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New Molecular Aspects and their Potential as Targets

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Foreword

Anti-hormonal drugs are a mainstay in the treatment of breast cancer and have impact in both primary and metastatic disease. A pervading problem, however, is therapeutic resistance which can either prevent initial response to anti-hormonal measures or be acquired during therapy. The molecular mechanisms underlying resistance are increasingly understood and this knowledge is leading to novel therapeutic approaches to more effective treat or delay the appearance of endocrine resistance.

The goal of the recent 3rd Tenovus/AstraZeneca Workshop in Cardiff was to ask "what's new" in endocrine resistance in breast cancer and assess the progress that is being made towards its treatment. This workshop comprised various talks from international experts and round-table discussion, culminating in the 10 articles within this book. The chapters within describe several key aspects of endocrine resistance and include the use of RNA interference screens to identify modifiers of sensitivity to hormonal therapy, the importance of coactivator and corepressor proteins to endocrine response and resistance and elucidating mechanisms of oestrogen receptor re-expression in ER-negative tumours. Furthermore, the intriguing concepts that antihormones themselves may promote adverse cellular features which sustain both an invasive, endocrine-resistant state and modify cellular interactions with the surrounding stroma together with the potential role of cancer stem cells in resistance are presented. Finally, novel therapeutic strategies in breast cancer such as heat shock protein inhibitors and pharmacological targeting of Src kinase are discussed together with a review of current treatment strategies that seek to combine signal transduction inhibitors with endocrine therapies.

The articles here add to our knowledge of molecular events that underlie hormonal resistance and strategies through which resistance may be circumvented. Continued success in this area will without doubt benefit current and future breast cancer patients and reduce the impact this disease has on its millions of suffers worldwide.

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Chapter 1
Experimental Endocrine Resistance: Concepts and Strategies

Robert I. Nicholson, Iain R. Hutcheson, Stephen Hiscox, Kathy M. Taylor and Julia M.W. Gee

Contents

Abstract Intensive research has been undertaken in order to understand the mechanisms that underlie the phenomenon of endocrine resistance with a view to identifying biomarkers predictive of antihormonal response and revealing potential therapeutic targets through which resistance may be delayed or prevented. Through these studies it is increasingly apparent that the tumour cells' ability to harness a variety of growth factor signalling pathways to drive proliferation in the presence of endocrine agents plays a major role in promoting a resistant phenotype. Importantly,

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the inappropriate activation of growth factor signalling cascades is now regarded to play a significant role in the promotion of antihormone failure in breast cancer cells and it is becoming clear that anti-hormones themselves can promote the expression of a number of growth factors and their receptors in the drug-responsive phase, which subsequently play key roles in the regulation of tumour growth during the drug-resistant phase. The importance of growth factor signalling in endocrine resistance is further revealed in that a high degree of interaction exists between intracellular signalling pathways downstream of the oestrogen receptor and growth factor receptors, further contributing to the development of an endocrine insensitive state. It is likely that our increasing knowledge in this area will ultimately lead to the development of inhibitory strategies targeted towards suppressing the activity of growth factor signalling pathways and their interplay with the oestrogen receptor to improve the outlook for breast cancer patients.

Keywords Oestrogen Receptor · Growth factor receptors · Cross-talk · Endocrine resistance

1.1 Introduction

It is self evident that the emergence of either de novo or acquired endocrine resistance in breast cancer cells must result from a subversion of the growth inhibitory activity of anti-hormonal drugs. Experimentally, this can take the form of radical cellular changes which drive mitogenic and survival signalling in breast cancer cells independently of oestrogen receptors (ER) and which can therefore operate in either ER positive or negative cells. Alternatively, however, it can also occur via more subtle changes in cellular pathways which facilitate ER signalling in the presence of anti-oestrogenic drugs or in a reduced oestrogen environment, leading to tumour cell growth (Nicholson et al., 2007).

The purpose of this chapter is to define several of the experimental mechanisms that are believed to underpin endocrine resistance in breast cancer cells and thereby provide a framework against which the more recent findings described in subsequent chapters can be viewed. Additionally, an attempt will be made to establish the therapeutic principals which have originated from the experimental studies and which are now being considered clinically as a means of more effectively treating or delaying the appearance of endocrine resistance. The chapter will primarily focus on the molecular cross-talk that exists between ERα and growth factor signalling pathways, an area that is thought to be a major contributor to the development of resistance to anti-hormonal treatments and which is rich in novel therapeutic approaches. For simplicities sake, the more recently identified actions of oestrogens on ERβ are not widely discussed in this chapter since they are considered by most not to be a major stimulus to growth in breast cancer cells. Indeed, their cellular levels fall as breast cancers progress and they may display growth retarding activity through their heterodimerisation with ERα (Hall and McDonnell, 1999).

1.2 Oestrogen Action and its Coupling to Growth Factor Signalling

The capacity of signalling molecules to induce cell growth has its roots in engaging the cell cycle and this is frequently coupled to the promotion of signals which enable cell survival. In many cell types this is achieved through the actions of locally and distally produced growth factors which act through cell surface receptors to drive established growth and survival pathways. In oestrogen dependent tissues, however, oestrogen receptors, alongside growth factors, are also key players in such events where they act as:

(i) Nuclear transcription factors able to directly engage the promoters of oestrogen regulated genes containing oestrogen response elements (EREs);
(ii) Binding proteins able to associate with other nuclear transcription factors to modulate their activity;
(iii) Cell membrane linked proteins able to facilitate growth signalling by enhancing the actions of several signal transduction pathways.

In each instance, the cellular actions of ERα (subsequently referred to as ER) are productively linked to growth factor signalling cascades to orchestrate growth and survival signalling. Interference with such ER signalling using anti-hormonal drugs has both anti-ER and anti-growth factor actions, while aberrant growth factor signalling can sustain breast cancer cell growth in the presence of anti-oestrogens and in an oestrogen withdrawn environment (Nicholson and Gee, 2000).

1.2.1 Interactions of the ER with EREs

The "classical" pathway associated with the cellular actions of oestrogen receptors (ERα) involves them functioning as nuclear transcription factors able to regulate the expression of genes containing oestrogen response elements (ERE) within their promoters. Genes possessing such EREs include a number of growth promoting growth factors (transforming growth factor alpha (TGFα), vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF), O'Lone et al., 2004), together with several survival factors, such as bcl-2. Importantly, ERs have two major activator functions, AF-1 and AF-2 which often act together to maximise transcriptional events. In some instances, however, substantial ER-regulated gene expression can be achieved through either AF-1 or AF-2 and this can be both promoter and tissue specific (O'Malley, 2005). Such differential responses are of great potential significance to ER action since AF-1 can be activated by oestrogen-independent mechanisms (often termed ligand-independent), while AF-2 responses have a more strict dependence on the presence of ER ligands (ligand-dependent). All aspects of ER signalling, therefore, are not wholly reliant on the presence of oestrogens and a degree of ER activation can be achieved through the activation of AF-1 by

alterative mechanisms. In this respect, it is noteworthy that ER phosphorylation is a critical event in ER activation and several intracellular kinases have been implicated in this process (Bunone et al., 1996; Joel et al., 1998; Kato et al., 1995; Lannigan, 2003). Indeed, ER phosphorylation of key sites within the AF-1 domain is thought necessary for ligand-independent ER transcription and may be achieved, for example, through growth factor induced activation of p42/p44 mitogen-activated protein kinase (MAPK) and AKT, possibly in a c-src dependent manner.

In addition to the ER, transcriptional activity arising from ER activation is modulated through multiple co-regulatory proteins which complex with the ER (Osborne and Schiff, 2005). These proteins can have histone-acetyltransferase (HAT) activity, required for chromatin decondensation (NCoA[nuclear receptor co-activator]1 or SRC1; NCoA2 or TIF2; NCoA3 or AIB1) or can recruit histone-deacetylase complexes (HDAC) to reverse this process (NCoR[nuclear receptor co-repressor]1 & 2) and are termed co-activators and co-repressors respectively. Such opposing actions, for example, can lead to the enhancement of AF-2 activity following the oestrogen-induced recruitment of co-activators or a reduction in AF-2 activity when co-repressors are present. Significantly, phosphorylation of co-activator proteins, alongside the ER, is critical for the activation of ER directed gene transcription and once again can be promoted by growth factor driven protein kinases, including MAPK and AKT (Shou et al., 2004). Clearly, a synergy exists between ER and growth factor signalling, where appropriate cellular growth and gene expression undoubtedly requires the measured activation of each.

1.2.2 Interactions of ER with Other Nuclear Transcription Factors

As stated above, ERs also act as binding proteins able to associate with other nuclear transcription factors to modulate their activity and this is considered to be contributory to oestrogen associated growth responses. They are able to achieve this through direct protein:protein interactions enabling them to effect other DNA regulator sequences and hence the expression of genes which do not necessarily contain EREs in their promoters. Such “non-classical” actions are known to impact on Jun/Fos activator protein 1 (AP-1) and specificity protein-1 (SP-1) sites in DNA and to influence the expression of growth factor receptors (IGF-1R), nuclear transcription factors (myc) and cell cycle regulatory proteins (cyclin D1), key components of proliferation and survival signalling (O’Lone et al., 2004; Shupnik et al., 2004).

1.2.3 Interactions of ER with Other Signalling Elements at the Cell Membrane

The final and most recent mechanism believed to be contributory to the growth promoting actions of ERs involves what has become known as “non-genomic” signalling whereby ERs become associated with the cell membrane through

cytoplasmic membrane anchors (Jacob et al., 2006) and are able to rapidly respond to oestrogens (Losel et al., 2003). Indeed such membrane initiated steroid signalling (MISS) occurs within minutes of oestrogen exposure and does not initially require transcriptional events. The importance of MISS is that it has been reported to interact with and/or activate several growth factor receptors (EGFR (Razandi et al., 2003), HER2 (Chung et al., 2002), IGF-1R (Kahlert et al., 2000)), signalling enzymes (Wong et al., 2002), adaptor proteins (Shc (Song et al., 2002)) and intracellular kinases (MAPK (Zhang et al., 2002), PI3K/AKT (Sun et al., 2001), c-src (Migliaccio et al., 2002)) that are intimately involved in cell growth and survival mechanisms and which are able, as described above, to drive a "feed forward" circuit of ER-induced transcriptional events involving both "classical" and "non-classical" nuclear steroid signalling pathways (Bedard et al., 2008). Indeed, in a recent model of steroid hormone signalling, O'Malley (2005) described membrane ER actions as an important means of activating several protein kinases which aid the actions of co-activators essential to ER mediated transcriptional events. Membrane associated ER effects may, therefore, be viewed as initiating ER signalling within responsive cells and promoting intracellular signalling cascades which augment the later nuclear actions of ERs. As such, membrane and nuclear ER signalling appear complementary in the induction of growth and survival mechanisms.

Interestingly, ER directed "non-genomic" responses (like nuclear ER responses) also rely on co-regulatory proteins that may be influenced by various signal transduction elements and one of these is believed to be over-expressed in some breast cancers. This protein (MNAR [modulator of non-genomic activity of ER]/PELP 1 [proline-, glutamic acid-, and leucine rich protein 1]) enhances ER directed nuclear and membrane signalling (Vadlamudi et al., 2001; Wong et al., 2002) and contains c-src activating domains which directly activate ERK/MAPK when in association with ER (Barletta et al., 2004). Controversially, other membrane associated proteins, such as GPR30, appear also to bind oestrogens with low affinity and instigate some signalling via EGFR transactivation. In breast cancer cells, however, GPR30 knockdown does not effect oestrogen signalling (Pedram et al., 2006) and their contribution of endocrine response and resistance is not considered further.

1.3 Anti-Hormone Action

By definition, all oestrogen targeted endocrine therapies have the common goal of depriving breast cancer cells of their required oestrogenic stimulation and reducing its productive cross-talk with interactive growth factor signalling elements to promote cell cycle arrest and induce cell loss. They achieve this either by lowering circulating oestrogen levels (using LH-RH analogues and aromatase inhibitors), or by antagonising their cellular actions by competition for ERs (using anti-oestrogenic drugs) (Nicholson and Johnston, 2005). Although such distortions of oestrogen mediated signalling have been described in terms of both "genomic" and "non-genomic" responses, characteristically, these actions are not simple and in some

instances vary between procedures associated with oestrogen withdrawal from ERs and those which involve ER occupancy by anti-oestrogenic drugs. Because of this, the different modalities used to treat breast cancer patients will be dealt with separately.

1.3.1 Selective Oestrogen Receptor Modulators (SERMs)

Early pharmacological studies using drugs such as tamoxifen, toremifene and raloxifene quickly established that they possessed mixed agonistic and antagonistic activity and that this varied considerably within oestrogen target tissues such as the breast, uterus and bone. In human breast cancer, SERMs are believed to be predominantly antagonists, although their limited agonistic activity has been linked to the phenomenon of tumour flare (Reddel and Sutherland, 1984) and may be exaggerated by excessive growth factor signalling to form a resistance mechanism (see below). Importantly, while SERMs are generally considered to be effective inhibitors of oestrogen-dependent AF-2 activity, they are considerably less effective on ligand-independent AF-1-mediated transcriptional responses (Tzukerman et al., 1994). The relative expression of AF-1 and AF-2 dependent genes within varying tissues (and potentially within breast cancer samples) may thus go some way to rationalising the mixed agonistic and antagonistic properties of SERMs, as may varying tissue availability of co-activators and co-repressors (Jordan and O'Malley, 2007).

1.3.2 Pure Anti-Oestrogens

The recognition that the agonistic activity of SERMs might limit their anti-tumour efficacy led to the development of a class of anti-oestrogenic drugs which, in many experimental settings, completely lack oestrogen-like properties. These drugs, which are epitomised by the oestradiol analogue fulvestrant (faslodex®), possess a long alkylsulphinyl side chain which disrupts cytoplasmic to nucleus ER translocation, ER dimerisation and binding to DNA and therefore severely limits both AF-1 and AF-2 responses (Osborne et al., 2004). Moreover, they also promote rapid degradation of ER following increased ER ubiquitinisation (Carlson, 2005), a novel property that must limit the capacity of ER to associate with other transcription factors and localise to the cell membrane. Because of their capacity to promote ER loss they are frequently termed selective oestrogen receptor down-regulators (SERDs). As testimony to the improved inhibitory actions of pure anti-oestrogens on ER, they are often considerably more effective than SERMs at promoting anti-tumour effects in several in vitro and in vivo models of human breast cancer (Carlson, 2005) and they often are active in tumours that have acquired a resistance to SERMs and oestrogen deprivation (see below).

