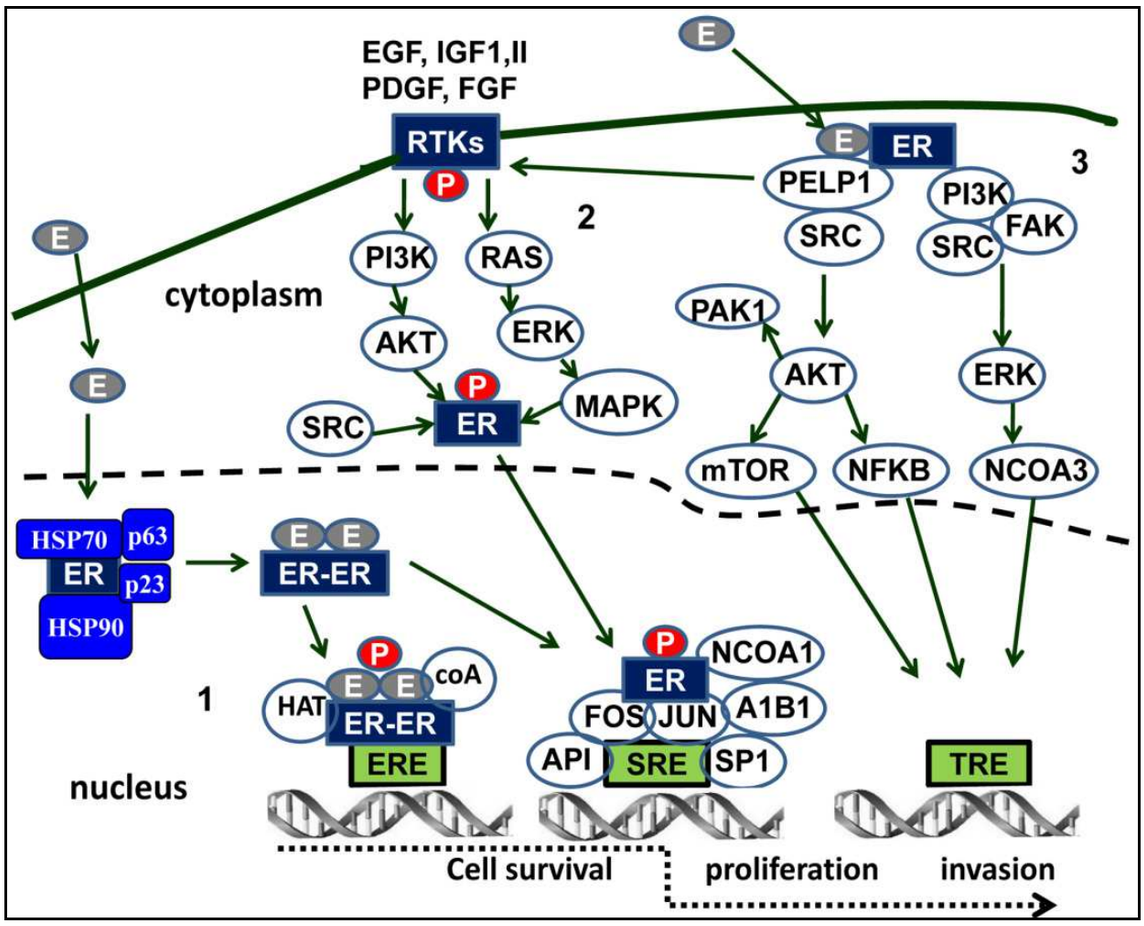


Fig. 2. Proposed cellular mechanisms mediated through the estrogen receptor. 1. Classical genomic mode of action, in which estrogen binds to an inactive ER complex, causing dissociation from heat shock and other cognate proteins, receptor dimerisation and phosphorylation (P). This can then interact directly with estrogen response elements (ERE) on target genes in concert with histone acetyl transferase (HAT) and several other co-activators (coA) or by association with the ubiquitous transcriptional factors FOS/JUN and with NCOA1 and AIB1 co-activators at API/SPI sites termed serum response element (SRE). 2. Cytoplasmically located ER can be phosphorylated by the action of AKT, SRC and ERK/MAPK serine/threonine kinases, downstream of signalling events initiated by various growth factors' interaction with their respective tyrosine kinase containing receptors and mediated through RAS or PI3K. This ligand- independent activated receptor can initiate transcription through the SRE. 3. Binding of estrogen to membrane–associated ER may induce assembly of complexes with either PI3K/FAK/SRC leading to activation through ERK of the transcriptional activator NCOA3 or with PELP1/SRC resulting in up-regulation of mTOR and NFKB through AKT. These mediate an action through other transcriptional response elements (TRE) on a variety of target genes without involving direct interaction of ER with chromatin. The latter mechanisms are referred to as the non-genomic pathways, that are postulated to explain those observed effects of estrogen which are too rapid to be accounted for by mechanism 1. Further 'crosstalk' between ER and RTKs may involve participation of PELP1.

had low levels of the metabolite when co-treated with paroxetine, a selective serotonin re-uptake inhibitor (prescribed to alleviate tamoxifen-associated hot flashes) that can inhibit CYP enzymes. Heterozygous patients showed a better outcome when treated with tamoxifen, as compared with untreated patients suggesting a role for cytochrome P450 enzyme variants in regulating the response to tamoxifen (Wegman et al., 2005).
The presence of ER variants was also hypothesized to have a role in endocrine resistance. A reduced response to endocrine therapy has been associated with the presence of a new truncated variant of ER, ER36, in addition to the full-length receptor (Shi et al., 2009).

3.2 Estrogen receptor β

It has been reported that ERß transcript levels were about 2-fold higher than those of ERα in tamoxifen-resistant as compared with tamoxifen-sensitive patients (Speirs et al., 1999) and that ERß bound to tamoxifen, raloxifen or the anti-estrogen ICI 164 384, increased transcription of AP-1-dependent genes (Paech et al., 1997). Other studies show that ERβ has a negative effect on ERα-promoted transcription (Hall & McDonnell 1999; Pettersson et al., 2000) or no correlation with response or resistance to endocrine treatment (Cappelletti et al., 2004). Development of antibodies distinguishing between the ER types and their variants has led to identification of responses in ERβ+ve but ERα-ve cancers and a potential role for the carboxy-terminally truncated variants of ERβ (ERβ2 and ERβ5) in tamoxifen responsiveness (Honma et al., 2008; Murphy and Watson, 2006). In addition to ERβ, the oestrogen-related receptor ERRγ was found to be over-expressed and mediated tamoxifen resistance in lobular invasive breast cancer models (Riggins et al., 2008).