1.3.3 Oestrogen Deprivation

Fundamentally, oestrogen deprivation of breast cancer cells differs from their treatment with anti-oestrogens since the latter involves ER occupancy while the former does not. This is not just esoteric since unoccupied ER is largely an inactive molecule bound to heat-shock proteins, whilst on ligand binding such proteins are released and the ER may, in the case of SERMs, be subject to varying degrees of activation. This not only applies to nuclear ERs, but also to membrane localised ER whose activation is believed to be highly dependent on ligands (Levin and Pietras, 2008). Theoretically, therefore, oestrogen deprivation should be a highly effective treatment for oestrogen dependent breast cancer, although it is currently unclear how complete oestrogen loss needs to be to maximise such responses and whether ER occupancy by other ligands (e.g. phyto-oestrogens or androgens) can compensate for the decreased availability of more classical oestrogens.

1.4 Mechanisms Associated with Endocrine Resistance

It is evident from the above that a complex bi-directional cross-talk exists in endocrine responsive breast cancer cells between ER and growth factors to sustain growth and survival signalling and that anti-hormonal drugs are able to differentially effect elements of such signalling to promote growth inhibition (Nicholson and Gee, 2000; Nicholson et al., 2007). Clinically, however, while disease control by anti-hormonal drugs offers disease free and survival benefits, they do not work in all patients and responses in others are at best transitory (Bedard et al., 2008). Experimentally, data is described which indicates that such resistance can occur in ER dependent and ER independent forms through both genetic (De Laurentiis et al., 2005) and drug-induced (Gee et al., 2006) alterations in growth factor signalling within breast cancer cells and more detailed descriptions of its individual components are described below.

1.4.1 ER Dependent Mechanisms: Growth Factor Pathway Switching

1.4.1.1 Anti-Oestrogens

Multiple studies have now shown that enhanced growth factor signalling can support elements of ER activity in the presence of anti-oestrogenic drugs and that such responses can be self perpetuating since the resultant increased nuclear and membrane ER activity can further reinforce growth factor activity through the induction and activation of growth factor signalling elements respectively (Massarweh and Schiff, 2007). Considerable work has been performed in this area using MCF-7 cells genetically engineered to over-express EGFR/HER2, where increased growth factor signalling augments both genomic and non-genomic ER actions in the presence

of tamoxifen, leading to de novo tamoxifen resistance (Benz et al., 1992; Shou et al., 2004). In this model, increased growth factor induced protein kinases, for example, are deemed responsible for the phosphorylation of the AIB1 co-activator which allows some nuclear ER signalling in the presence of tamoxifen (agonism) (Font de Mora and Brown, 2000; Osborne et al., 2003), while high levels of membrane associated EGFR/HER2 facilitate the non-genomic oestrogen-like activities of the tamoxifen/ER complex (Chung et al., 2002; Shou et al., 2004). These data are paralleled clinically, in that the co-expression of HER2 and AIB1 confers a poor outlook to patients receiving adjuvant tamoxifen therapy (Osborne et al., 2003). Significantly, other studies have suggested that additional co-activators, such as NCoA-1/SRC-1, may also promote a resistant phenotype in tumours over-expressing HER2 (Fleming et al., 2004). Clearly, increased ER/growth factor signalling at tumour cell membranes, coupled to the activation of nuclear ER components appears to radically alter the pharmacological properties of SERMs in favour of agonistic and growth promoting activity. Critically, this new and intricate cross-talk is sensitive to fulvestrant (Nicholson et al., 1995; Dowsett et al., 2005) and gefitinib (McClelland et al., 2001, Knowlden et al., 2003) and is much less evident in non-transfected cells which express only modest levels of EGFR/HER2, where the antagonistic properties of tamoxifen predominate.

As mentioned above, a feature of breast cancer cells expressing high levels of growth factor receptors is that they often show evidence of an increased activity of several downstream protein kinases involved in signal transduction. These include MAPK (Knowlden et al., 2003), AKT (Jordan et al., 2004; Beeram et al., 2007), PAK-1 (Holm et al., 2006, Rayala et al., 2006), PKA (Michalides et al., 2004) and c-src (Chu et al., 2007; Hiscox et al., 2006c), which are able to directly or indirectly promote the activation of ER signalling components. They may also show elevated DNA binding activity of transcription factors, such as AP-1 which, depending on the balance of nuclear co-regulators, can use tamoxifen ER complexes to aid signalling through alternative response elements (see Section 1.2.2). Since cells genetically engineered to express highly activated forms of these signalling elements often show de novo resistance to tamoxifen (PAK1, Rayala et al., 2006; AKT, Clark et al., 2002, Beeram et al., 2007;Yoo et al., 2008, MAPK, Donovan et al., 2001), while their inhibition may restore response in acquired resistance models (Knowlden et al., 2003), they clearly play a central role in the development of endocrine resistance and may, in some instances, directly substitute for the over-expression of growth factor receptors. Certainly, elevated activity of MAPK (Gee et al., 2001), AKT (Kirkegaard et al., 2005) and AP-1 (Johnston et al., 1999) in clinical breast cancer samples has been linked to anti-oestrogen resistance.

In addition to a role of EGFR/HER2 in de novo tamoxifen resistance, these growth factor receptors also play a critical part in the development of acquired resistance to tamoxifen in MCF-7 cells (Nicholson et al., 2007). This arises because oestrogens, in addition to inducing several growth factor signalling elements (see Section 1.2.1), also act to repress the expression of others, including EGFR/HER2 (see Gee et al., 2006). Consequently, blockade of ER signalling upregulates EGFR/HER2 expression in a time dependent fashion reaching after 3

months treatment in vitro approximately a 40-fold rise in their membrane expression levels (Knowlden et al., 2003). These dramatic changes are accompanied by an increased formation and activation of EGFR/HER2 heterodimers, which once again serve to drive ER-dependent tamoxifen-resistant proliferation and survival through recruitment and activation of MAPK (Knowlden et al., 2003), AKT (Jordan et al., 2004) and c-src (Hiscox et al., 2006c). In our own studies, phosphorylation of serine 118 within the ER by MAPK appears of key importance, since it allows the recruitment of several co-activators (e.g. p68 RNA helicase) to the tamoxifen ER complex (Britton et al., 2006). Concurrent reporter gene construct studies in tamoxifen resistant cells indicate that EGFR/MAPK-promoted ER/AF-1 phosphorylation enhances the agonistic activity of the tamoxifen/ER complex and re-instigates the expression of several ERE-containing genes. Significantly, this reactivation of ER was found to be associated with the increased production of key ligands that promote EGFR, HER2 and IGF-1R signalling, including transforming growth factor β, amphiregulin, epiregulin and IGF-II, with chromatin immunoprecipitation (ChIP) assays demonstrating that ER is bound to a consensus ERE within the amphiregulin promoter in tamoxifen resistant MCF-7 cells. Critically, neutralising antibody studies against several EGF-like ligands established that ampiregulin is indeed the essential element driving the elevated EGFR/HER2 signalling in these cells (Britton et al., 2006). Although the temporal sequence of these events remains to be established during the development of acquired resistance to tamoxifen, we have postulated that EGFR/HER2/MAPK/ER driven increases in the expression of ampiregulin may serve to establish a self-propagating autocrine signalling loop allowing the emergence and maintenance of efficient EGFR-promoted resistant growth. An additional feature of this loop involves the increased IGF-II production noted above, which facilitates a Src-dependent cross-talk between the IGF-1R and the EGFR (Knowlden et al., 2005). In this model, increased ER driven IGF-II production results in increased IGF-1R promoted Src phosphorylation which then phosphorylates tyrosine 845 on the EGFR to enhance the kinase activity of the EGFR. The activation of this phosphorylation site on the EGFR is necessary for them to respond to EGF-like ligands (Biscardi et al., 1999). Clearly, retained nuclear ER signalling in tamoxifen resistant cells offers a considerable boost to the activation of growth factor signalling elements and is entirely complimentary to an increased redistribution of ER to extranuclear sites in tamoxifen resistance (Fan et al., 2007), where it produces parallel productive interactions with membrane associated EGFR/HER2/IGF-1R leading to the further activation of several signal transduction cascades (Fan et al., 2007; Massarweh and Schiff, 2007). Significantly, c-src appears central to the relocation of ER to the tumour cell membranes as the process is reversed by a src kinase inhibitor (Fan et al., 2007).

Interestingly, although the mechanisms which lead to the induction of EGFR and HER2 in endocrine resistant cells vary at the transcriptional level, they are reported to involve a negative regulatory element within the first intron of their genes (Wilson and Chrysogelos, 2002; Newman et al., 2000). Additionally, however, Hurtado et al., (2008) have implicated the Paired Box gene 2 product (Pax2), in a novel role, as a critical mediator of ER repression of HER2 by oestrogen which paradoxically is

shared by tamoxifen. Critically, however, the capacity of Pax2 to repress HER2 in tamoxifen treated cells is reversed by AIB-1 which competes out Pax2 binding to a HER2 cis-regulatory element, with now AIB-1 driving increased HER2 expression. These data suggest that Pax2 functions as a repressive protein which competes with an activating protein for the regulation of the HER2 gene. As such, either a decrease in Pax2 expression or an increase in AIB-1 levels would overcome the initial repressive effects of tamoxifen on HER2 transcription. Importantly, in our tamoxifen resistant cells although the former appears to predominate, Hurtado et al., (2008) demonstrated that this allows effectively allows more AIB-1 to associate with the HER2 cis-regulatory element to drive increased HER2 expression. Clinically, they also observed that increased Pax2 was associated with lower HER2 expression and with improved survival.

In a recent study, Soni et al. (2008) have demonstrated that tamoxifen resistant MCF-7 cells in vitro also over-express the focal adhesion docking protein encoded by the breast cancer anti-oestrogen resistance-1 (BCAR-1, also known as p130cas) gene. BCAR-1, first identified by Dorssers et al. (1993) using a functional assay to detect genes involved in oestrogen-independent growth of breast cancer cells, has several important cellular functions, including an ability to aid membrane ER signalling (Cabodi et al., 2004), together with a capacity to relocate the guanine nucleotide exchange factor (GEF) BCAR-3/AND-34 to the cell membrane where it activates numerous small GTPases (Cai et al., 2003). Critically, Soni et al. (2008) demonstrated that blocking the activity of BCAR-1 in tamoxifen resistant cells reduced EGFR levels and attenuated EGFR signalling onto ERK and PI3K/AKT, leading to an inhibition of cell proliferation and increased apoptosis i.e. re-sensitises the cells to the growth inhibitory actions of tamoxifen. Evidently, BCAR-1 is an essential element in regulating growth factor driven signalling in this model of tamoxifen resistance, an observation concordant with the report of its increased expression in human breast cancers where patients have a reduced overall survival and intrinsic resistance to tamoxifen (van der Flier et al., 2000). BCAR-1 also docks with c-src kinase leading to the phosphorylation and activation of both src and BCAR-1 (Soni et al., 2008). BCAR1/C-src kinase complexes, therefore, appears to play a dual role in promoting EGFR signalling, firstly by directly phosphorylating tyrosine 845 in the EGFR and secondly by enabling growth factor downstream signalling through the activation of several small GTPases.

Significantly, over-expression of BCAR3 in ZR-75-1 breast cancer cells also readily confers anti-oestrogen resistance and detailed evaluation of its downstream signalling components has shown that it activates several Rho family GTPases, including Cdc42 and Rac leading to increased kinase activity of the Cdk42/Rac-responsive serine/threonine kinase PAK-1 and cyclin D1 promoter activation (Cai et al., 2003). In this study, Cai and his colleagues also showed an increased association of BCAR3 and BCAR1 in 578-T cells, an oestrogen independent cell line, and demonstrated that loss of anti-oestrogen response in ZR-75-1 cells was recapitulated by transfection of a constitutively active form of Rac1, supporting a critical role for BCAR1, BCAR3 and Rac1 in anti-hormone resistance.

Finally, over-expression of several growth factors has been show to promote tamoxifen resistance (and/or oestrogen independence) in breast cancer cells in vitro and in vivo. These notably include PC cell-derived growth factor (Tangkeangsirisin et al., 2004), also known as progranulin, vascular endothelial growth factor (Guo et al., 2003), which stimulates breast cancer cell proliferation in vitro, together with angiogenic responses in vivo, keratinocyte growth factor (Chang et al., 2006), whose capacity to override the inhibitor actions of tamoxifen in endocrine responsive MCF-7 cells is reversed by silencing of the keratinocyte growth factor receptor (Rotolo et al., 2008) and heregulins (Tang et al., 1996), which promote the formation of HER3/HER2 heterodimers and strongly promote growth and survival pathways through the activation of MAPK and Akt (Hutcheson et al., 2007). Interestingly, Folgiero et al. (2008) have recently shown that $\alpha 6\beta 4$ integrin is also capable of inducing HER3 in breast cancer cells to maintain the PI3K/Akt survival pathway and tamoxifen resistance, while Liu et al. (2007) have shown that HER3 silencing abrogates HER2-mediated tamoxifen resistance via the inactivation of the PI3K/Akt pathway. Taken together with the parallel identification of colony-stimulating growth factor, fibroblast growth factor 17, platelet derived growth factor receptor α and β, Akt1 and Akt2 as signalling molecules able to promote resistance following retroviral insertion mutagenesis (Meijer et al., 2006; van Agthoven et al., 2008), clearly indicate that there are many potential ways to achieve breast cancer growth in the presence of anti-hormonal drugs. The likely common denominator, however, being, the recruitment and activation of signalling transduction cascades which drive growth and survival signalling.

1.4.1.2 Oestrogen Deprivation

An important distinction between the cellular actions of anti-oestrogenic drugs and oestrogen deprivation is that the latter invariably promotes substantial increases in ER levels which appear particularly sensitive to altered growth factor signalling. This has been described in several breast cancer models employing oestrogen deprivation and can lead to adaptive hypersensitivity to oestrogens and resistant growth (see review by Nicholson et al., 2004). Once again, both membrane-initiated steroid signalling (MISS) and nuclear initiated steroid signalling (NISS) have been implicated in this form of resistance, with increased signalling through MAPK and AKT being provided by increased levels of HER2 and IGF-1R.

Significantly, the oestrogen deprived model used by Richard Santen's group is highly dependent on the increased levels of membrane associated ER being activated by minute levels of oestrogens (10^{-13}M), leading to the rapid growth factor dependent activation of the Ras/Raf/Mek/MAPK and PI3K/AKT signalling cascades which then promote increased nuclear signalling events at the level of cell cycle regulators, such as E2F1 (Yue et al., 2007). Provocatively, however, in another model of oestrogen hypersensitivity, greater emphasis is placed on the capacity of ER and growth factor directed pathways to converge on the regulation of nuclear ER activity which is dependent upon MAPK, p90RSK and AKT (Martin et al., 2005). Despite

these differences, cross-talk between ER and growth factor signalling elements once again underpin the resistant states since the cells remain sensitive to fulvestrant and appropriate signal transduction inhibitors. Interestingly, studies from our own group have produced a third model of resistance to oestrogen deprivation which does not gain adaptive hypersensitivity (Staka et al., 2005). Although this model, unlike those described above, is derived from breast cancer cells cultured in a reduced oestrogen and growth factor environment, shows no evidence of using EGFR/HER2 and IGF-1R signalling, ER and AKT remain critical to the growth of the cells and productive cross-talk between these elements is suggested by inhibitor studies.

1.4.1.3 Anti-Hormone Induced Changes in Growth Factor Signalling

Recently Gee et al. (2006) has evaluated in some detail the capacity of anti-oestrogens to induce gene expression during the early phase of their inhibitory response and has concluded that multiple genes, alongside EGFR/HER2, may attenuate growth inhibition leading to anti-hormone resistance, including NFkB, Bag1, 14-3-3, and tyrosine kinases, such as Lyn (Gee et al., 2006; see also Chapter 4). Interestingly, additional induced genes appear to confer other adverse features to the breast cancer cells in an appropriate cellular environment, with CD59 facilitating evasion of immune surveillance and RhoE, αcatenin and c-src promoting a more invasive phenotype when intercellular contacts are compromised. These data may go some way to explain the emerging relationship between the development of resistance and the gain of a more aggressive breast cancer phenotype (Hiscox et al., 2006c; also see Chapter 8).

1.4.2 ER Independent Endocrine Resistance

Based on the above, exaggerated ER/growth factor cross-talk can play a very dominant role in the development of several experimental forms of endocrine resistance. However, there is also experimentally derived data demonstrating that when more extreme, aberrant growth factor signalling can drive tumour cell growth in a manner dislocated from steroid hormone receptors. As such several mechanisms may contribute to ER independent signalling, including genetic or phenotypic changes that alter the expression of key genes effecting growth factor signalling. Certainly, the original study by Dorrsers and colleagues (Dorssers et al., 1993, van Agthoven et al., 1998) identified BCAR-1 and BCAR-3 as genes that were able to support resistant growth in an ER-independent manner, presumably by maximising the efficiency of growth factor signalling. This concept is supported by a more recent study by Riggins et al. (2006) who described the capacity of BCAR-1 to dock with c-src, an interaction which led to the phosphorylation and activation of both proteins and an activation of the EGFR (via phosphorylation of tyrosine 845 on the EGFR) in manner that did not require ER signalling. Indeed, in that study they also described a

src-dependent activation of signal transducer and activation of transcription (STAT) 5b which did not require ER signalling and was thought to be involved in the development of the endocrine resistant state. Similarly, Iorns et al. (2008) have shown that CDK10 silencing in an RNAi screen of breast cancer cells increases ETS2-driven transcription of c-RAF, resulting in MAPK pathway activation and loss of tumour cell reliance upon oestrogen receptor signalling (see Chapter 9). Such data are consistent with ER being merely supportive of the dominant growth factor pathway in anti-hormone-resistant cells, rather than always being essential for it.

As an alternative to the above, ER-independent breast cancer growth can also be readily achieved by providing hormone sensitive or endocrine resistant cells with exogenous growth factors, circumventing the need for ER driven growth factor production (or making the consequences of it redundant). Several classes of growth factor appear to be able to promote this effect, including heregulins which, although only sparing expressed in MCF-7 cells and its resistant variants, are able drive sustained growth factor receptor driven downstream kinase activity and negate growth inhibitory effects of ER blockade (Tang et al., 1996). In this instance, the primary mechanism of growth response to heregulins is mediated through their binding HER3 and promotion of its heterodimerisation with both EGFR and HER2 (Hutcheson et al., 2007). Failure to respond to anti-hormonal drugs may thus be affected by growth factors originating from multiple paracrine and endocrine sources (see Chapter 5).

As a caveat to such externally mediated responses on breast cancer cells, it is now evident that this may also arise indirectly through the capacity of tumour cells to express growth factors for receptors which they do not themselves possess, yet which are present on surrounding tissue elements. Thus, for example, while several of our breast cancer models produce vascular endothelial growth factor (VEGF) they lack the VEGFR which is present on endothelial cells (see Chapter 8). A capacity of breast cancer cells to promote angiogenic responses may thus aid tumour growth independent of ER signalling and thereby potentially subvert tumour derived endocrine response mechanisms. Certainly, tumour cells promoting strong angiogenic responses in vivo have been suggested to show diminished responses to anti-hormonal drugs (Manders et al., 2003).

The final ER-independent mechanism associated with endocrine resistance is ER loss and several experimental studies have suggested that this may be a by-product of the long-term activation of growth factor signalling pathways. Certainly, an inverse relationship between the expression of ER and EGFR/HER2 is commonly noted in clinical breast cancer specimens (Nicholson et al., 2001), with total ER loss being seen in approximately 30% of patients. Preclinical transfection studies suggest that elevated signalling through MAPK (Creighton et al., 2006; see also Chapter 3) and src (see Chapter 8) can promote the transcriptional repression of the ER gene, possibly through the activation of NFkB (Van Laere et al., 2007) and may eventually lead to ER silencing through promoter methylation of CpG islands (Giacinti et al., 2006; Fleury et al., 2008). Importantly, this phenomenon is also observed in ER positive acquired anti-hormone resistant models where prolonged tamoxifen and fulvestrant treatment ($>$ 2 years) leads to ER negativity (see Chapters 4 and 8).

1.4.3 Mechanisms Leading to Increased Growth and Survival Signalling in Breast Cancer Cells

1.4.3.1 Zinc Transporters and Endocrine Resistance

Recent studies within our group have begun to define a previously unidentified role for zinc in contributing to ER dependent and independent forms of endocrine resistance through its capacity to sustain the activity of growth factor signalling. Zinc is a metal ion which is involved in the regulation of many cellular processes, including proliferation and survival, and increased zinc levels have been shown to inactivate several phosphatases involved in the dephosphorylation and inactivation of EGFR, HER2, IGF-1R and c-src (Haase and Maret, 2005). Exposure of our tamoxifen resistant cells to zinc, therefore, serves to activate signalling from these growth factor receptors and raise the intracellular activity of MAPK and AKT to promote tumour cell growth and motility (Taylor et al., 2008). Significantly, we have shown that acquired tamoxifen resistance in vitro is associated with increased basal zinc levels and increased expression of a key zinc transporter, ZIP7 (HKE4/SLC39A7), which facilitates the release of zinc from its intracellular stores. Silencing of this gene through the use of siRNA to ZIP7, reduces zinc levels, blocks growth factor responses and inhibits cell growth, providing evidence for a central importance of zinc in resistance (Taylor et al., 2008). Importantly, the expression of a further family member located on tumour cell membranes, LIV-1 (Taylor et al., 2007), has been previously shown by our group to predict breast cancer spread to the regional lymph nodes and may link zinc transport to other features of tumour progression noted in our endocrine resistant cells.

1.4.3.2 Hypo- and Hyper-Methylation of DNA in Endocrine Resistance

In general terms, the hyper-methylation/hypo-methylation of gene promoters can have a profound effect on gene transcription, leading, at its extremes, to gene silencing or increased gene expression respectively. In anti-oestrogen resistant MCF-7 cells, a recent study by Fan et al. (2006) has suggested that a hypo-methylated state predominates and results in the increased expression and activation of multiple growth regulatory pathways, including EGFR/HER2 and related proteins, PKA signalling elements, cytokines and cytokine receptors, Wnt/β-catenin, Notch signalling elements and IFN signalling components. Characteristically, however, considerable differences between the induced gene expression profiles of SERMs and SERDs exist, emphasising once again the individual nature and diverse mechanisms of actions of endocrine agents which target the same receptor.

Interestingly, although DNA hyper-methylation was not as evident as hypo-methylation in the study of Fan et al. (2006), it is of some potential importance in endocrine resistance since it is an established mechanism for inactivating tumour suppressor and pro-apoptotic genes which would subsequently allow growth factor associated growth signalling to occur more efficiently. Certainly, several investigations have described the capacity of anti-oestrogenic drugs to promote gene

inactivation through the hyper-methylation of CpG islands in oestrogen regulated genes (Jensen et al., 1999; Leu et al., 2004). In our own studies (see Chapter 4), we have shown that exposure of MCF-7 cells to either tamoxifen or fulvestrant for prolonged periods (> 2 years) effectively silences a substantial cohort of oestrogen regulated growth tumour suppressor genes which when re-expressed by treatment of the cells with 5-AZA are predominantly growth inhibitory in the presence of oestradiol. Evidently, the remodelling of chromatin structure by anti-hormonal drugs, through the hypo- and hyper-methylation of DNA, appears to provide complimentary signals to promote resistant growth, acting to stimulate positive growth regulatory signals, while inhibiting negative ones.

1.4.3.3 Epithelial Mesenchymal Transition (EMT) in Endocrine Resistance

Importantly in our models, endocrine resistance is frequently hall-marked by a partial epithelial mesenchymal transition (EMT) which characteristically involves a reprogramming of cells towards a less differentiated, more invasive, phenotype. Studies from our own group have highlighted the breadth of induced genes which may contribute to this altered phenotype (Gee et al., 2006). Among these are CD44, notably encompassing the CD44v3 isoform (see Chapter 8), which acts as a co-receptor for erbB family members (Yu et al., 2002; Ghatak et al., 2005) and c-Met, the pro-invasive tyrosine kinase receptor target for HGF/scatter factor (Orian-Rousseau et al., 2002). Since, in the case of long-term fulvestrant resistance, such poorly differentiated cells are largely unresponsive to EGFR/HER2 blockade and express only modest levels of these growth factor receptors, their aggressive phenotype must rely on an entirely different cohort of signalling pathways from those identified in earlier forms of resistance (see Chapter 4).

1.4.3.4 Loss of Tumour Suppressors in Endocrine Resistance

A recent enhanced retroviral mutagen study has revealed that a disruptive insertion into the allele coding for the p27 cyclin-dependent kinase (CDK) inhibitor created oestrogen-independent and anti-oestrogen resistant breast cancer cells that still contained functional ER. Several notable changes were observed to the signalling pathways of the cells, including increased CDK2 activity, hyper-phosphorylation of AIB1 which enhanced its co-activator activity on the transcription of E2F1 and growth factor binding protein 2-associated binder 2 (Gab2) and Akt activity were increased following E2F1 over-activation (Yuan et al., 2007). Similarly, loss of the retinoblastoma tumour suppressor (RB) protein, a common aberration in breast cancer (Dublin et al., 1998) also leads to increased CDC2 activity (Varma et al., 2007), increased E2F-regulated gene expression (Bosco et al., 2007), cell cycle progression and an endocrine resistant phenotype in vitro (Bosco et al., 2007, Varma et al., 2007), as does loss of the cell cycle inhibitor p21 (Cariou et al., 2000). Evidently, loss of cell cycle inhibitors allow passage of anti-hormone treated cells through the cell cycle, together with enhancing aspects of pathways positively regulated by growth factor signalling.

1.5 New Generalised Model of Endocrine Resistance

Although the development of endocrine resistance is clearly complex and can be achieved through numerous mechanisms, several trends centring round the regulation of growth factor signalling cascades are evident from the above. As such, endocrine resistance can be achieved by:

1 Increased growth factor activation resulting from increased availability of growth factors (available from autocrine or paracrine sources) and increased expression of growth factor receptors. This process appears to be aided by the concurrent up-regulation of several growth factor signalling facilitators (e.g. membrane ER, CD44, ZIP7, BCAR1 and possibly BCAR3).
2 Increased activation of several pathways downstream of growth factor receptors and small GTPases (e.g. Ras, Raf, MEK, MAPK, PI3K/AKT, PKC, PKA, Rac1 and c-src).
3 Increased expression/activation of growth factor directed nuclear transcription factors (e.g. ER, AP-1, ETS2, E2F) and their co-activators (AIB-1/SRC-1).
4 Loss of negative effectors of the above events (e.g. PAX2 (HER2 expression), RASAL-1 (GEF activation; see Chapter 4), CDK10 (ETS and Raf expression), pTEN (AKT activation), Rb and p27kip1 (E2F and cell cycle activation) and ER co-repressors; see Chapter 2).

Since each of these elements are mechanistically linked to the regulation of the cell cycle and the survival of breast cancer cells, it is not surprising that they are often shared between de novo and acquired endocrine resistant models, are brought about by either genetic aberrations in growth factor signalling cascades or through adaptive mechanisms and result in either ER dependent forms of endocrine resistance, where the ER is harnessed to the altered growth factor signalling mechanism, or ER independent forms, where it is not.

1.6 Therapeutic Strategies to Treat or Delay/Prevent Endocrine Resistance

1.6.1 ER Positive Disease

The retention of functional ERs in breast cancer models resistant to SERMs often allows a subsequent response to either fulvestrant or oestrogen withdrawal and most likely stems from the capacity of these treatments to lower nuclear and membrane ER signalling and their cross-talk with growth factor signalling elements. Unfortunately, such treatment responses are short-lived and the development of resistance involving altered growth factor signalling appears inevitable with all forms of endocrine therapy in experimental models. Improved responses, however, are often achieved with anti-growth factor therapies which can bring about growth inhibition lasting many months in vitro (Nicholson et al., 2007) and in vivo (Massarweh et al., 2008). To date success in this area has been achieved using inhibitors of growth

factor receptors and their downstream signalling elements (Nicholson and Johnston, 2005) and this is reflected in the large number of clinical studies emerging around this new strategy (Leary et al., 2007; see Chapter 10). Of great potential importance to this approach is the observation made by several groups that such anti-growth factor treatments are capable of restoring anti-hormone responses by reducing the oestrogen-like activity of the growth factor primed ER/co-activator complex. Continued anti-hormonal measures, alongside anti-growth factor treatments, may thus prove more effective in the treatment of endocrine resistance than single anti-growth factor therapies in anti-hormone withdrawn patients.