3.3 Adaptation to estrogen withdrawal

Breast cancer cells can acquire a state of hypersensitivity to estrogen that renders them resistant to endocrine therapy. MCF7 cells cultured in estrogen-free medium to produce long-term estrogen deprived cells (LTED) mimics the effects of ablative endocrine therapy (Santen et al., 2003) and produces cells that are highly sensitised to substantially lower concentrations of estrogen as compared with wild-type MCF-7 cells (Masamura et al., 1995). Growth factor signalling and ER expression was significantly higher in these cells. Treatment with estrogen resulted in rapid association of ER and phosphorylation of SHC, an adaptor protein involved in tyrosine kinase receptor signalling, and increased activation of both SRC and the RAS/RAF/MEK/MAPK signalling pathways (Song et al., 2002a,b; Song et al., 2004). Exposure of these cells to fulvestrant blocked MAPK activation indicating that this pathway may be a downstream effector of the ER non-genomic pathway (Santen et al., 2003; Song et al., 2002a,). However, a high AKT and MAPK level in LTED cells was associated with increased resistance to endocrine therapy and a worse outcome.
In another version of MCF7 LTED cells, enhanced transcriptional activity of ER was associated with increased activation of growth factor pathways that in turn trans-activate ER (Johnston & Dowsett, 2003). After prolonged culture in the absence of estradiol, the ER in these cells functions independently from exogenous estradiol, which was suggested to be due to a super-sensitivity of LTED to residual estrogen present in the medium (Chan et al., 2002; Martin et al., 2003). These cells also showed increased levels of phosphorylation of ER at serine 118, a known target for several intracellular kinases. Furthermore, IGF-1R and ERBB2 signalling was significantly increased in these cells concurrently with increased MAPK activation. Interestingly, the phosphorylation of ER at serine 118 was blocked by

MAPK or EGFR/ERBB2 blockade but not by blocking MEK/MAPK or PI3K/AKT signalling, indicating that additional kinases might be involved in this hypersensitive state. Nicholson et al., (2004) also developed an MCF7 cell line (MCF-7X cells) that is resistant to estrogen withdrawal but not hypersensitive to it. These cells could be growth inhibited by fulvestrant, implying that the ER pathway is still involved in their proliferation. However, the PI3K/AKT pathway was demonstrated to be the main factor promoting their growth without the involvement of EGFR/ERBB2 or IGF-1R signalling, suggesting that the adaptation to estrogen withdrawal can occur in the absence of increased sensitivity to estrogen and does not require activation of classical growth factor receptors.

3.4 Estrogen receptor and co-regulators

Since ER action is mainly controlled through transcriptional factors and co-regulator molecules, it seems likely some of these may be implicated in endocrine resistance. For example, increased AP1 and NFKB transcriptional activity has been associated with endocrine resistance (Johnston et al., 1999; Zhou et al., 2007). And similarly when ER co-activators are over-expressed or phosphorylated. For example, NCOA3 (A1B1 or SRC3) over-expression leads to constitutive ER-mediated transcription, which confers resistance both *in vitro* and in xenograft models and is associated with reduced responsiveness to tamoxifen in patients (Ali & Coombes, 2002; Osborne et al., 2003; Ring & Dowsett, 2004). Another ER co-activator associated with resistance is PELP1 (Fig 2) which is a cytoplasmic scaffold protein that modulates ER interaction with SRC, leading to activation of SRC and the ERK family kinases and also promotes oestrogen activation of PI3K (Gururaj et al., 2006). Interestingly, ER cytoplasmic complex composed of ERα, PI3K, SRC and focal adhesion kinase (FAK; also known as pTK2) is formed as a result of the transient methylation of ER at R260 by protein arginine N-methyltransferase 1 (pRMT1). This complex activates AKT and could confer resistance to endocrine therapy but this methylation event which is frequent in breast cancer has yet to be linked to resistance (Le Romancer et al., 2008).

3.5 Growth factor receptor pathways

Perhaps the most important factors that affect the response to endocrine therapy are those that can modulate alternative proliferation and survival in the tumours in which the ER signalling pathway is effectively inhibited. These alternative growth pathways can do so by the establishment of a bidirectional cross talk with ER signalling. These pathways will act as ER-independent drivers of cancer proliferation and survival and are involved in both *de novo* and *acquired* resistance (Normanno et al., 2005). Increased expression of EGFR, ERBB2 and IGF1R along with their downstream components such as ERK and PI3K can modulate tamoxifen resistance (Faridi et al., 2003; Hutcheson et al., 2003; McClelland et al., 2001). ERBB2 has been reported to be over-expressed in association with down regulation of the X-linked tumour suppressor forkhead box p3 (FOXP3) and the zinc finger transcription factor GATA4 (Hua et al., 2009; Zuo et al., 2007). Other factors that might affect ERBB2 expression are the presence of the paired-domain transcription factor PAX2 and the ER co-activator NCOA3 which compete for binding and regulating ERBB2 transcription and, in turn, responsiveness to endocrine therapy. However, like GATA4 and FOXP3, PAX2 was also shown to be down-regulated in tamoxifen resistant breast cancers in the presence of NCOA3 and an over-expressed ERBB2 (Hurtado et al., 2008). The SRC substrates BCAR1 and BCAR3 have both been reported to elicit endocrine resistance *in vitro* (Dorssers et al., 1993). BCAR1

binds and activates SRC leading to phosphorylation of EGFR and the signal transducer and activator of transcription 5B (STAT5B) (Riggins et al., 2007). On the other hand, BCAR3 is believed to activate RAC and p21-activated kinase 1 (pAK1), which is a mediator of endocrine resistance itself through ER phosphorylation, and through the activation of SRC in association with BCAR1 (Cai et al., 2003; Rayala et al., 2006; Riggins et al., 2003; van Agthoven et al., 1998).

The de-regulation of several growth pathways including EGFR, ERBB2 and IGF1R are implicated in endocrine resistance (Faridi. et al., 2003; Miller et al., 2009). Many events might trigger this de-regulation such as activating mutations in PIK3CA and loss of heterozygosity or methylation of PTEN, activation of AKT, over-expression of ERBB2 and activation of IGF1R and ERBB3 following the loss of PTEN (Arpino et al., 2008; Miller et al., 2009; Riggins et al., 2007). However, following de-regulation of these pathways acquisition of endocrine resistance might be effected by a number of possible activities as summarised by Musgrove & Sutherland, (2009): "decreased ER expression mediated by ERK activation; loss of ER-mediated repression of EGFR and ERBB2 and consequent activation of mitogenic signalling cascades; ligand-independent activation of ER or its co-activators through phosphorylation; up-regulation of key cell cycle regulators, for example MYC and the D and E-type cyclins, through constitutive activation of mitogenic signalling pathways; and the inhibition of apoptosis through constitutive activation of survival signalling".

3.6 Cell cycle signalling molecules

In order for cancer cells to bypass the inhibition of cell proliferation elicited by endocrine agents, one would expect down-regulation of effector molecules involved in the induction of apoptosis while those involved in proliferation, especially during G1 phase, are up regulated. Over-expressed cell cycle regulators include MYC, cyclin E1, cyclin D1, cyclin D1b, as well as p21 and p27, and a de-activated RB gene (Prall et al., 1998; Wang et al., 2008). Over-expression of MYC and cyclin D1 leads to an abundance of CDK complexes that are directly associated with increased cellular proliferation and/or relief of the inhibitory effects of the negative cell cycle regulators p21 and p27, a phenomenon that is also achieved through activation of ERBB2, AKT and SRC (Caldon et al., 2009; Chu et al., 2008; Hui et al., 2002; Perez-Tenorio et al., 2006). Cyclin D1 can also interact with several transcription factors including ER and STAT3 (Coqueret et al., 2002). Tamoxifen actually enhances the binding of cyclin D1 to ER at the expense of STAT3, hence activating both transcription factors and consequently establishing endocrine resistance (Ishii et al., 2008). Other important molecules are those involved in apoptosis. In particular, the pro-apoptotic molecules such as BIK (BCL2-interacting killer) and caspase 9 are down regulated in endocrine resistant cancers while those which are considered as anti-apoptotic molecules such as BCL-XL and its second messenger ceramide, are up regulated (Mandlekar et al., 2001; Riggins et al., 2005) . The expression of these molecules is also affected by signalling through PI3K/AKT, TNF, IFN and NFKB.