Studies on the development of acquired anti-hormone resistance from endocrine responsive cells have shown that altered growth factor signalling within the first few weeks of anti-hormonal treatment is important to the subsequent development of the resistance state (Gee et al., 2003; 2006). This is because the expression of growth factor receptors, like EGFR and HER2, is switched on by anti-hormonal treatments and this enables survival signals which limiting their anti-tumour activity. This observation has provided a strong rationale for the combination of anti-hormonal treatments with anti-growth factor therapies in endocrine responsive disease where increased rates of growth inhibition and cell kill have been reported in vitro and in vivo (Leary et al., 2007). Indeed, so effective are these combinations that in our own laboratory we have been able to largely prevent the development of anti-oestrogen resistance in vitro through the co-targeting of ER with gefitinib (Gee et al., 2003), an EGFR selective tyrosine kinase inhibitor, and AZD0530 (Hiscox et al., 2008), a c-src kinase inhibitor, while other laboratories have used anti-hormones plus farnesyl transferase inhibitors (Martin et al., 2007) and PI3K/mTOR inhibitors (Steelman et al., 2008) as a means of enhancing anti-hormone induced cell death. Again these experimental observations are reflected in several current clinical studies and trials (Leary et al., 2007; see Chapter 10). The future identification of other regulatory elements in the resistant or responsive phenotype will undoubtedly provide equivalent and increasingly effective therapeutic approaches and it is likely that these will also encompass strategies to co-targeting additional anti-hormone-induced or inherently expressed paracrine and angiogenic influences on tumour cells.

Interestingly, while attempts to target growth factor signalling elements has inevitably lead to the development of highly selective inhibitors, such as gefitinib and trastuzumab, there is currently great interest in using less selective drugs. This is based on the known plasticity of cancer cells, which allows them to readily switch between growth factor signalling pathways and the induction of compensatory signalling by highly selective inhibitors (Gee et al., 2006; Jones et al., 2006; Hutcheson et al., 2006), each of which can promote anti-growth factor resistance (Jones et al., 2006). As such, it is now perceived that drugs which target multiple growth factor receptors (e.g. lapatinib which targets EGFR and HER2), or common convergence points arising from multiple growth factor receptors (e.g. farnesyl transferase inhibitors which targets ras signalling) or multiple signal transduction elements (e.g. 17-AAG which down-regulates ER, together with several signal transduction elements including AKT (see Chapter 7) may avoid such obstacles and provide more effective treatments alone and in combination with anti-hormones. In our own experience, while the single use of gefitinib in tamoxifen resistant breast cancer cells

effectively blocks EGFR signalling, it serves to facilitate IGF-1R signalling and cell survival via an IRS-1 dependent mechanism (Knowlden et al., 2007). The simultaneous blockade of the EGFR and IGF-1R negates these compensatory actions, greatly increasing cell kill and significantly delaying the development of gefitinib resistance. Similarly, tamoxifen resistant cells are inherently more sensitive to 17-AAG than endocrine responsive cells with the drug down-regulating the cellular levels of ER and AKT, together with EGFR and HER2 (Madden TA, personal communication).

1.6.2 ER Negative Endocrine Resistant Breast Cancer

Current strategies for the treatment of ER negative endocrine resistant breast cancer, outside of conventional chemotherapy, are extremely limited. Some success has recently been derived from the increasing use of trastuzumab in women with HER2 over-expressing tumours, although this is a relatively small cohort of patients. Disappointingly, responses to gefitinib are similarly restricted, despite the elevated expression of the EGFR in many ER negative tumours.

At presence two further approaches are being pursued. Firstly, considerable effort is being made to define the growth and survival pathways being used by these cells and our microarray and protein analysis have revealed a potential role for c-Met in ER negative, faslodex resistant breast cancer cells (see Chapter 8). This increase in c-Met confers a greatly increased sensitivity to exogenous HGF, which promotes their further invasiveness when stimulated by the exogenous ligand or by co-culturing cells with fibroblasts that produce large quantities of this growth factor (Hiscox et al., 2006a). Such data place the Met receptor centrally in invasiveness during adaptation to faslodex and its relevance to other forms of resistance is currently being investigated. Secondly, equal effort is being made to coax ER negative cells to re-express ERs, including the use of signal transduction inhibitors to signalling elements thought to reduce ER expression (e.g. parthenalide, an NFκB inhibitor) and agents designed to reverse ER gene silencing (e.g. 5-AZA, a methylase inhibitor). It is hoped that ER re-expression will restore endocrine response in patients not normally considered for endocrine measures and limit other adverse features associated with ER negative disease. Interestingly, the use of 5-AZA in ER positive tamoxifen resistant breast cancer cells in vitro promotes the expression of previously silenced tumour suppressor genes to halt cell growth (see Chapter 4), suggesting a value for this agent in ER positive and ER negative endocrine resistant breast cancer.

1.7 Conclusions and Future Perspectives

Endocrine therapy is a well established and valuable approach to the treatment of breast cancer. Although resistance mechanisms to these therapies emerge during breast cancer development and treatment, they appear to be dominated by aberrations in growth factor signalling cascades which can drive breast cancer growth

in an ER dependent or independent manner. As our molecular knowledge of these events expands, so too will our capacity to intervene. Inevitably this will improve the survival of breast cancer patients.

Abbreviations

ER:	oestrogen receptors
ERE:	oestrogen response elements
AF-1:	activator function 1
AF-2:	activator function 2
TGFα:	transforming growth factor alpha
VEGF:	vascular endothelial growth factor
VEGFR:	vascular endothelial growth factor receptor
HGF:	hepatocyte growth factor
NFkB:	nuclear factor of kappa light polypeptide gene enhancer in B-cells
5-AZA:	5-aza-2′-deoxycytidine
CDK:	cyclin-dependent kinase
bcl-2:	B-cell lymphoma protein 2
MAPK:	mitogen-activated protein kinase
AKT:	protein kinase B
HAT:	histone-acetyltransferase
HDAC:	histone-deacetylase complex
NCoA:	nuclear receptor co-activator
NCoR:	nuclear receptor co-repressor
AP-1:	Jun/Fos activator protein 1
SP-1:	specificity protein-1
IGF-1R:	insulin-like growth factor receptor 1
MNAR:	modulator of non-genomic activity of ER
PELP 1:	proline-, glutamic acid-, and leucine rich protein 1
EGFR:	epidermal growth factor receptor
SERM:	selective oestrogen receptor modulators
SERD:	selective oestrogen receptor down-regulators
HER2:	human epidermal growth factor receptor 2
TGFβ:	including transforming growth factor β
GEF:	guanine nucleotide exchange factor
PDGFR:	platelet derived growth factor receptor
MISS:	membrane-initiated steroid signalling
NISS:	nuclear initiated steroid signalling

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Chapter 2
Transcriptional Coactivators and Corepressors in Endocrine Response and Resistance in Breast Cancer

Simak Ali

Contents

Abstract Regulation of gene expression by sequence-specific DNA binding proteins involves the co-ordinated action of a repertoire of transcriptional coregulator complexes, which together act to modify chromatin at gene promoters, thereby facilitating gene expression. The mechanisms by which such coregulators are recruited to the promoters of estrogen-responsive genes by estrogen receptor-α have been well studied in breast cancer cells. These studies have highlighted coactivator and corepressor proteins that appear to be critical for the agonist and antagonist actions of estrogen and anti-estrogens, and indicate that altered levels and/or activities of these proteins is an important feature of response and resistance to endocrine treatments in breast cancer.

Keywords Anti-estrogens · estrogen receptor · coactivators · corepressors · gene regulation

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2.1 Introduction

The majority of breast tumours express estrogen receptor-α (ERα) and adjuvant endocrine treatment is used to inhibit ERα action. Endocrine agents fall into two main classes, anti-estrogens such as tamoxifen that act as ligands by direct binding to ERα, or drugs that block local and systemic estrogen biosynthesis (LHRH agonists and aromatase inhibitors). Although these are relatively successful treatments, an important clinical problem is de novo and acquired endocrine resistance that is not associated with loss of ERα, such that recurrence is ERα-dependent in many of these cases (Ali and Coombes 2002; Johnston and Dowsett 2003; Carpenter and Miller 2005; Schiff and Osborne 2005). In this scenario, a central working hypothesis is that activation of ERα by estrogen-independent pathways, increased sensitivity of ERα to residual estrogens following estrogen ablation therapies or, increased agonist activity in the case of anti-estrogens, could feature in endocrine resistance. Clearly, a detailed understanding of the mechanisms of ERα action in breast cancer cells is important for the development of strategies for overcoming endocrine resistance.

2.2 Mechanisms of Estrogen Receptor-α Action

ERα is a member of the nuclear receptor (NR) superfamily of transcription factors that acts primarily as a sequence-specific DNA binding protein to regulate the expression of estrogen-responsive genes (Mangelsdorf et al. 1995; Chawla et al. 2001). Regulation of gene expression by ERα requires its recruitment to estrogen-regulated genes by direct binding to estrogen response elements (ERE), or indirectly through interaction with other transcription factors, for example AP1 and Sp1, prominent examples of the latter type of estrogen-regulated genes being the cyclin D1 and c-Myc genes (Bjornstrom and Sjoberg 2005). Recent studies using chromatin immunoprecipitation (ChIP) microarray (ChIP-chip) analysis, in which DNA sequences to which ERα is bound following estrogen stimulation in the MCF-7 breast cancer cell line are profiled using genomic DNA microarrays, show that as few as 4% of the ERα binding sites map to the proximal promoter region, with the majority of ERα binding sites located at considerably greater distances from the transcription start sites, some ERα binding sites being located more than 150 kb distal to the gene promoter (Carroll et al. 2005; Carroll et al. 2006). These findings cast some doubt on the in vivo significance of the reported recruitment of ERα to some gene promoters through indirect binding. For example, the ChIP-chip studies have revealed a previously unidentified ERα binding site 67 kb upstream of the c-Myc promoter, and in the case of the cyclin D1 gene, highlighted a cell-type specific enhancer downstream of the coding region of the cyclin D1 gene (Carroll and Brown 2006; Carroll et al. 2006; Eeckhoute et al. 2006). The ChIP-chip studies do, however, highlight the importance of other transcription factors, in particular FoxA1, for ERα recruitment to EREs.

These studies, together with gene expression microarray analyses show that ERα regulates the expression of a large number of genes in breast cancer cells. An early

gene expression microarray study carried out on 12,000 genes reported that more than 400 genes showed estrogen regulation (Frasor et al. 2003; Frasor et al. 2004), whilst our own data using recently available microarray platforms providing total genome coverage, show that the expression of 1,128 genes are altered within 24 hours following estrogen addition (Buluwela and Ali, unpublished). These studies are in good agreement with ChIP-chip analyses, which identified 3,665 ERα binding regions in MCF-7 cells (Carroll et al. 2006). Hence, the estrogen-stimulated growth of breast cancer cells appears to require the concerted action of a large number of genes. It is also important to note that these studies have shown that in addition to stimulating gene expression, ERα represses the expression of many genes in breast cancer cells, for example the expression of several pro-apoptotic and growth inhibitory genes is repressed by estrogen.

2.3 Transcriptional Coactivators and Corepressors in Mediating Gene Regulation by Estrogen Receptor-α

In eukaryotes, the genomic DNA is wrapped around an octamer composed of dimers of histone proteins, histones H2A, H2B, H3 and H4, each octamer unit having 147 bp of DNA wrapped around it, forming the nucleosome (Luger et al. 1997). The nucleosomes are in turn further compacted to form the chromatin, allowing the large amount of genomic DNA to be accommodated in the nucleus. In addition to providing compaction of the DNA, the chromatin restricts access of promoter regions to the transcriptional machinery. For gene expression to proceed, extensive chromatin remodelling and reversible post-translational modification of the nucleosomal histones is required. The amino-terminal tails of the core histones are extensively modified on lysine, arginine, serine and threonine residues by protein kinases, phosphatases, histone acetyltransferases (HATs), histone deacetylases (HDACs), histone methyltransferases (HMTs), histone demethylases, ubiquitin and SUMO ligases (Rosenfeld and Glass 2001; Rosenfeld et al. 2006). Many of these modifications are associated with actively transcribed genes and others with repressed genes, leading to the concept of the "histone code" (Strahl and Allis 2000), in which specific histone modifications engendered by one factor provoke the sequential recruitment of other transcription factors or coregulators, thereby providing combinatorial and dynamic aspects to gene regulation. ATP-dependent chromatin remodelling complexes allow relative movement of the nucleosomes on the DNA, again regulating transcription factor access. Additionally, the methylation status of the DNA is important for the transcriptional activity of a gene, with CpG methylation which, together with histone deacetylation and lysine methylation, provide potent gene silencing and the formation of heterochromatin (Hermann et al. 2004; Metivier et al. 2006).

In common with other nuclear receptors, upon binding to its response element, ERα regulates gene expression by recruiting transcriptional coregulators and components of the basal transcription machinery. Almost 300 potential NR coregulators have been reported to date (Lonard et al. 2007). However, clear and important roles

in transcription initiation by NR have been established for relatively few of these proteins, although the large numbers of potential coregulators reported do provide a basis for tissue, developmental and temporal regulation of NR function in vivo. The kinetics of the chromatin changes associated with transcription initiation and coregulator recruitment are best defined for ERα in MCF-7 cells. Detailed ChIP-based analysis of ERα and coregulator recruitment, histone modification and chromatin remodelling at the promoters of the estrogen-regulated genes cathepsin D, cyclin D1, c-Myc and the pS2/TFF1 gene have shown that ERα and RNA polymerase II (PolII) are recruited and dissociated from the promoters of these genes in a cyclical manner, with a periodicity of 40–60 minutes (Shang et al. 2000; Reid et al. 2003; Liu and Bagchi 2004; Park et al. 2005). The most detailed analysis is available for the pS2 promoter, where ERα recruitment is followed in a sequential manner by the recruitment of SWI/SNF ATP-dependent chromatin remodelling complexes, HMTs and HAT, with concomitant nucleosome re-phasing, histone methylation and acetylation (Metivier et al. 2003). The latter study demonstrated that nucleosome re-phasing, histone demethylation and deacetylation through the recruitment of SWI/SNF proteins and HDACs follow ERα dissociation. The first cycle of ERα and coregulator recruitment is not transcriptionally productive, with subsequent cycles of ERα recruitment leading to transcription (Metivier et al. 2006).

2.4 Transcriptional Coactivators and Corepressors in Breast Cancer

Of the large numbers of transcriptional coregulators recruited by ERα to estrogen-regulated gene promoters, the best characterised are the related Steroid Receptor Coactivators (SRC) or NCoAs, SRC1/NCoA1, TIF-2/GRIP1/NCoA2 and AIB1/NCoA3 (Glass and Rosenfeld 2000), termed coactivators because their over-expression stimulates NR activity. They are recruited to the ligand binding domains of agonist-bound NR, where they appear to function predominantly as platform proteins for the recruitment to gene promoters of the histone acetyltransferases CBP, p300 and P/CAF (p300/CBP associated factor). Further, SRCs recruit the arginine methyltransferases, CARM1 and PRMT1 (Stallcup et al. 2003). Targeted deletion of SRC1 and AIB1 results in reduced mammary gland development (Xu et al. 1998; Xu et al. 2000), underscoring their importance for hormonal action in the breast.

Many NRs repress gene expression in the unliganded state by recruiting transcriptional corepressors. In particular, the nuclear receptor corepressor (NCoR) and the related factor SMRT (Silencing Mediator of Retinoid and Thyroid receptors), act as molecular scaffolds for the recruitment of HDACs, but have also been identified in association with the SWI/SNF chromatin remodelling complex that also contains KAP-1, a corepressor that has been linked to heterochromatin silencing (Glass and Rosenfeld 2000). In addition, anti-estrogens such as tamoxifen and faslodex facilitate the recruitment of corepressors, in particular NCoR and SMRT, by ERα and

consequent repression of estrogen-responsive genes. Furthermore, in the absence of ligand, ERα can be recruited to the pS2 promoter, and facilitates NCoR/SMRT recruitment under certain circumstances (Metivier et al. 2004).

A great deal of in vitro and in vivo evidence has emerged regarding the importance in particular of NCoAs and NCoR/SMRT in breast cancer progression. Indeed, NCoA3, also known as AIB1 (Amplified in Breast Cancer 1) was originally identified following its cloning from a region of chromosome 20 that is amplified in breast cancer cells, and the AIB1 gene has been shown to be amplified in 5–10% of breast tumours (Anzick et al. 1997). Furthermore, transgenic mice over-expressing AIB1 develop mammary tumours, with 85% of the tumours being ERα-positive (Torres-Arzayus et al. 2004), suggesting that AIB1 is an oncogene.

A role for these coregulators in tamoxifen resistance is indicated by several in vitro studies. Firstly, Lavinsky et al. (Lavinsky et al. 1998) showed that long-term treatment of MCF-7 cells transplanted in nude mice with tamoxifen, results in the development of tamoxifen-resistant tumours, the tumours being characterised by reduced levels of NCoR. Moreover, in fibroblasts obtained from mice in which the NCoR gene had been deleted, tamoxifen acted as an agonist (Jepsen et al. 2000). Additionally, RNA interference (RNAi)-mediated silencing of NCoR and SMRT resulted in tamoxifen-stimulation of MCF-7 cell growth (Keeton and Brown 2005).

Tamoxifen is a so-called selective estrogen receptor modulator (SERM), which is an antagonist in some tissues, such as the breast, whilst having agonist properties in other tissues, such as the uterus. A potential mechanism to explain the tissue-specific agonist/antagonist activities of tamoxifen was provided by the demonstration that SRC1 levels are low in breast cells and high in uterine cells (Shang and Brown 2002). These authors showed that in both cell types the tamoxifen-bound ERα recruits NCoR/SMRT to promoters of genes to which ERα is directly recruited through binding to estrogen response elements. However, high levels of SRC1 in uterine cells results in coactivator recruitment by the tamoxifen-bound receptor to promoters of genes to which ERα is recruited through interaction with other transcription factors, whereas in breast cells, where SRC1 levels are low, NCoR/SMRT is recruited to these gene promoters. Together, these studies offer a model for resistance to endocrine therapies, in which relative levels of the corepressors NCoR/SMRT and the coactivators SRC1/AIB1 contribute to response and resistance to endocrine treatments.

SRC2/TIF2 and CBP overexpression have been reported in breast cancer, compared with the normal breast (Kurebayashi et al. 2000; Girault et al. 2006). SRC1 levels have also been associated with shorter disease-free survival in breast cancer (Myers et al. 2004). Immunohistochemical analysis of 290 ERα-positive primary breast cancers showed that high levels of AIB1 are associated with a greater likelihood of relapse and decreased overall survival (Jiang and Ali, unpublished), a finding also reported by several other groups (Shou et al. 2004; Lonard et al. 2007). AIB1 levels are higher in tamoxifen-resistant lines derived from MCF-7 cells, but not in MCF-7 derived lines selected on the basis of growth in the absence of estrogen (our unpublished data). Further, AIB1 levels were also elevated in a series of 21 breast cancer biopsies taken from patients after local recurrence following

tamoxifen treatment, when compared with the pre-treatment biopsies ($p = 0.034$; Wilcoxon signed-rank test) (Jiang and Ali, unpublished). Conversely, low levels of NCoR appear to be associated with shorter disease-free interval (Girault et al. 2003) and SMRT negativity has been reported to be associated with a shorter disease-free interval and overall survival (Green et al. 2007). These findings are in agreement with the hypothesis that the balance between these coactivators and corepressors may define response to endocrine treatments.

2.5 Cross-Talk Between ER-α Coregulators and Growth Factor Receptor Signalling Cascades

There is now substantial evidence linking growth factor receptor signalling, in particular via the EGF receptor, HER-2/neu and IGF receptor pathways, with endocrine resistance in ERα-positive breast cancer (Dowsett et al. 2005; Gee et al. 2005; Osborne et al. 2005). These findings have led to the proposal that inhibitors of cell surface receptor activation, for example using the EGFR inhibitor gefitinib/Iressa, lapatinib or herceptin, or using inhibitors of downstream protein kinase cascades, in particular the PI3K/AKT and MAPK pathways may be valuable treatments for endocrine resistant breast cancer. Interestingly, patients with ERα-positive breast cancer with high-level expression of AIB1 that are also HER2-positive have the worst prognosis (Shou et al. 2004). A similar relationship has been reported for EGFR/HER1 with AIB1, as well as for HER3 with AIB1 (Kirkegaard et al. 2007). Together, these data suggest that crosstalk between cell surface receptors, ERα and AIB1 is important for ERα activity and response to endocrine therapies and indicating that ERα and AIB1 phosphorylation may be important in breast cancer progression, and support in vitro data showing that growth factors, particularly EGF and IGF1 stimulate ERα activity, often in a ligand-independent manner, in large part through activation of ERK1/2 MAPKs. MAPK phosphorylates ERα at several sites, to stimulate ER activity upon estrogen binding, as well as the activity of unliganded and tamoxifen-bound ERα; presumably by facilitating NCoA recruitment or corepressor dissociation. However, recent findings (Murphy et al. 2004; Sarwar et al. 2006) show that high level ER phosphorylation at a key residue, ser-118 (phosphorylated by ERK1/2), correlates with markers of better prognosis including low tumour grade. However, phosphorylation of ser-118 was higher after relapse following tamoxifen treatment. Phosphorylation at another important site, ser-167 (phosphorylated by p90RSK, AKT), predicts for better survival ($n = 310$; $p < 0.05$) (Jiang et al. 2007). These and other findings raise the possibility that in addition to ERα phosphorylation, coregulator phosphorylation is important for mediating the effects of growth factor signalling cascades on ERα activity.

Cytokines, steroid hormones and epidermal growth factor induce phosphorylation of SRC1 and AIB1. Both coactivators are phosphorylated at multiple sites, and phosphorylation appears to be required for optimal activity, including the regulation of interaction with the histone acetyltransferases p/CAF and CBP, and alters the affinity for nuclear receptors (Wu et al. 2004; Wu et al. 2005; Zheng et al. 2005;

Wu et al. 2007). Conversely, phosphorylation of NCoR by AKT and SMRT by MEK leads to their export to the nucleus, consequently reducing their ability to repress gene expression (Hermanson et al. 2002a; Hermanson et al. 2002b; Jonas and Privalsky 2004; Lonard and O'Malley 2007). Hence, stimulation of growth factor signalling pathways, as frequently observed in endocrine resistance could involve phosphorylation-mediated stimulation of coactivator activities and/or loss of corepressor activities at promoters of estrogen-regulated genes.

As mentioned above, phosphorylation of ERα at several sites causes ligand-independent ERα activity. The mechanisms by which such stimulation of ERα activity comes about are unclear. One possibility is suggested by the report that p68 RNA helicase, is preferentially recruited to ERα phosphorylated at ser-118 (Endoh et al. 1999). p68 RNA helicase may stimulate ERα activity by facilitating the recruitment of SRC coactivators, as well as the RNA coactivator SRA (Watanabe et al. 2001), thus providing another mechanism by which crosstalk with growth factor signalling could lead to preferential coactivator recruitment by ERα.

2.6 Involvement of Other Transcriptional Coregulators in Estrogen Signalling

Approximately 300 NR coregulators have been described in the literature. In addition to the coactivators and corepressors discussed above, one or more of these potential coregulators may play important roles in breast cancer progression. Likely to be important for estrogen action in the breast are the corepressors RIP140 and L-CoR. Unlike NCoR/SMRT, RIP140 and L-CoR are recruited through interaction with the NR ligand binding domain through α-helical motifs having the consensus sequence Leu-Xaa-Xaa-Leu, normally found in coactivators such as SRC1. Hence, these corepressors are recruited by the estrogen-bound ERα. Although there is no evidence to indicate that RIP140 and L-CoR are required for the regulation of estrogen-responsive genes whose expression is stimulated by ERα, it is important to note that the majority of estrogen-regulated genes in breast cancer cells are those whose expression is repressed by estrogen. Another ERα corepressor, ZNF366, which binds to the ERα DNA binding domain, acts as a repressor by recruiting the corepressor CtBP, RIP140 and through direct interaction with histone deacetylases (Lopez-Garcia et al. 2006). ZNF366 is interesting as its expression is considerably lower in breast cancer cells, compared with normal breast epithelial cells, suggesting that it acts to reduce ERα activity.

2.7 Transcriptional Coregulators as Targets in Breast Cancer Treatment

Gene expression by ERα requires multiple chromatin changes, changes that are mediated by transcriptional coregulator complexes. The last few years have shown

that several coactivators and corepressors are likely to be important for endocrine response and resistance in breast cancer, in particular, the coactivators SRC1, AIB1 and the corepressors NCoR, SMRT. The importance of these proteins in crosstalk with growth factor signalling indicates that they may be important downstream targets of growth factor receptor and protein kinase inhibitors currently being evaluated in the clinic. In addition, SRC1 and AIB1 are subject to other modifications, in particular acetylation, ubiquitination and SUMOylation (Lonard and O'Malley 2007; Lonard and O'Malley 2008), with the modifications determining their activity and turnover, a better understanding of which may provide additional methods for drug development. As SRC1 and AIB1 appear to act by facilitating the recruitment of histone acetyltransferases, namely CBP/p300 and P/CAF, and the histone methyltransferases CARM1 and PRMT1, small molecule inhibitors of these enzymes may provide additional agents for the treatment of endocrine resistant breast cancer. An interesting additional possibility is provided by the recent observation that expression of estrogen-regulated genes requires topoisomerase IIß and PARP-1-mediated double-strand DNA break (Ju et al. 2006), raising the possibility of using PARP inhibitors for blocking ERα activity, which have already been proposed for use in the treatment of breast cancers in which BRCA1 or BRCA2 genes are mutated, due to its usual role in DNA repair (Bryant et al. 2005; Farmer et al. 2005).

Abbreviations

NR:	nuclear receptor
ERE:	estrogen response element
ChIP:	chromatin immunoprecipitation
HAT:	histone acetyltransferase
HDAC:	histone deacetylase
HMT:	histone methyltransferase
SMRT:	silencing mediator of retinoid and thyroid receptors
NCoR:	nuclear receptor corepressor
AIB1:	amplified in breast cancer 1
EGF:	epidermal growth factor
HER-2/neu:	human epidermal growth factor receptor 2
IGF:	insulin-like growth factor

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Chapter 3
The Re-Expression of Estrogen Receptor in Estrogen Receptor-Negative Breast Cancer and Restoration of Anti-Estrogen Responses

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Contents

Abstract Loss of estrogen receptor α (ERα) in breast cancer correlates with a more aggressive, tamoxifen resistant phenotype. ERα-negative tumors often display overexpression or amplification of growth factor receptors of the erbB family, particularly EGFR and erbB-2, and consequently, elevated growth factor signaling and resultant MAP kinase (ERK) activity. We have previously shown that overexpression/hyperactivation of EGFR or erbB-2, or the downstream effectors Raf or MEK, in ERα+, estrogen-dependent MCF-7 cells results in the acquisition of estrogen-independence and loss of ERα expression. We have shown that the common downstream effector of ERα downregulation in all our model cell lines is hyperactive MAPK and that inhibition of this hyperactive MAPK restores ERα

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expression. Microarray expression profiling of these hyperactive MAPK model cell lines revealed a hyperactive MAPK signature that correlates with ERα-breast cancer and not ERα+ breast cancer. We have more recently extended these observations to established ERα-breast cancer cell lines and primary cultures from ERα-breast tumor specimens. Inhibition of MAPK in these ERα-breast cancer cells restores ERα expression and associated with this re-expression of ERα is the acquisition of anti-estrogen responses. These data demonstrate the dynamic nature of ERα expression in breast cancer cells and the ability to impact ERα expression by altering cellular signaling pathways. Further, they suggest a potential novel therapeutic strategy for ERα-breast cancer: inhibition of MAPK activity to restore both ERα expression and anti-estrogen responses.