4. Epithelial mesenchymal transition

The phenomenon of epithelial cells undergoing a transition towards a mesenchymal phenotype was first identified as programmed events occurring during embryonic developmental processes (Greenberg & Hay, 1982). Since then EMT has since been described in various pathological conditions. During the process of cancer metastasis, a minority of

epithelial cells lose their apico-basal polarity, detach from adjacent cells, scatter and acquire increased motility and are able to invade into the extracellular matrix with subsequent penetration into the vasculature. This process is facilitated by a morphological transformation into a fibroblastoid structure that has all the hallmark features of EMT, Both processes share remarkable similarities, with characteristic phenotypic changes. These include the loss of cell-cell adhesion as a result of reduced E-cadherin in adherens junctions, occludins (OCLN) and claudins (CLDN) in tight junctions and desmoplakin (DSP) in desmosomes and down regulation of epithelial cytokeratins (KRT8, KRT18, and KRT19) and up-regulation of mesenchymal proteins most notably vimentin (VIM) and fibronectin and sometimes alpha smooth muscle actin (ACTA2) along with many other changes.
Fig 3 depicts the changes occurring during EMT. Multiple molecular mechanisms underlie EMT initiation and its reversal process, MET, which cancer cells are thought to undergo at sites where they form metastases, in order to re-establish cohesive colonies and initiate neo-vascularisation.

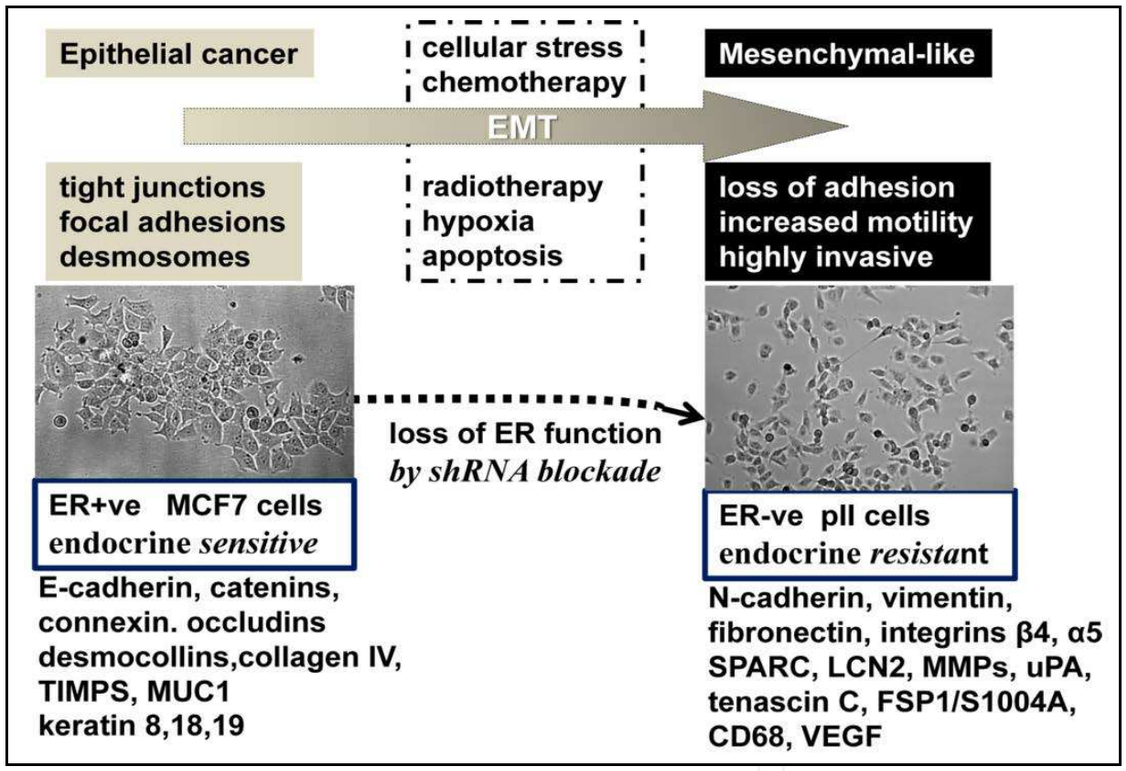


Fig. 3. Epithelial to mesenchymal transition. Loss of epithelial characteristics and breakdown of tissue architecture through dissolution of cell-cell junctions and loss of apico-basal polarity by detachment from the basement membrane can be initiated through a variety of diverse cellular insults which lead to transformation into a cell type that displays mesenchymal–like features. At a molecular level there is a certain uniformity of changes. Cells that have lost ER function and consequently acquired endocrine independence, in this case by shRNA- induced down-regulation (Al Saleh, 2010), show both the morphological appearance as well as the phenotypic changes that are characteristic of cells undergoing EMT. Several differences are indicated between MCF7 and pII cells that parallel those seen during EMT.

The transformation of epithelial cells into a mesenchymal-like form requires the participation of a complex network of both extra- and intra-cellular signals., Amongst the many identified are TGFβ, HGF, FGF, EGFR family members, IGF1 and 2, and PDGF (Thiery et al., 2002). An array of embryonic transcription factors such as the homeobox protein GOOSECOID (GSC), TCF3 (E47), the zinc-finger proteins SNAIL1 and SNAIL2 (previously SLUG), the basic helix-loop-helix protein TWIST1, the forkhead box proteins FOXC1 and FOXC2 , and the zinc-finger E-box-binding proteins ZEB1 and ZEB2 (SIP1) , are generated by the activity of these growth factor pathways, each of which is capable, on its own, of inducing an EMT.

There is increasing evidence of extensive crosstalk between these molecules, permitting the formation of an extensive signalling network responsible for establishing and maintaining a mesenchymal phenotype. (Moreno-Bueno et al., 2008; Peinado et al., 2007). In addition, some of these transcriptional activators such as TWIST are pivotal factors in overcoming cellular senescence (Ansieau et al., 2008) and in generating tumourigenic cancer stem cells (Mani et al., 2008). Interestingly, EMT-inducing transcription factors also confer stem cell characteristics on epithelial cells. For example, the receptor KIT which is an important factor for maintaining the stem cell state in the haematopoietic system has been shown to induce SNAIL2 expression in both mice (Perez-Losada et al., 2002) and humans (Sanchez-Martin et al., 2002). Many of these transcription factors exert repressive functions by binding to conserved E-box sequences in the promoter regions of such critical genes as CDHI (Gilles et al., 2003; Pieper et al., 1992).

4.1 Transforming Growth Factor β

TGFβ can independently promote an EMT phenotype in mouse mammary epithelial cells (Thuault et al., 2006; Waerner et al., 2006). This cytokine induces EMT by both SMAD-dependent and independent signalling events (Berx et al., 2007; Das et al, 2009; Santisteban et al., 2009). In advanced disease, TGF-β can stimulate invasion and metastasis of tumours that have become TGF-β insensitive which can be inhibited by ectopic expression of dominant negative TGF-β receptors (Ozdamar et al., 2005). 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 several genes (Onder et al., 2008). This type of signalling that depends on SMAD, up-regulates the expression of many transcription factors such as SNAIL1, SNAIL2, TWIST, and members of the ZFH family, ZEB1 and ZEB2 (Sarrio et al., 2008; Vandewalle et al., 2005; Yang et al., 2004) that are considered to be primary transcriptional inducers of EMT. TGFβ can also phosphorylate certain cytoplasmic proteins regulating cell polarity and tight junction formation. These include RAS/MAPK (Xue et al., 2003), integrin β-1 (Blanco et al., 2002), integrin-linked kinase (Hartwell et al., 2006), p38 MAPK (Mani et al., 2007), RHOA kinase (ROCK) (Moody et al., 2005), PI3K (Martin et al., 2003), JAGGED1/NOTCH (Come et al., 2006), SARA (Laffin et al., 2008), NFKB (Lester et al., 2007), PAR6 (Berx et al., 2001; Storci et al., 2008), pAR66A and ERK (Wu et al., 2009). Furthermore, EMT induced by the oncogenic stimulation by RAS and/or RAF activation in mammary, kidney and skin epithelial tissue was found to depend almost completely on TGF-β signaling (Moustakas and Heldin, 2009). TGFβ can also induce the activation of other signalling pathways that

might participate in initiation of EMT such as the WNT and NOTCH pathways (Polyak and Weinberg, 2009). Figure 4 illustrates the major events that are thought to be critical in the trans-differentiation of epithelial cells.

4.2 AXL

As mentioned earlier, receptor tyrosine kinase activity is altered in breast cancer and is considered to be an important factor in endocrine resistance. These molecules are also implicated in EMT since they already play a pivotal role in embryogenesis. One interesting member of the TAM (Tyro-AXL-MER) receptor tyrosine kinases is AXL which exerts diverse effects in regulating cellular responses that include cell proliferation, cell survival, migration, autophagy, angiogenesis, natural killer cell differentiation and platelet aggregation (Linger et al., 2008). AXL was reported to be associated with EMT since it is activated in many signal transduction pathways including AKT, MAPK, NFKB, and STAT. (Hafizi et al., 2006). Furthermore, AXL expression alone is considered as a predictive marker for poor overall patient survival. It has also been reported that elevated AXL levels are needed for maintaining breast cancer invasiveness, growth in foreign microenvironments and metastatic potential. Endocrine-resistant breast cancer cells show highly elevated expression of AXL (Al Saleh et al., 2010).

4.3 E-cadherin and its transcriptional repressors

E-cadherin is a critical switch in EMT during early embryonic development. Its down-regulation in epithelial cells triggers acquisition of a fibroblastic phenotype, dissociation from the epithelium sheets and migration, vital steps in gastrulation, neural crest formation and organ development (Thiery, 2003). E-cadherin expression is often lost in aggressive breast cancers acquiring EMT which would result in the disassembly of inter-cellular adhesion complexes, loosening contacts between neighbouring epithelial cells and thus disrupting the overall tissue architecture. E-cadherin loss also causes the liberation of β-catenin to the nucleus and its subsequent activation of WNT signalling of other EMT inducers as described above. Furthermore, E-cadherin loss mediates EMT through 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 (Onder et al., 2008).

An interesting connection between endocrine resistance and EMT is established through the connection between SNAIL, E-cadherin and metastasis-associated protein 3 (MTA3). MTA3, which is directly activated by ER, is a repressor of SNAIL, thereby also repressing EMT (Al Saleh et al., 2011). We have recently shown that down-regulation of ER in MCF7 cells leads to a reduction in both MTA1 and MTA3 and a concurrent rise in SNAIL2 **(**Al Saleh et al., 2011a).

Reduction of E-cadherin expression correlates with poor differentiation, invasiveness, aggressive metastatic behaviour, and an unfavourable prognosis (Berx et al., 2001; Wheelock et al., 2003); experimental knockdown of E-cadherin is sufficient to establish metastasis but not fully reverse EMT by itself. Interestingly, the down regulated expression of E-cadherin during EMT is a reversible process that arises through hypermethylation of the E-cadherin promoter or transcriptional repression although many lobular breast cancers appear to have lost the expression of E-cadherin through inactivating mutations and loss of heterozygosity (Berx et al, 2001).

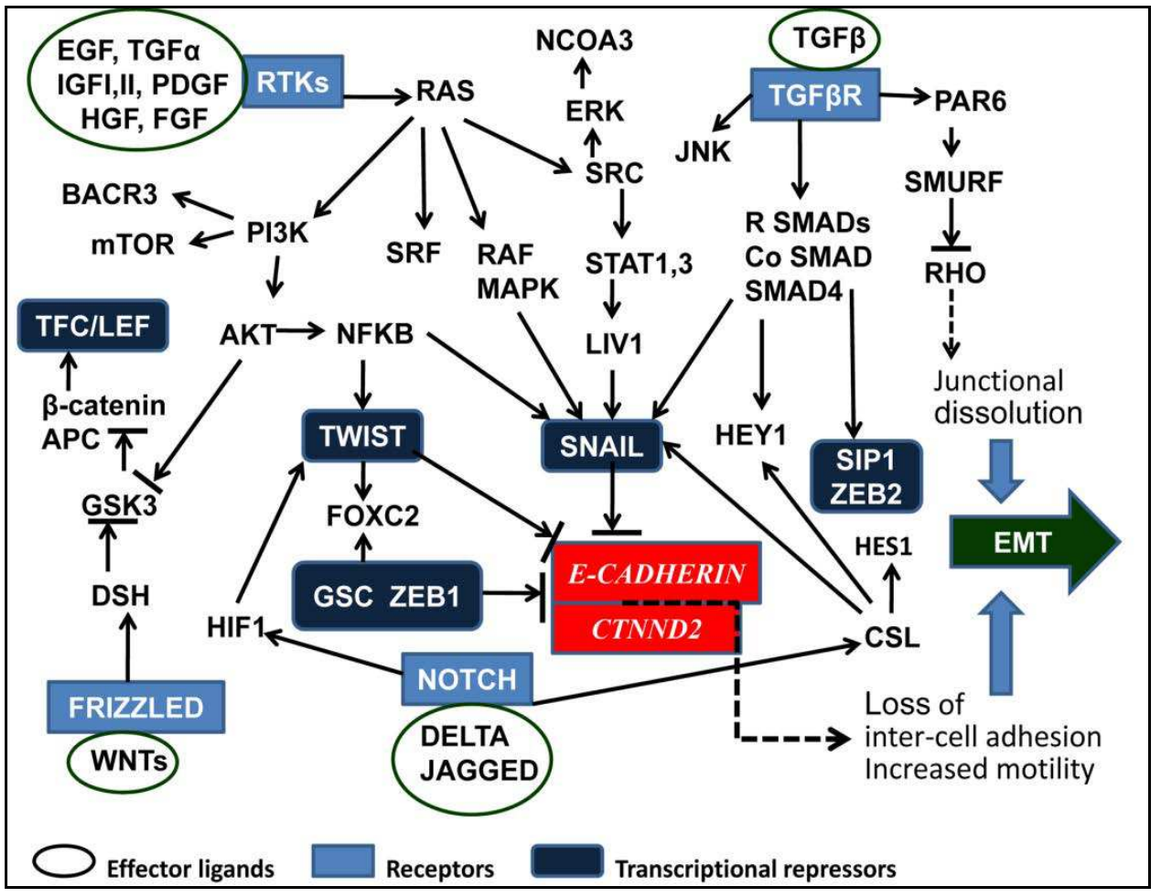


Fig. 4. Transduction pathways and effectors contributing to processes leading to EMT. A variety of growth factors (EGF, TGFα, IGFI, II, PDGF, HGF, FGF) binding to receptor tyrosine kinases (RTK) activate the central RAS pathway to promote transcription of SNAIL through the RAF/MAPK, the PI3K/AKT/NFKB or the SRC/LIV pathways. AKT, as well as WNTs acting through the FRIZZLED receptor, promote inhibition of GSK3 through DSH to promote re-localisation of β-catenin and generate TCF/LEF that also increases SNAIL. DELTA/JAGGED signalling through NOTCH also increases SNAIL via CSL as well as TWIST through HIF1. TGFβ signals through its receptor to increase SMAD family members that co-operatively promote both SNAIL as well as SIP1/ZEB2. It also acts through PAR6 to up-regulate the ubiquitin ligase SMURF that degrades RHO which is a key promoter of tight junctions, The transcriptional repressors SNAIL, TWIST, GSC, ZEB1,2 and TFC/LEF effectively down-regulate E-cadherin and associated molecules, which leads to loss of cell adhesion, permitting cell scattering, cellular motility and invasion through the action of up-regulated proteases. Not shown here, for clarity, is HEDGEHOG signalling which through GLI integrates with the RTK and WNT pathways to up-regulate SNAIL family members Evidence for the interactions illustrated is summarised in excellent reviews by Huber et al., 2005; Moustakes & Heldin, 2007 and Sabbah et al., 2008 and references therein.