Keywords Estrogen receptor loss · MAPK · Microarray profiling

3.1 ERα Expression in the Normal Breast and Breast Cancer

In the normal female breast, only a fraction of the cells composing the glandular epithelium express estrogen receptor α (ERα) at the protein level. In some studies, approximately 6–12% of the glandular epithelial cells are ERα-positive (Anderson et al., 1998), while others place the fraction a little higher at 10–30% (Jacquemier et al., 1990; Koerner et al., 2001; Petersen et al., 1987). Significantly, all the reports are consistent in that ERα is expressed exclusively in the glandular epithelium, and never in any other cell type in the breast. ERα is synthesized throughout the cell cycle and maintained through S phase (Ballare et al., 1989), though maximum synthesis occurs in G1 and G2 phases (Jakesz et al., 1984), corresponding with the maximum expression seen in late G1 (Rostagno et al., 1996). However, there is a near-complete dissociation between ERα expression and cell proliferation in the normal adult breast – ERα expression is rarely, if ever, seen in proliferating cells in multiple dual labeling immunohistochemical analyses where breast cells were stained for ERα and Ki-67. Samples of normal breast tissue revealed that ERα and Ki-67 are rarely co-expressed in the same cell (Ballare et al., 1989; Shoker et al., 1999a), or that cells expressing either protein are separate populations entirely (Anderson et al., 1998; Clarke et al., 1997; Russo et al., 1999). These data are further supported by observations from local estrogen treatment to the mammary glands of ovariectomized mice where an increase in ERα expression in nearby cells occurs, but not in the cells of the terminal end bud, which proliferate rapidly upon estrogen stimulation (Daniel et al., 1987). While the ERα-positive cells in normal breast tissue rarely proliferate, they are seen in close proximity to the proliferating cells, suggesting that ERα-positive cells act as a sensor and regulate growth of the surrounding epithelium through paracrine/juxtacrine mechanisms. The lack of proliferation in the ERα+/PR+ ductal epithelium also suggests a link between ERα expression and terminal differentiation in the normal breast. These data have led to the conclusion that ERα+ and ERα-tumors arise from distinct cell lineages. However,

more recent data from Cheng et al. demonstrates that ERα+ cells do proliferate in response to estradiol but lose receptor expression post treatment suggesting this is why ERα+ /Ki67+ cells are normally not seen (Cheng et al., 2004). These data strongly suggest that perhaps receptor expression is a dynamic event dependent on other signaling events and not a static on/off situation whereby a cell is only ERα+ /−.

In the progression to breast cancer, cells increase their level of ERα expression, and ERα levels seen in breast cancer are consistently higher than those seen in normal breast (Panahy et al., 1987; Silvistrini et al., 1979). Increased ERα expression is seen in the earliest stages of ductal hyperplasia, and increases even more with progressing atypia – in cases of atypical ductal hyperplasia and in low to intermediate grade ductal carcinoma in situ (DCIS), most of the ductal epithelium stains ERα-positive (Allred et al., 2001; Shoker et al., 1999b). Another early change observed in the progression to malignancy is the loss of the inverse relationship between ERα expression and cell proliferation, especially as ERα expression becomes more widespread (Shoker et al., 1999a). Atypical ductal hyperplasia (ADH) and low grade CIS demonstrate strong ERα-positivity, with immunohistochemical analysis revealing contiguous ERα-positive cells in a majority of the lesion (Roger et al., 2000; Shoker et al., 1999b). In fact, Allred and colleagues have reported that in 95% of ADH cases, 90% of the cells are ERα-positive (Allred et al., 2001). Progressing lesions then begin to lose ERα expression, and only about 78% of high grade CIS are ERα-positive (Roger et al., 2000). As CIS progresses to invasive carcinoma, ERα expression continues to decrease, as does the expression of its transcriptional cofactors (Kurebayashi et al., 2000; Terenius et al., 1974). Overall, approximately 50–70% of all breast tumors are ERα-positive, 60–65% of DCIS cases are ERα-positive, and only about 55% of invasive carcinomas are ERα-positive (Ballare et al., 1989; Fanelli et al., 1996; Harvey et al., 1999; Kobayashi et al., 1992; Wittliff et al., 1972). ERα-positive breast cancer is more common in post-menopausal women (Silvistrini et al., 1979; Walker et al., 1992), and tumor ERα level corresponds directly with patient age (Pujol et al., 1994). The level of ERα expression in breast cancer correlates inversely with the proliferative index measured by Ki-67 staining (Vollmer et al., 1989), agreeing with clinical observations of slower growth in ERα-positive tumors. In ERα-positive breast cancer, more DNA synthesis occurs in ERα-negative cells than in those expressing ERα. However, unlike cells in the normal breast, ERα-positive breast cancer cells do proliferate (Clarke et al., 1997).

Clinically, breast cancer presents as either ERα positive or as ERα negative. The presence of ERα is correlated with a better prognosis both in terms of increased disease-free survival and overall survival, and predicts for response to hormonal therapies such as tamoxifen (Clark and McGuire, 1988; De Sombre et al., 1986; Knight et al., 1977; McGuire et al., 1990). Tamoxifen, the standard of care in hormonal therapies, as an adjuvant therapy is effective in both pre-and post-menopausal patients with ERα+ tumors (Fantl et al., 1993). However, 25–35% of all ERα+ tumors do not respond to tamoxifen (de novo resistance), and even those that do initially respond, ultimately develop resistance (acquired resitance) (Johnston, 1997). 20–50% of patients with ERα+ primary tumors that relapse following adjuvant

tamoxifen therapy have recurrent tumors where ERα expression is lost (Johnston, 1997; Johnston et al., 1995; Newby et al., 1997). In patients who have failed on tamoxifen, second-line hormonal therapies result in only a 10–30% response rate (Cheung et al., 1997; Johnston, 1997; Johnston et al., 1995; Newby et al., 1997). Tamoxifen has not been demonstrated to have a therapeutic benefit in ERα-negative patients (Early Breast Cancer Trialists Collaborative Group; Harvey et al., 1999). Because expression of ERα is required for response to anti-estrogen therapies, understanding the generation of the ERα negative phenotype and finding ways to restore ERα expression and response could lead to a novel therapeutic strategy for ERα-breast cancer.

3.2 The ERα-Negative Phenotype is Associated with Up-Regulation of ErbB Family Members

ERα-tumors are characterized by a more aggressive phenotype, a poor prognosis, and a lack of response to hormonal therapies. It has been demonstrated that most ERα-negative breast cancer cell lines such as MDA-MB-231 cells, MCF-7/Adr cells, and MDA-MB-468 cells exhibit site specific methylation of CpG islands in the ERα promoter, and that reversion of this ERα-negativity requires treatment with a demethylating agent such as 5-aza-cytidine (Ferguson et al., 1995; Ottaviano et al., 1994). About 25% of ERα-negative breast tumors were found to exhibit hypermethylation of the ERα promoter (Lapidus et al., 1996). More recent data using a highly sensitive methylation-specific PCR assay determined that 100% of these same ERα-negative tumors displayed some degree of methylation, however a number of ERα-positive tumors also showed similar degrees of methylation (Lapidus et al., 1998). More recently, it has also been demonstrated that histone deacetylation can maintain repression of ERα and this can be reversed with histone deacetylase inhibitors (Keen et al., 2003; Yang et al., 2000, 2001), although this has only been observed in cell lines.

ERα-negative tumors tend to overexpress certain growth factor receptors. Two such receptors that are up-regulated in ERα-negative tumors are the epidermal growth factor receptor (EGFR) and c-erbB-2. They are also important prognostic indicators. For example, in breast cancer cells, the overexpression of EGFR is inversely correlated with ERα (Sainsbury et al., 1985), and EGFR+ tumors have a poor prognosis independent of ERα status (Nicholson et al., 1988, 1989, 1991; Sainsbury et al., 1987; Toi et al., 1991). Double-label immunohistochemical detection of ERα and EGFR in breast tumor specimens and breast cancer cell lines confirms the inverse correlation of expression (Sharma et al., 1994a, 1994b; Van Agthoven et al., 1994). Furthermore, in ERα+ /EGFR+ tumors, individual tumor cells express high levels of only ERα or EGFR, but not both (Sharma et al., 1994a; Van Agthoven et al., 1994). The EGFR+ cells in these tumors are also associated with a higher growth rate than the ERα+ /low EGFR cells (Toi et al., 1990, 1994). Similarly, tumors that overexpress c-erbB-2 have a poorer prognosis and tend to be ERα-negative (Gusterson, 1992; Perren, 1991; Slamon et al., 1987). It has been

estimated that about 10–30% of c-erbB-2 overexpressing tumors are ERα+; importantly, these tend to have reduced ERα levels. Using phospho-erbB-2 specific antibodies, it has been demonstrated that those erbB-2 overexpressing tumors that also exhibited activation of that c-erbB-2 were most likely to be ERα-/PR- (DiGiovanna et al., 2002) indicating that downstream signaling via this receptor is associated with the ERα-phenotype. In keeping with this data, higher levels of activated MAPK, a downstream effector of both EGFR and c-erbB-2 signaling, have been found in ERα-breast cancer compared to ERα+ breast cancer. These data suggest that overexpression of EGFR or c-erbB-2 is actively involved, and not just associated, with the ERα-phenotype in breast cancer.

3.3 Hyperactivation of MAPK Results in Down-Regulation of ERα Expression and This Down-Regulation is Reversible

Using cell line models obtained by the stable transfection and overexpression of various signal transduction factors into ERα+ MCF-7 breast cancer cells we have shown that hyperactivation of MAPK, as a result of EGFR or c-erbB-2 overexpression/activation, results in the downregulation of ERα protein and mRNA (El-Ashry et al., 1996; Liu et al., 1995; Miller et al., 1994; Oh et al., 2001). This downregulation is a dynamic event, reversible through the abrogation of MAPK (ERK 1/2) signaling – either via pharmacologic inhibition, through expression of dominant negative forms of ERK1 and ERK2 (Oh et al., 2001), or as a result of knockdown of ERK1,2 expression with siRNAs specific to each ERK. Shown schematically in Fig. 3.1 and quantitatively in Table 3.1 is the ERα and MAPK activity status of these cell lines. These cell lines, expressing a constitutively active c-Raf-1 (yielding (ca)Raf cells), a constitutively active MEK-1 construct (yielding (ca)MEK cells), a wild type EGFR which can be activated by ligand (EGFR + EGF) cells), or a wild type c-erbB-2 (a clone with constitutively high

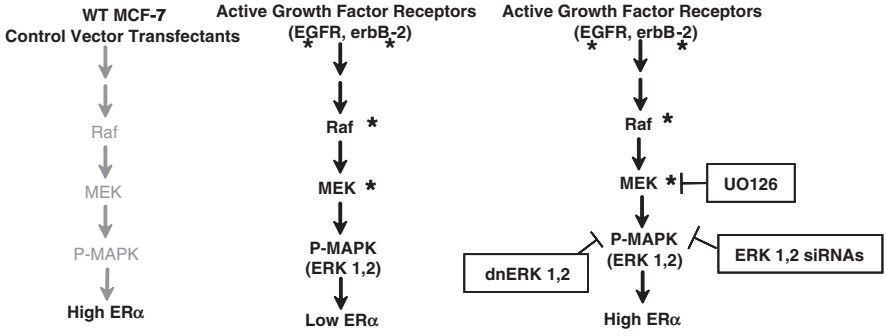

Fig. 3.1 Schematic representation of signal transduction molecules overexpressed in MCF-7 cells (*) and the downstream signaling pathways hyperactivated (*bold*) compared to the low, basal activation (*gray*) in control-transfected MCF-7 cells. The effects of hyperactivation of MAPK or inhibition of MAPK activity on ERα are highlighted in bold

Table 3.1 ERα content of the various cell lines was measured by ligand binding assay, and MAPK acitivity was determined by Western blotting for P-MAPK. Results are expressed as fmol of ERα per mg total protein and as relative MAPK activity

Cell line	MAPK activity	ER levels
co-MCF-7	+	∼120
co-MCF-7/lt-E2	+	∼ 400
(ca)Raf	++++	∼6–10
(ca)MEK	++++	∼4–7
EGFR + EGF	+++++	∼8–10
(ca)erbB-2	++++	∼20

levels of autophosphorylation and constitutive downstream signaling, (ca)erbB-2 cells) all grow in the absence of estrogen and express between four and twenty fmol of ERα/mg protein, a significant reduction when compared to the control transfected cell lines which exhibit about 120 fmol/mg protein when growing in the continuous presence of estrogen (co-MCF7) or about 400 fmol/mg protein when growing in the continuous absence of estrogen (co-MCF7/lt-E2). When ligand binding assays were used clinically, the cutoff for ERα was in some cases anything less than 10 fmol/mg protein and in others, anything less than 3 fmol/mg protein, thus our cell lines exhibit reductions in ERα expression that place them in the very low to ERα- category. Thus, specific hyperactivation of ERK1,2 through MEK, Raf, erbB-2, or ligand-induced EGFR induces a potent down-regulation of ERα protein expression.

This MAPK induced down-regulation of ERα expression is reversible via the inhibition of MAPK activity and the return in ERα protein expression upon inhibition of MAPK corresponds to restoration of ERα transcriptional activity as measured through transient transfection assays with ERE reporter constructs (Holloway et al., 2004; Oh et al., 2001). Figure 3.2 demonstrates both the restoration of ERα protein expression upon MAPK inhibition via the pharmacologic inhibitor U0126, and the ability of dnERK1 and 2 constructs to significantly increase ERE-luc activity in all 4 hyperactive MAPK cell lines to levels comparable to the co-MCF7 cells; the dnERK constructs do not affect ERE-luc activity in the co-MCF7 cells, either in the absence or presence of estrogen. Thus, it is the hyperactivation of MAPK induced by EGFR, erbB-2, Raf, and MEK that is specifically responsible for downregulation of ER expression and this downregulation is reversible upon inhibition of that MAPK.