The appearance of another mesenchymal marker, N-cadherin (CDH12) and/or cadherin-11 (CDH11), in a process termed 'cadherin switching', is also a well documented event in EMT (Gjerdrum et al., 2010; Sarrio et al., 2008; Sphyris and Mani, 2009; Wheelock et al., 2008). The expression of these mesenchymal markers during EMT is induced by SNAIL, ZEB2/SIP1 and SNAIL2 (Cano et al., 2000; Sarrio et al., 2008; Vandewalle et al., 2005). N-cadherin is reported to be highly expressed in invasive and metastatic human breast cancer cell lines and tumours and to correlate with aggressive clinical behaviour. Nevertheless, N-cadherin expression can be triggered in E-cadherin expressing cells and it could in fact cause EMT, impacting on their epithelial phenotype, suggesting a dominating role for this cadherin over the other, possibly in synergy with FGF2 (Hazan et al., 2000, 2004). MCF7 cells that have acquired endocrine independence through induced loss of ER expression also display cadherin switching which is accompanied by increased motility, F-actin cytoskeletal rearrangement and the loss of cellular adhesion molecules. It is suggested that endocrine resistance is a major event influencing the cells to move and invade into the surrounding tissues (Al Saleh, 2010; Al Saleh et al., 2011a).

4.4 Vimentin

A marker that is commonly used to characterise EMT is vimentin, a component of type III intermediate filaments and the archetypal mesenchymal marker (Trimboli et al., 2008). Elevated vimentin expression correlates well with increased cell migration, invasion and EMT induction in several breast cancer cell lines (Al Saleh, 2010; Al Saleh et al., 2011a; Gilles et al., 2003) in co-ordination with other mesenchymal markers such as tenascin C (Dandachi et al., 2001; Polette et al., 2007), which has been associated with over-expressed ERBB2 and down-regulated ER. The molecular events triggering vimentin expression during EMT are less well delineated in comparision to the mechanisms inducing E-cadherin down-regulation. The expression of vimentin is considered to be a late occurrence in EMT in a temporal sequence of genetic events starting from loss of epithelial markers followed by appearance of mesenchymal markers (Polette et al., 2007). Direct activation of vimentin expression in human breast tumour cells (Gilles et al., 2003) by β-catenin/T-cell factor/lymphocyte enhancer factor-1 is consistent with the activation of β-catenin as a downstream event from consequential loss of E-cadherin. The indirect promotion of vimentin expression by ZEB2/SIP1 during EMT in a β-catenin-independent manner (Bindels et al., 2006) suggests the existence of some trans-activators driving EMT which are associated with vimentin expression.

4.5 Matrix metalloproteinases and lipocalin

In order for cancer cells to metastasise, they need to penetrate into and through the extracellular matrix (ECM). This process is facilitated by the activity of matrix metalloproteinases (MMPs). A family of more than 28 MMPs have been reported to be up-regulated in nearly every tumour type and are closely involved in cancer progression through cleavage and release of bioactive molecules that inhibit apoptosis and stimulate cancer invasion and metastasis. For example, treatment of cells with MMP-3 results in an increased expression of the activated splice variant RAC1b, elevating the levels of cellular reactive oxygen species which, in turn, lead to increased expression of SNAIL and EMT initiation (Orlichenko et al., 2008). An MMP-9 associated protein, Lipocalin2 (LCN2), was

also found to play a major role in cell regulation, proliferation, differentiation and regulation of EMT. It's over-expression in human breast cancer cells can cause up-regulation of vimentin and fibronectin while E-cadherin is down regulated (Yang et al., 2009). Furthermore, LCN2 over-expression significantly increases cell motility and invasiveness in previously non-invasive MCF-7 cells. Interestingly, siRNA-mediated LCN2 silencing inhibited cell migration and development of the mesenchymal phenotype in aggressive breast cancer cells. It was also reported that reduced expression of ER and increased expression of SNAIL2 was correlated with LCN2 expression while over-expression of ER in LCN-2 expressing cells was able to reverse EMT and reduce SNAIL2 expression, suggesting that ER negatively regulates LCN2-induced EMT (Yang et al, 2009).

4.6 Hypoxia

An interesting physiological mechanism that can cause EMT is hypoxia. It has been reported that tumour progression and metastasis is promoted by the stabilisation of the hypoxia-inducible factor-1α (HIF-1α). This transcription factor was shown to be associated with TWIST in inducing both EMT and tumour metastasis by hypoxia or over-expression of the former. Furthermore, the expression of TWIST was found to be regulated by HIF-1 binding to the hypoxia-response element (HRE) in the TWIST proximal promoter and is associated with it in inducing EMT or metastasis (Yang et al, 2008). Interestingly, the HIF-1α null mice phenotype resembles TWIST deficient mice. In addition, patients with head and neck cancer whose tumours co-express TWIST and HIF-1 had very poor prognosis suggesting a major role for these two genes in regulating EMT.

4.7 HOX genes

Another important set of genes in regulating EMT is the homeobox (HOX) gene family, master players in regulating embryonic development and maintaining homeostasis through strictly regulated expression in various tissues and organs during adult life. Several studies have demonstrated the association of HOX genes in the pathogenesis of multiple cancers. For example, HOXA7 and HOXD13 have been associated with lung cancer (Lechner et al., 2001), HOXC4 and HOXC8 in prostate cancer (Miller et al., 2003), HOXB7 in ovarian cancer (Naora et al, 2001) and HOXA10 in endometrial cancer (Yoshida et al., 2006). In one study 60% of their breast cancers had no HOXA5 expression (Raman et al., 2000) which causes p53-dependent apoptosis. HOXA5 was reported to cause cell death through the activation of the caspase pathways in HS578T cells expressing mutant p53 (Chen et al., 2004). HOXD10 was extensively reduced as malignancy increased in epithelial cells, and restoring its expression in MDA-MB-231 could significantly reduce the migration capacity of these highly aggressive cells (Carrio et al., 2005). HOXB13 over-expression was associated with increased MCF10A cell motility and invasion *in vitro*, while its ratio to interleukin-17β receptor was predictive of tumour recurrence during adjuvant tamoxifen monotherapy. HOXB7 is involved in tissue remodeling of the normal mammary gland (Ma et al., 2004) and is expressed at higher levels in metastatic breast tumours (Care et al., 1998, 2001). Furthermore, regulation of the expression of several growth and angiogenic factors, including basic FGF, VEGF, IL8, ANG1, ANG2, and MMP9 in SKBR3 breast cancer cells, depends on the over-expressed levels of HOXB7 which can result in the formation of vascularised tumours when grown as xenografts in nude mice. HOXB9 like HOXB7 can lead to increased cell motility and EMT (Hayashida et al., 2010).

4.8 NOTCH

DELTA/JAGGED acting through the NOTCH pathway are implicated in both cell fate in the normal human mammary gland (Raouf et al., 2008) and regulation of cancer stem cells (CSCs) in both ductal carcinoma *in situ* and in invasive carcinoma of the breast (Dontu et al., 2004; Stylianou et al., 2006). This pathway is known to be transcriptionally induced by TGFβ/SMAD signalling and contributes to EMT (Zavadil et al., 2004). This pathway is cell type specific and can be either oncogenic through activation of the NKFB pathway or it can be tumour suppressive. Wang et al., (2006) provided evidence demonstrating that NOTCH receptor signalling regulates SNAIL 1 and 2, ZEB1 and vimentin.

4.9 WNT

The WNT signalling pathway mediates several vital processes such as cell proliferation, migration, differentiation, adhesion and death (Vincan et al., 2008). In addition, this pathway can promote migration and EMT in breast cancer cells through the stabilisation or increased expression of SNAIL1 and 2 and TWIST (Onder et al., 2008; Vogelstein et al., 2004). SNAIL has been implicated in regulating WNT-1-induced EMT in MCF-7 cells. Furthermore, WNT signalling can also lead to the translocation of β-catenin to the nucleus where it can drive the expression of several EMT inducing transcription factors through the WNT induced inhibition of glycogen synthase kinase-3β (GSK3β)-mediated phosphorylation. However, β-catenin alone usually is not enough to induce EMT although in colorectal cancer WNT is indeed a silencer of its negative regulators SOX17 (Zhang et al., 2008), SFRPS18, 19 and DKK1 (Aguilera et al., 2006). Interestingly, both SFRP1 and DKK1 are frequently silenced by methylation in breast cancer.

4.10 miRNA

It is well established that non-protein coding micro (mi) RNAs play a significant role in regulation of gene expression and cellular protein levels. They are now also being increasingly recognised as major regulators of EMT and metastasis, specifically the miR-200 family (miR-200a, miR-200b, miR-200c, miR-141, miR-429 and miR-205 (Gregory et al, 2008; Park et al., 2008). Members from the miR-200 family and miR-205 are associated with increased expression of E-cadherin and decreased vimentin. In addition, these miRNAs also target the expression of ZEB1 and ZEB2, the E-cadherin transcriptional repressors. Expression levels of miR-205 and of some members of the miR-200 family were also found to vary inversely with vimentin expression in primary serous papillary carcinomas of the ovary (Park et al., 2008). In another study, EMT was induced through either TGFβ or the tyrosine phosphatase pEZ in Madin–Darby canine kidney (MDCK) cells. The levels of both miR-205 and miR-200 family members was down-regulated after EMT induction while their ectopic expression induced MET (Gregory et al., 2008).

One way that natural antisense transcripts can play a major role in EMT is by targeting the regulation of ZEB2 expression. This was documented when EMT was induced in a human colorectal cancer cell line by SNAIL. ZEB2 levels were found to be directly increased after EMT initiation which was explained as the result of the action of a natural antisense transcript that prevented the splicing of a large intron in the 5′ untranslated region (UTR) that contains an internal ribosomal entry site which lowers ZEB2 levels in epithelial cells through the inhibition of ribosome scanning. During EMT activation, the antisense transcript levels are increased. They bind to the 5′UTR and inhibit splicing, preserving the

internal ribosomal entry site sequence and thereby increasing the translational efficiency of ZEB2 which then directly inhibits E-cadherin expression, maintaining an EMT state (Beltran et al., 2008).

Although these RNA molecules are associated with the regulation of EMT and MET, other miRNAs such as miR-10b are reportedly associated with metastasis and invasion. It inhibits HOX10 translation while increasing RHOC when induced by TWIST (Ma et al., 2007). Another miRNA that seems to increase the metastatic potential of cancer cells is miR-29a; up-regulated in a mesenchymal metastatic RASXT mammary cell line compared to epithelial EpRas cells. In addition, over-expression of miR-29a suppresses expression of tristetraprolin, a regulator of epithelial polarity and metastasis, and leads to EMT and metastasis through RAS signalling. This correlates with data from breast cancer patients showing enhanced miR-29a and reduced tristetraprolin levels (Gebeshuber et al., 2009). In contrast to miR-10b, miR-335 was found to be a suppressor of invasion and metastasis through modulation of the expression of the 'six gene signature' set: COL1A1, MERTK, PLCB1, PTPRN2, TNC and SOX4 which are considered predictive markers of metastasis and invasion. miR-335 was also reported to suppress invasion and metastasis in MDAMB231, a highly metastatic and invasive ER-ve breast cancer cell line (Tavazoie et al., 2008).

4.11 Epithelial to mesenchymal transition and breast cancer stem cells

An interesting idea that has emerged recently suggests the possibility that cancer cells undergoing EMT acquire stem cell-like characteristics. The breast cancer stem cell (BCSCs) hypothesis contends that breast cancer is derived from a single tumour initiating cell with stem cell-like properties.

BCSCs are characterized as $CD24^{-/low}$ and $CD44^{+}$ cells which are associated with basal subtype breast cancer. It was first reported by Al-Hajj et al., (2003) when they showed that a $CD44^{+}/CD24^{-/low}$ sub-population of breast cancer cells could produce tumours in a xenograft model more effectively. These cells are regarded as the 'metastatic component' of the cancer, particularly in breast neoplasms as they are the only subset of cells with potential to initiate new tumour growth. This was further supported by analysis of genetic profiles of $CD44^{+}$ breast cancer cells which showed enrichment with stem-cell markers and displayed activated TGFβ signalling with lung metastasis and poor clinical outcomes (Sheridan et al., 2006; Shipitsin et al., 2007). Furthermore, it has been reported that metaplastic and claudin-low breast cancers are enriched with markers of EMT and display stem cell characteristics suggesting that cancer cells undergoing EMT exhibit stem cell-like characteristics (Prat et al., 2010). In addition to that, inducing EMT in immortalized human mammary epithelial cells with either TGFβ, SNAIL1 and TWIST confers stem cell characteristics with increased formation of mammospheres in three dimensional culture and ductal outgrowths in xenotransplants (Mani et al., 2008; Morel et al, 2008). Interestingly, BCSCs isolated from primary tumors and normal breast tissue showed an increased expression of the mesenchymal markers TWIST1 and 2, FOXC2, SNAIL1, ZEB2, vimentin and fibronectin while epithelial cells (which are $CD44^{-}/CD24^{+}$) isolated from differentiated carcinoma do not (Mani et al., 2008). Furthermore, hypoxia-induced SNAIL2 expression has also been associated with acquisition of a basal-like breast cancer phenotype with high levels of the stem cell regulatory genes CD133 and BMI1 (Storci et al., 2008). 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 (DiMeo et al., 2009) in a mouse model of breast cancer metastasis to the lung.

It has also been reported that a $CD24^{-/low}/CD44^{+}$ *in vivo* tumour out- growth which is enriched with EMT markers results from CD8 T-cell-mediated immune response to epithelial breast cancer which would develop characteristics of aggressive carcinomas including potent tumourigenicity, ability to re-establish an epithelial tumour, and enhanced resistance to drugs and radiation (Sheridan et al., 2006; Santisteban et al., 2009). Moreover, breast cancer cells disseminated into the circulation and bone marrow are enriched with $CD44^{+}CD24^{-}$ antigen phenotype (Balic et al., 2006)

EMT induction may be a contributory factor to the decreased efficacy of chemotherapy in breast (Cheng et al., 2007), colorectal (Yang et al., 2006) and ovarian cancer (Kajiyama et al., 2006) while introduction of TWIST into breast cancer cells has been shown to induce paclitaxel resistance. In addition, AKT2 expression, which was amplified in breast cancer has also been correlated with acquired paclitaxel resistance (Cheng et al., 2007). Interestingly, acquisition of enhanced EGFR/ERBB2 signalling in ER+ breast cancer with tamoxifen resistance has been suggested to result from the selection of a more stem cell-like phenotype. EGFR expression is seen in stem cells of the normal mammary gland in both mice and humans (Asselin-Labat et al., 2006; Hebbard et al., 2000) whilst ER is predominantly expressed in the more differentiated luminal cells (Hebbard et al., 2000; Shipitsin et al., 2007). Furthermore, the EGFR pathway is also activated in CSCs of DCIS of the breast and 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 (Ginestier et al., 2007). The CSC populations of four ERBB2+ breast cancer cell lines have been shown to express more ERBB2 mRNA and protein in comparison to the non-CSC population. Furthermore, trastuzumab was also shown to reduce mammosphere-forming ability and tumourigenicity on serial xenotransplantation (Magnifico et al., 2009). Interestingly, ERBB2+ tumours that received treatment with lapatinib showed decreased EMT related genes in comparison to $CD24^{low/-}/CD44^{+}$ post treatment tissues from patients that received standard anthracycline-taxane chemotherapy. In addition, the γ secretase inhibitor DAPT or a NOTCH 4 neutralizing antibody significantly reduced mammosphere formation in DCIS. NOTCH pathway antagonism has been reported to enhance the reduction of mammosphere formation in ERBB2 over-expressing cell lines induced by trastuzumab (Magnifico et al, 2009).