3.4 Expression Profiling of Breast Cancer Cells with Hyperactivated MAPK Reveals Their Close Identity with ERα-Breast Cancer

We have developed gene expression profiles for our hyperactive MAPK cell lines using the Affymetrix gene array system (Creighton et al., 2006). Comparison of the gene expression profiles from the hyperactive MAPK cell lines to that from control

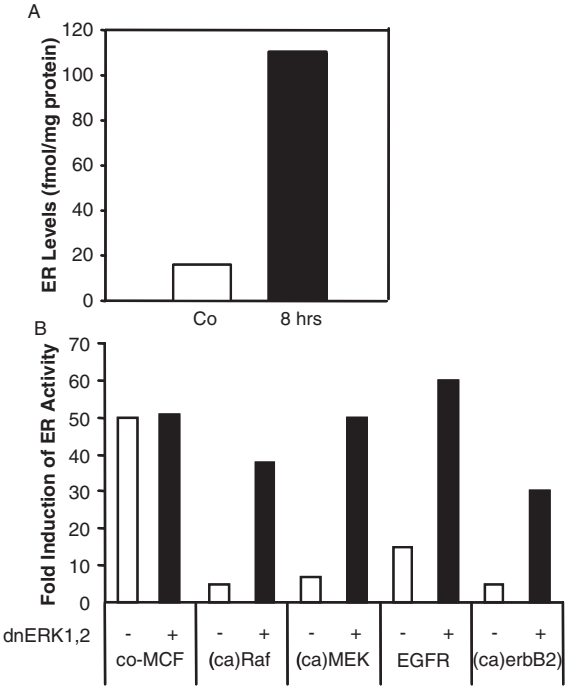

Fig. 3.2 Inhibition of MAPK activity restores both ERα expression and transcriptional activity. (**A**) (Ca)Raf cells were treated or not with 10 μM U0126 for 8 hours. Whole cell extracts were prepared and ERα measured by ligand binding. Values are expressed as fmol/mg protein. (**B**) Co-MCF-7 cells or the indicated cell lines with hyperactive MAPK were transfected with ERE-luc or NON-luc in the presence of the control vector pCEP4L (−) or equal amounts of dnERK1 and 2(+). Luciferase activity was determined 48 hours after transfection and data is presented as fold induction of ERE-luc by estrogen over vehicle control normalized to the NON-luc results

MCF-7 cells (co-MCF-7/lt-E2) generated a MAPK gene profile signature, as well as erbB-2, MEK, EGFR, and Raf specific profiles (Fig. 3.3A). Not surprisingly, several of the genes in the profile are known estrogen regulated genes such as PR, GREB1, SDF-1, and Myb, which are oppositely regulated by hyperactive MAPK (Fig. 3.3B) indicating not only the down-regulation of ERα itself, but also of its entire signaling cassette. However, a large number of genes with altered expression levels are unrelated to ER expression and function. These include many transcription factors, genes involved in neoplastic transformation, cell survival and viability, angiogenesis, and regulation of mitogenic signaling molecules such as erbB2, all processes expected from the known role of MAPK downstream signaling. Among these are genes such as the ets transcription factors ELF-4 and ETV5, RelB, VEGF, and PDCD4. Thus the MAPK signature gene set appears to impact every aspect of cellular function, thereby implicating high levels of MAPK activity in the growth and survival of breast tumors lacking ERα signaling.

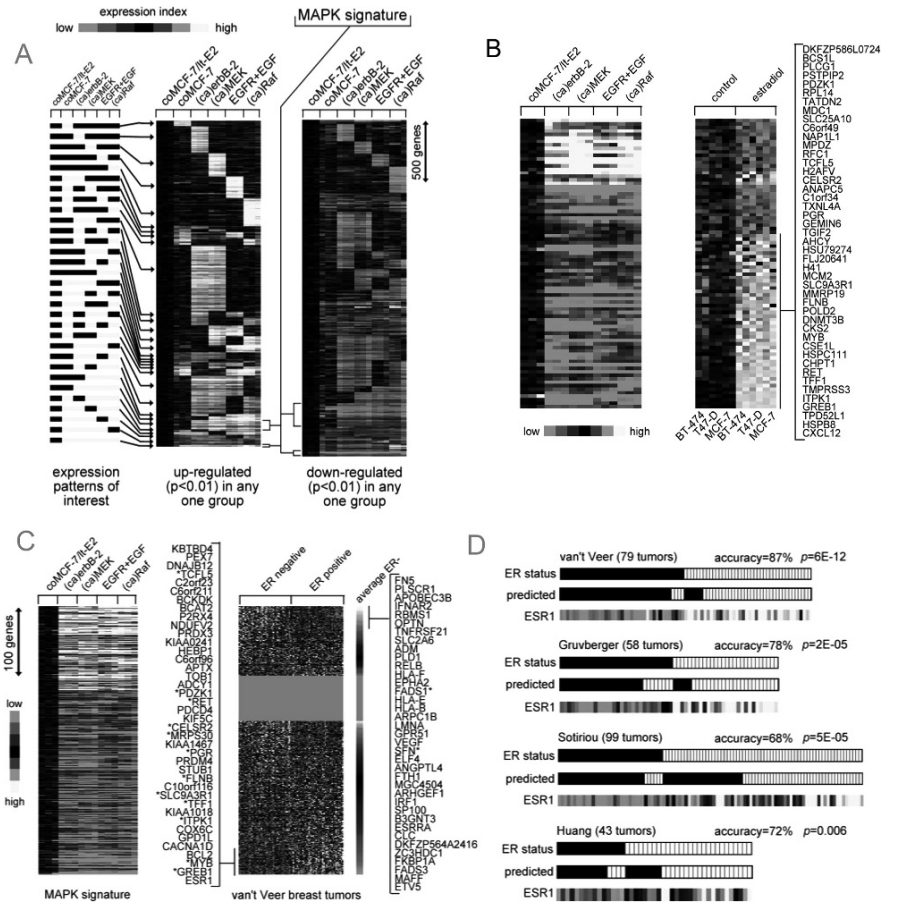

Fig. 3.3 A MAPK gene profile generated from in vitro cell line models predicts ERα-breast cancer. (**A**) Supervised clustering of expression values for genes showing significant up- or down-regulation ($p < 0.01$) in at least one MAPK+ cell lines, relative to coMCF-7/lt-E2 cells. Genes significantly expressed were evaluated against a set of pre-defined expression patterns to determine clusters of co-expressed genes. The level of expression of each gene relative to coMCF-7/lt-E2 is represented using a *grey scale*. MAPK gene signature is genes that show consistent up-or down-regulation in all MAPK+ cell lines. (**B**) The MAPK gene signature is inversely correlated with an mRNA-expression signature of estrogen induced breast cancer cells. (**C**) The mRNA expression signature of MAPK activation shares significant similarities with mRNA signatures of ERα-breast tumors. *Grey* denotes genes not represent in the van't veer breast tumor profiling. (**D**) Use of the MAPK signature to classify breast tumors from 4 independent datasets as ERα+ or ERα-. For a given tumor dataset, each profile was compared with the MAPK signature for similarity or dissimilarity. *White bars* represent ER+ tumors and *black bars* represent ER-tumors. ER status in tumor datasets was determined by pathology. ER mRNA (ESR1) levels of the tumor datasets is also shown

We went on to examine the expression patterns of these genes in vivo in public mRNA profile datasets of human breast tumors. When examining a dataset of 79 tumors from (Van't Veer et al., 2002), we found that most of the genes that

were up-regulated in the MAPK signature were also over-expressed in ERα-breast tumors relative to ERα+ tumors (Fig. 3.3C). Similarly, most of the genes down-regulated in the MAPK signature were under-expressed in ERα- relative to ERα+ tumors, including *ESR1* (the mRNA for ER) and several estrogen-inducible genes. The MAPK mRNA signature was found to be so similar to the ER-negative breast tumor signature that the in vitro MAPK signature expression pattern could be used to distinguish between ER-negative and ER-positive tumor profiles with 87% accuracy. When each tumor profile was classified (using the Pearson correlation) as being similar or dissimilar to an "idealized" MAPK pattern (+1 if up-regulated in the MAPK signature, −1 if down-regulated), tumor profiles that were similar to the MAPK signature were highly enriched ($p = 6.3\text{E} - 12$) for ER-negative tumors. The MAPK signature was able to classify ER-status with comparable accuracy in three other independent breast tumor datasets (Gruvberger et al. (2001) with 78% accuracy, $p = 2.4\text{E} - 05$; Sotiriou et al. (2003), 68% accuracy, $p = 4.6\text{E} - 05$; and Huang et al. (2003), 72% accuracy, $p = 0.006$) as shown in Fig. 3.3D. This is striking not only because the in vitro cell line profile is closely matching in vivo tumor profiles, but all the more so as this MAPK profile was generated from 4 stably transfected cell lines all derived from a single parental breast cancer cell line. Even more remarkable is that this single parental cell line is an ERα + /PR+ cell line that was converted to an ERα- phenotype by the overexpression of various members of the MAPK signaling pathway. The close correlation between the gene expression changes seen in our cell line models and those seen in ERα negative tumors highlights the validity of these cell lines as in vitro models of ERα-breast cancer, and further validates the role of MAPK signaling in both the down-regulation of ERα expression and the biology of ERα-breast cancer.

3.5 Restoration of ERα Expression and Anti-Estrogen Responses in ERα-Breast Cancer Cell Lines and Primary Cultures via MAPK Inhibition

Using established ERα-breast cancer cell lines, the SUM 229s which overexpress EGFR, SUM 190s which overexpress both EGFR and erbB-2, and SUM 149s which model inflammatory breast cancer and have very high levels of RhoC leading to hyperactivation of NFkB in addition to EGFR overexpression, we have recently demonstrated that inhibition of MAPK activity could result in restoration of ERα expression (Bayliss et al., 2007). In fact, inhibition of MAPK activity via the pharmacologic MEK inhibitor U0126 results in significant levels of ERα expression in each of the 3 cell lines (Fig. 3.4A–C). To extend these data further, we have also established primary cultures from ERα-breast tumors. In all 3 ERα- primary tumor cell cultures we examined, inhibition of MAPK activity (even the relatively short inhibition in DT5s) is sufficient to restore ERα expression in these cells (Fig. 3.5). In DT13s, which overexpress ErbB-2, herceptin is also effective in restoring ERα

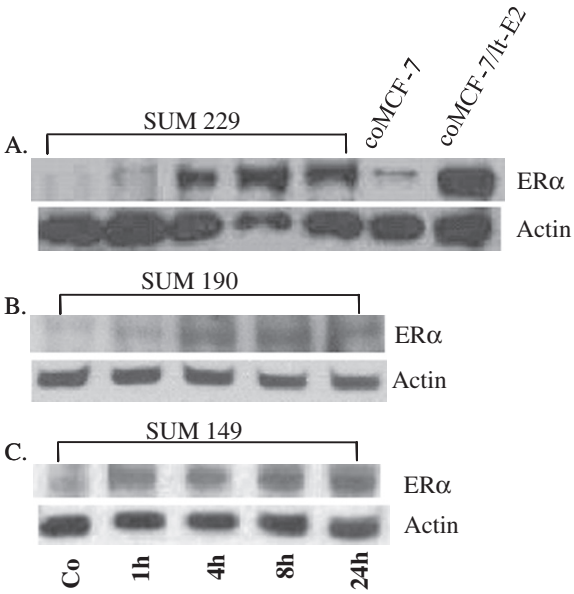

Fig. 3.4 ERα re-expression upon MAPK inhibition in ERα-breast cancer cell lines. ERα- SUM 229 (**A**), SUM 190 (**B**), and SUM 149 (**C**) breast cancer cell lines were treated with 10 μM U0126 or DMSO (co) for the indicated times and western blotted for ERα and actin as a loading control

expression (Fig. 3.5) even though the inhibition of MAPK by herceptin occurs early and does not last much beyond 4 hours.

This re-expressed ERα is able to restore anti-estrogen responsiveness in a subset of these ERα-breast cancer cell lines and primary tumor cell cultures. SUM 229s were analyzed in 6-day WST-1 growth assays with 1 μM U0126 which was the maximum tolerated daily dose for these cells. While 1 μM U0126 does not result in the same large increase in ERα expression that 10 μM does, it is still effective in restoring some ERα expression in SUM 229 cells (Fig. 3.6A). Growth assays were then performed where the effects of 4-hydroxy-tamoxifen (4HT) and the pure antiestrogen ICI 182,780 (faslodex) at 10^{-7} M alone, 1 μM U0126 alone, or the combination of 4HT or ICI and U0126 on cell proliferation were assessed using a WST-1 assay at 6 days. 1 μM U016, while having no growth inhibitory effects on its own, restored growth inhibitory effects of both 4HT and ICI (Fig. 3.6B, right-hand graph). For comparison, the growth inhibitory effects of both 4HT and ICI at 6 days on ERα+ MCF7s is shown in Fig. 3.6B (left-hand graph). SUM 149 cells, on the other hand, were extremely resistant to growth inhibitory effects of U0126 even though MAPK activity was inhibited. In this cell line where 10 μM U0126 resulted in a significant increase in ERα expression (Fig. 3.4A), this re-expressed ERα could not restore the growth inhibitory effects of anti-estrogens (Fig. 3.6C). This is most likely due to the hyperactivation of NFkB exhibited by these cells, a well established inducer of anti-estrogen resistance (Degraffenried et al., 2004; Nakshatri et al., 1997; Riggins et al., 2005). These 2 cell lines thus serve as examples of 2 different subsets of

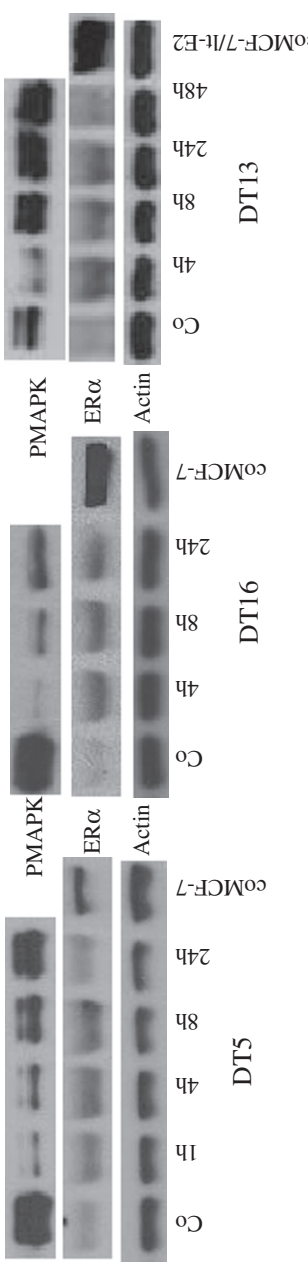

Fig. 3.5 Inhibition of MAPK in ERα-breast tumors restores ERα protein expression. P-MAPK, ERα and actin loading control in ERα- DT5 (*left-hand panel*), DT16 (*center panel*), and DT13 (*right-hand panel*). DT5 and DT16 were treated with 10 μM U0126 for indicated times and DT13 was treated with 500 ng/ml herceptin or the vehicle 1.1% benzyl alcohol (Co) for indicated times