Colorectal and lung tumours undergoing EMT display decreased sensitivity to EGFR kinase inhibitors, possibly by the activation of downstream targets PI3K and AKT (Barr et al., 2008). In breast cancer, $CD44^{+}/CD24^{-/low}$ CSCs acquire resistance against the chemotherapeutic agents docetaxel, doxorubicin and cyclophosphamide (Li et al., 2008). Furthermore, a proportion of $CD44^{+}/CD24^{-/low}$ cells increase in breast cancer patients following treatment with these anti-cancer drugs suggesting that breast cancer cells may acquire resistance to both conventional and targeted therapies upon conversion to a mesenchymal-like phenotype. This in turn would suggest that any EMT inducing factors such as TWIST and ERBB2 are crucial players in inducing cancer stem cells.

An analysis of a panel of breast cancer cell lines of luminal, intermediate and basal phenotypes showed a significant increase in the fraction of CSCs ($CD44^{+}/CD24^{low/-}/ESA^{+}$) in basal type breast cancers compared to hormone-sensitive luminal cancers (Fillmore & Kuperwasser, 2008). In addition, the number of CSCs and cell line tumourigenicity in *in vivo* models was correlated positively (Fillmore et al., 2008).

A functionally redundant ER in endocrine-resistant breast cancer might promote a more mesenchymal stem-cell-like phenotype based on the observation that ER negatively regulates the expression of the key EMT transcription factors including SNAIL1 and SNAIL2 (Dhasarathy et al., 2007; Ye et al., 2008). Furthermore, tamoxifen resistant MCF7 cells have been reported to show an enhanced mammosphere formation capacity in comparison to the tamoxifen sensitive cells which suggests an increased CSC fraction (Storci et al., 2008). EMT may facilitate the generation of CSCs with mesenchymal and self-renewal properties necessary for dissemination and initiation of metastasis. (Hollier et al., 2009; Mani et al, 2008). An immunohistochemical analysis of 479 invasive breast carcinomas showed a high expression of the EMT-induced markers vimentin, α-smooth muscle actin, N-cadherin, CDH1, SPARC, laminin and fascin, in comparison to the low expression of E-cadherin in these $CD44^+/CD24^-$ basal-like breast tumours. These tumours have the ability to form distant metastases hence exhibiting a worse prognosis (Perou et al., 2000; Sorlie et al., 2001). In a study on 117 samples of primary invasive breast carcinomas, nuclear staining of the EMT inducing transcription factor FOXC2 showed a significant correlation with $CD44^+CD24^-$ basal-like subtypes (Mani et al., 2007). Another study on 226 blood samples from 39 patients with metastatic breast cancer showed that the majority of the circulating tumour cells (CTCs) exhibited EMT and CSC characteristics (Aktas et al., 2009). CTCs were present in 69 of 226 (31%) blood samples taken from patients with metastatic breast cancer to investigate the expression of TWIST, AKT2, and PI3Kα and ALDH1 which is considered to be a stem cell marker. In the CTC-positive group, 62% were positive for the EMT markers and 69% for ALDH1, while in the CTC-negative group the proportions were 7 and 14%, respectively (Aktas et al., 2009). The CTCs have also been shown to have a reduced expression of epithelial-specific cytokeratins (Pantel et al., 2008). Interestingly, disseminated tumour cells (DTCs) over-expressed TWIST. Assessment of occurrence of bone marrow metastases indicated that TWIST+ cells were present prior to chemotherapy and this was significantly associated with relapse (Watson et al., 2007).

EMT undergoing CTCs have also been shown to resist apoptosis. One study reported that following the induction of EMT by TGFβ in the EpH-4 and nMuMG murine mammary epithelial cell lines, they tended to acquire resistance to ultraviolet light induced apoptosis (Robson et al., 2006). Likewise, down regulation of the expression of LET-7 miRNA in breast cancer cell lines increased their metastatic potential and the resistance to therapy, in association with the acquisition of stem cell characteristics and EMT-associated gene expression profiles (Yu et al., 2007). Furthermore, the factors that can induce a full EMT; TGFβ, WNT, HEDGEHOG, NOTCH, and RAS signaling pathways, are all considered to be involved in the induction and maintenance of stem cell niches (Fuxe et al., 2010). There is however some data showing that TGFβ stimulation of transformed human breast epithelial cells can result in the loss of stem cell-like properties including the ability to form mammospheres (Tang et al., 2007).

5. Endocrine resistance and EMT

It is becoming increasingly apparent that acquired endocrine resistance is a multi-factorial stepwise progression that can be triggered through a number of distinct pathways, that *in vitro*, can be manipulated. Whether it is the actual loss of ER due to transcriptional or translational down-regulation, or functional redundancy of ER (which seems to be the more frequent occurrence *in vivo*), either scenario would have the same end result in terms of

independence from estrogen. It is therefore pertinent to ask what happens to a cell that experiences loss of ER. As described in preceding sections this issue has been addressed by various cell models that have been made endocrine resistant by exposure to antiestrogens or by deprivation of estradiol, but rarely by the direct prevention of ER synthesis.

We have explored this avenue by modifying MCF7 cells by transfection with shRNA generating plasmids targeting the ER mRNA (Al Azmi, 2006; Luqmani et al., 2009; Al Saleh et al., 2011a). As expected, stably transfected cell lines with constitutive reduction of ER (termed pII) exhibit a loss of response to either estradiol or tamoxifen/fulvestrant and hypersensitivity to EGF and IGF1 (Salloum, 2010). There is reduction in the classical ER-regulated markers such as pS2, cathepsin D, PR and PRLR. Like the tumour-derived naturally ER-ve MDAMB231 cell line, these (acquired) endocrine resistant cells show increased motility and ability to invade simulated components of the ECM mimicking the behaviour of aggressive ER-ve/EGFR+ve tumours. Both of these activities as well as cellular proliferation are reduced by various tyrosine kinase inhibitors that are known to block, in particular, EGFR and VEGFR phosphorylation (Al Saleh, 2010) supporting the data mentioned in preceding sections. However, the most striking features of pII cells was initially noted in their morphological appearance (see Fig 3), assuming a more elongated spindly shape and failure to form the compact colonies characteristic of MCF7 cells, with re-arrangement of the actin cytoskeleton giving rise to increased incidence of lamellipodia and microspikes, features closely associated with cellular motility (Parker et al., 2002).

Microarray analysis confirmed that pII cells had assumed a phenotype that is generally seen for mesenchymal cells, with transcriptional loss of genes normally associated with epithelial cells. Lack of colony formation can be explained by loss of E-cadherin and many other factors responsible for normal cell-cell adhesion including catenins, laminin, type IV collagen, desmogleins, desmocollins, occludins, connexion 2b claudins and MUC1. Likewise, archetypical epithelial components such as keratins 8, 18 and 19 and tissue inhibitors of metallo-proteinases are all reduced. On the other hand, we observed an increased expression of mesenchymal markers such as N cadherin, vimentin, fibronectin, integrins β4 and α5, tenascin, SPARC, PLAU, VEGF, CD68, FSP1/S100A4, LCN2 and various metalloproteinases In short, we are seeing all the hallmarks of cells undergoing EMT with acquisition of the phenotype characterising the group of basal-like 'claudin low' tumours such as the triple negative (ER-ve, PR-ve, ERBB2-ve) metaplastic tumours described by Hennessy et al., (2009). A similar conclusion was reached by Gadalla et al., (2005) who observed an EMT-like transition with loss of E-cadherin and reduction in CD24 induced by ER silencing. However, they did not observe the increase in CD44 that we and others have widely reported.

An interesting molecule whose expression was found to be substantially repressed in our pII cells **(**Al Saleh et al., 2011a) is GATA3, a zinc finger transcription factor that plays an important role as a regulator of mammary gland formation and development (Kouros-Mehr et al., 2008) and has been implicated in both EMT and breast cancer metastasis. GATA3 is a positive transcriptional regulator of ER expression whilst simultaneously itself being a target gene for the ER complex. Its expression has been linked to favourable outcome of endocrine therapy (Parikh et al., 2005). Several studies have shown association of GATA3 with ER+ tumours (eg, Mehra et al., 2005). Yan et al., (2010) recently demonstrated that not only was GATA3 expression abolished in ER-ve cell lines but also correlated with E-cadherin. siRNA-induced silencing of GATA3 resulted in fibroblastic-like transformation of MCF7 cells. On the other hand, restoration of GATA3 expression in ER-ve cells led to

renewal of epithelial characteristics as typified by increased levels of E-cadherin and decrease of N-cadherin, vimentin and MMP9 with parallel reduction of tumour forming capacity of MDAMB231 cells injected into xenografted mice. These studies elegantly support the notion that ER regulated events is intimately involved in the same processes that lead to EMT and very crucially, that these events are reversible.

Another significant group of genes variously implicated in EMT that is elevated in pII cells is included in the '24 gene signature' of genes proposed as predictive of invasiveness (Zajchowski et al., 2001): integrin, TIMP-2 and TIMP-3, MT1-MMP, PAI-1, Osteonectin/SPARC, thrombospondin-1, collagen (VI) α1 and collagen (I) α2. pII also display the '9 gene signature' of down-regulated or low expressing genes (E-cadherin, CLDN7, CRB3, KRT8, TACSTD1, IRF6, SPINT2, MAL2 and MARVELD3) that was found by Katz et al., (2011) to be common between their C35 transfected cells and claudin-low tumours. Evidence that the latter represent EMT is now substantial and supported by *in vitro* observations (Prat et al., 2010; Taube et al., 2010).

Substantial reduction in ER expression has been observed in modified MCF7 sub-lines resistant to the mitotic inhibitors paclitaxel and docetaxel and the anthracycline doxorubicin (Iseri et al., 2011). Microarray analysis showed up-regulation of SNAIL2, CDH2, VIM, CLDN1, CLDN11, EGFR, FGFR1, SMAD3 and TGFBR2 and down-regulation of E-cadherin, OCLN, CLDN3, CLDN4, and CLDN7. This data bears remarkable resemblance to the profile for pII cells with the common denominator being loss of ER.

This brings us finally to the group of transcriptional repressors that have been coined as the 'mediators of EMT' and discussed above, so far a relatively smaller group that unify a much larger and diverse array of signalling molecules involved in their regulation. Of the key factors identified in cadherin switching, ZEB1, ZEB2/SIP1 and SNAIL2 (Onder et al, 2008) are all significantly elevated in our endocrine resistant pII cells. These observations lead us to conclude that there is a high degree of synonimity between endocrine resistance and EMT, both effected by functional loss of ER and both resulting in increased propensity for tumour dissemination through the actions of a common set of mediators. The repression of SNAIL by the ER dependent MTA3 (Fujita et al., 2003), a subunit of the Mi-2/NuRD histone deacetylase complex, which could well be regarded, among others, as a guardian of the epithelial phenotype (?) may be worthy of further attention. Interestingly, another family member, MTA1, is described as a potent inhibitor of nuclear ER function through cytoplasmic sequestration of the receptor and this may provide an explanation for resistance in ER+ cells as MTA1 would indirectly reduce the levels of MTA3 thereby relieving SNAIL repression.

There have also been intriguing suggestions regarding the origin of the mesenchymal-like cells, with the attractive view of these as a possibly slow growing pre-existing CSC sub-population within the tumour (Lim et al., 2010; May et al., 2011). In such a scenario there is no induced EMT as such, but a gradual emergence of a group of cells already bearing these properties, to become the dominant group. Similar ideas have often been suggested to explain the re-emergence of 'drug–regressed' tumours as an expansion of a pre-existing intrinsically resistant cell population once the sensitive cells have been eliminated. However, attractive as this may be, in the alternative scheme elaborated by May et al., (2011) there would be a reversion of such 'MaSCs' back to an epithelial phenotype at the site of metastatic growth in a reverse MET transition, which raises the question that If cells can undergo MET then why not EMT, and there is no necessity to postulate the existence of *a priori* mesenchymal cells. Moreover, the *in vitro* data demonstrates quite clearly that an

actual EMT transition does take place as the initial population of cells is relatively homogeneous with respect to being epithelial in nature. Most if not all of the cells in culture can simultaneously undergo EMT whereas it is very likely that only a very small fraction of cells in a tumour acquire all of the characteristics enabling them to undergo a full transition, which may be why such mesenchymal-like cells have not been routinely reported by pathologists (Thompson et al., 2008).

6. Summary

The persistent problem of drug resistance and in particular the therapeutic failure of endocrine agents presents serious therapeutic issues especially in view of the success of this type of intervention in a significantly large proportion of women with breast cancer. Many studies have focused on elucidating the mechanisms responsible for *de novo* and *acquired* independence from estrogen. Consensus of opinion favours the view that signaling pathways mediated through a variety of peptide growth factors is largely responsible for the aggressive proliferation of tumours that have ceased to depend upon the ER, although no single unifying or even major factor has been identified. Somewhat in parallel, the last few years have witnessed an increasing number of reports describing the relatively recently recognized phenomenon of EMT, highlighting its similarity to the events leading to tumour invasion and vascular dissemination. Many of the key mediators of EMT particularly the transcriptional repression of E-cadherin by SNAIL appear to be critical steps in tumour progression. The association of mesenchymal-like features such as cadherin switching, loss of adhesion proteins and CD24, increased vimentin and fibronectin, with ER-ve tumours, have been sporadically, almost anecdotally reported in the literature over the last decade or more. We have now found evidence to show that the acquisition of endocrine independence, due to induced ER loss, by previously ER+ breast cancer cells, is accompanied by all the hallmark features of EMT. Although it is still far from clear whether the two processes are occurring side by side or whether either is causal of the other, it seems reasonable to conclude that loss of ER can directly trigger EMT. It remains to be seen whether restoration of ER in the trans-differentiated cells can reverse EMT and allow the cells to regain estrogen dependence.

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8. References

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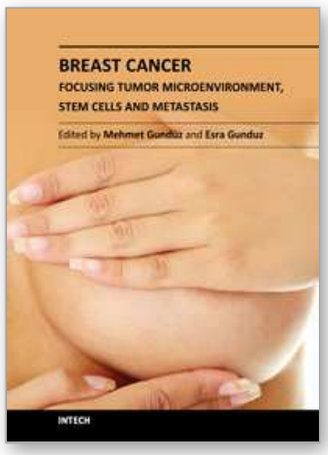
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Cancer is the leading cause of death in most countries and its consequences result in huge economic, social and psychological burden. Breast cancer is the most frequently diagnosed cancer type and the leading cause of cancer death among females. In this book, we discussed characteristics of breast cancer cell, role of microenvironment, stem cells and metastasis for this deadly cancer. We hope that this book will contribute to the development of novel diagnostic as well as therapeutic approaches.

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