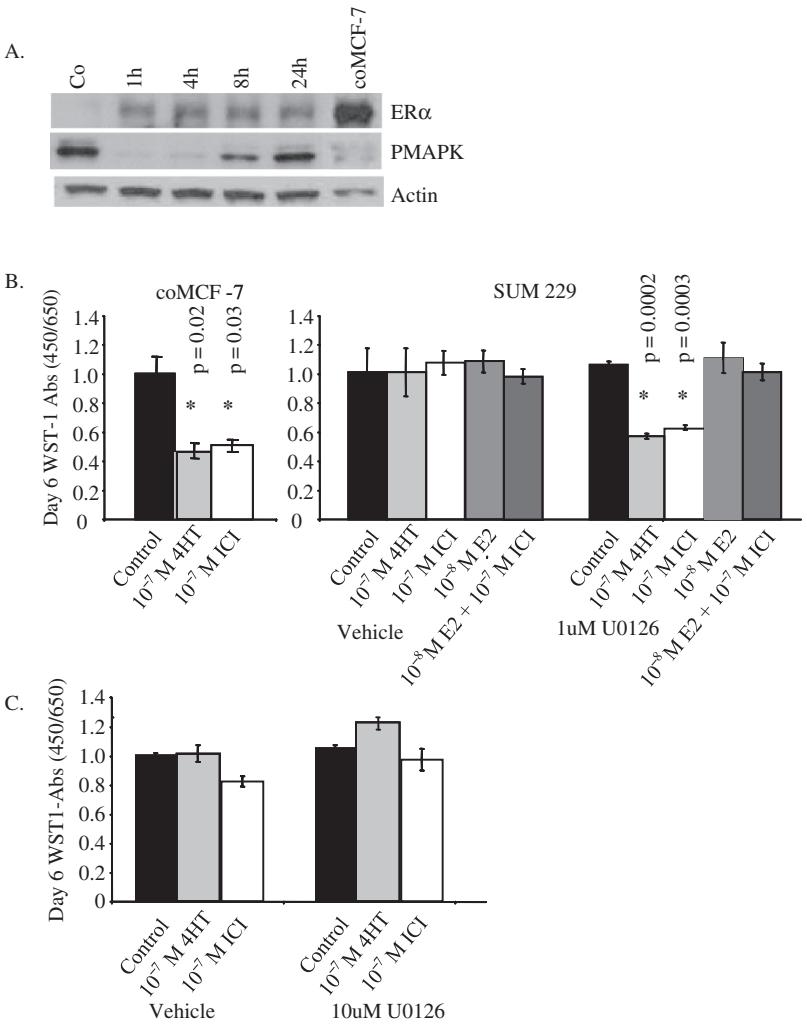

Fig. 3.6 Re-expression of ERα can restore anti-estrogen responses in ERα-breast cancer cell lines. (**A**) Sum 229 cells were treated with 1 μM U0126 or DMSO vehicle (co) for indicated times and then western blotted for ERα and actin. (**B**) Proliferation of coMCF-7 cells treated with 4-hydroxy-tamoxifen and ICI 182,780 or not (*left-hand panel*) or of sum 229 cells treated daily with U0126 and every other day with an anti-estrogens (*right-hand panel*) at 6 days measured by WST-1 assay. (**C**) Proliferation of sum 149 cells treated as in (B), except a higher U0126 dose of 10 μM was used

ERα-breast cancers: those in which EGFR/erbB-2 driven MAPK plays a role in cell proliferation and thus the concomitant inhibition of MAPK and restoration of ERα expression restores ERα driven growth pathways and anti-estrogen sensitivity vs. those in which pathways/factors other than growth factor receptor/MAPK drive growth and thus while inhibition of MAPK restores ERα expression, these other pathways/factors bypass ERα and maintain the anti-estrogen resistant phenotype.

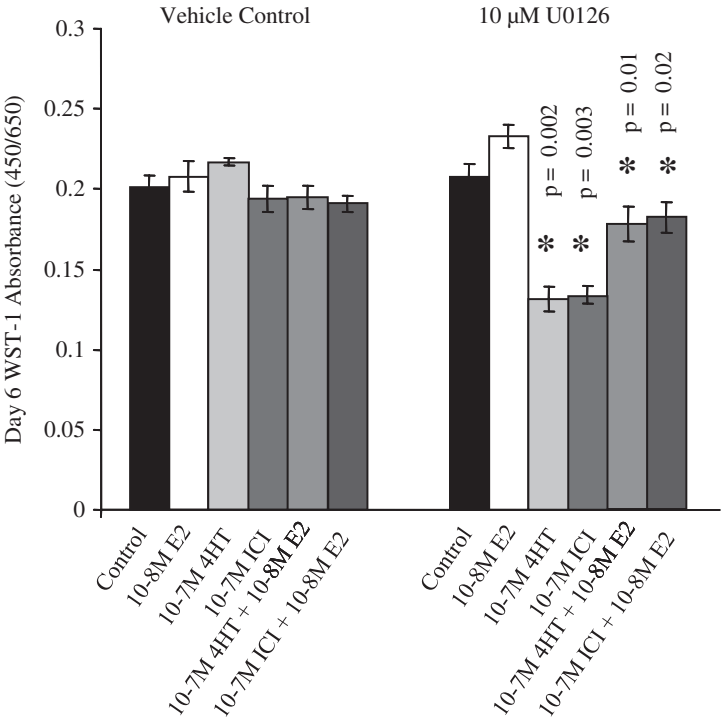

Fig. 3.7 MAPK inhibition in DT16 cells restores anti-estrogen sensitivity. Proliferation of DT16 cells treated with indicated concentrations of U0126 and/or estradiol, 4-hydroxy-tamoxifen, or ICI 182,780 measured by WST-1 assay at 6 days

The ability of restored ERα to mediate an anti-estrogen response in ERα- primary cultures from tumors was assessed in the DT16 cells in a 6 day growth assay where cells were treated with 10 μM U0126 every 48 hours. As seen with the established ERα- cell lines, in these dissociated ERα-tumor cells, re-expression of ERα upon inhibition of MAPK does restore responses to anti-estrogens (Fig. 3.7). These anti-estrogen responses are specific as estrogen at 10^{-8} M E2 is able to partially reverse the 4HT and ICI effects. Combined, these data suggest that re-expressed ERα, upon inhibition of MAPK activity, is capable of mediating the growth inhibitory effects of anti-estrogens in at least some ERα-breast cancers.

3.6 Breast Cancer Cell Lines Exhibiting a Basal Phenotype Do Not Exhibit MAPK-Dependent Re-Expression of ERα

We hypothesized that a third subset of ERα-breast cancers would exist: those in which inhibition of MAPK would not result in re-expression of ERα. It is well established that a subset of ERα-breast cancers exhibit hypermethylation of the ERα promoter resulting in the permanent repression of ERα (Ottaviano et al., 1994;

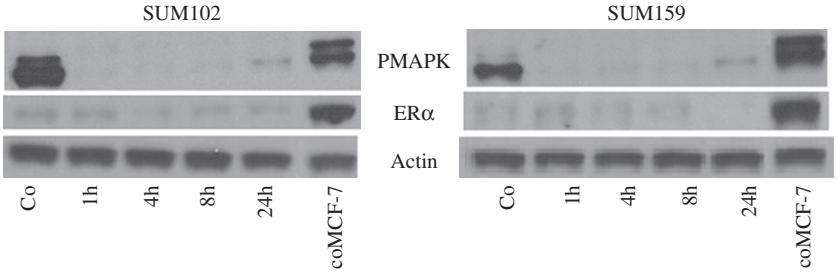

Fig. 3.8 Failure of MAPK inhibition to restore ERα expression in ERα-breast cancer cells with a basal phenotype. SUM102 and SUM159 were treated with 10 μM U0126 or DMSO vehicle (co) for indicated times and analyzed by western blotting for P-MAPK, ERα, and actin expression

Ferguson et al., 1995; Lapidus et al., 1998), and thus MAPK inhibition alone would not be expected to restore ERα expression in this case. More recently, breast tumors have been defined as having luminal cell properties or basal cell properties, with the basal cell phenotype correlating with lack of ERα expression, in some cases with BRCA mutation, and in many cases EGFR overexpression (Perou et al., 2000; Sorlie et al., 2001, 2003). We therefore examined two ERα-breast cancer cell lines (SUM 102 and SUM 159) that have been demonstrated by microarray analyses to display the basal phenotype (Neve et al., 2006) for the ability of MAPK inhibition to restore ERα expression. While both cell lines exhibit hyperactive MAPK and U0126 is able to effectively inhibit this MAPK activity, no restoration of ERα expression could be observed in these cells (Fig. 3.8). These 2 cell lines in fact turn out to exhibit hypermethylation of the ERα promoter (data not shown). These data suggest that additional mechanisms operate to repress ERα expression in at least 2 cell lines exhibting a basal phenotype, hypermethylation of the ERα promoter, such that MAPK inhibition alone is not sufficient to restore ERα expression.

3.7 Where Do We Go From Here in the Treatment of ERα-Breast Cancer?

We have demonstrated the ability to re-express ERα in ERα-breast cancer cells via the inhibition of hyperactive MAPK resulting from overexpression of EGFR or erbB-2 in both established ERα-breast cancer cell lines as well as ERα-tumors. This re-expression of ERα can be achieved via either direct inhibition of MAPK or via inhibition of the upstream growth factor receptor (EGFR or erbB-2) that is driving its hyperactivation. We have also established that the restoration of ERα expression is sufficient to induce anti-estrogen responses in a subset of these ERα-breast cancer cells.

Clearly then, strong signaling via MAPK directly represses ERα expression and ERα signaling is known to repress the expression of both EGFR and erbB-2. It is apparent that in breast cancer, the co-expression of high level signaling from both

receptor types is mutually exclusive. A clue for why this co-expression is not tolerated by breast cancer cells comes from our own studies as well as those of others. Upon the generation of our high growth factor signaling transfected lines, they were originally expanded in estrogen-containing media and exhibited a high rate of apoptosis (El-Ashry et al., 1996). Only upon shut-down of expression of the transfected gene (which occurred if cells were growing in the presence of estrogen) or down-regulation of ER expression by MAPK (as we've shown occurs when cell are grown in the absence of estrogen) did the cells not undergo apoptosis. Complimentary to these data, several investigators have forced the expression of ERα in ERα-breast cancer cell lines like MDA-MB-231 cells and have demonstrated that estrogen now induces apoptosis in these cells with high growth factor receptor signaling (Bayliss et al., 2007; Huang et al., 2003; Sotiriou et al., 2003). Collectively, these data indicate that high level signaling through both receptor systems is not tolerated by cells, although the mechanisms underlying this are not well understood.

The re-expression of ERα in established ERα-breast cancer cell lines had only been previously demonstrated via inhibition of DNA methylation or histone deacetylation in those cell lines in which the ERα promoter has been shown to be methylated (Ferguson et al., 1995; Ottaviano et al., 1994; Zhou et al., 2007). The methylation of ERα promoter is presumably a means of permanent repression secondary to some other down-regulating event. The down-regulation of ERα expression by hyperactive MAPK is a more direct mechanism and is dynamic and reversible – that is the down-regulation is reversed by the inhibition of MAPK activity and occurs again shortly after return of MAPK activity. And as we found with the two cell lines in which the ERα promoter turned out to be hypermethylated, despite the very high levels of MAPK exhibited by these cells and the effectiveness of U0126 in inhibiting MAPK, ERα expression could not be restored. Our data indicates that in addition to hypermethylation of the ERα promoter, hyperactivation of MAPK resulting from overexpression of EGFR or erbB-2 can also be directly responsible for the lack of ERα expression in ERα-tumors. Importantly, this MAPK meditated down-regulation of ERα expression can be targeted to result in re-expression of ERα. In support of our in vitro and ex vivo data, it has recently been shown that in a small study of 10 ERα-/erbB-2+ patients treated for various lengths of time with Herceptin, that 3 of these re-expressed ERα (Munzone et al., 2006). A more recent study by Massarweh et al suggests this mechanism can be exploited in ERα + /erbB-2+ tumors that lose ERα expression during treatment. They found that resistance to estrogen deprivation/fulvestrant in an ERα + /erbB-2+ MCF-7 xenograft model was accompanied by upregulation of MAPK activity and loss of ERα expression, and subsequent co-treatment with Iressa resulted in inhibition of MAPK activity and increased ERα expression (Massarweh et al., 2006).

Regardless of the different potential mechanisms for down-regulating/restoring ERα expression, the re-expressed ERα must be not only functional upon re-expression, that is induce the regulation of estrogen-responsive genes, but must also be able to regulate growth in response to estrogen/anti-estrogens in order to be clinically relevant. In studies where demethylation of the ERα promoter or use of histone deacetylase inhibitors restored ERα expression, this ERα was functional in

that it could regulate ERE-luciferase activity as well as the expression of specific estrogen regulated genes such as the progesterone receptor (PR) (Yang et al., 2000, 2001; Zhou et al., 2007). And in our previous studies in our hyperactive MAPK cell line models, re-expresion of ERα upon inhibition of MAPK also restored ERα's transcriptional activity (Bayliss et al., 2007; Holloway et al., 2004; Oh et al., 2001). The ability of the re-expressed ERα upon MAPK inhibition to mediate the growth inhibition of anti-estrogens in both established ERα-breast cancer cell lines and in dissociated tumor cell cultures demonstrates clearly for the potential for a novel therapeutic strategy for ERα-breast cancer. In these studies, three different cell line types were observed. In the cell lines (SUM 229 and SUM 190) which were quite sensitive to MAPK inhibition in terms of growth inhibition, restoration of ERα expression correlated with restoration of response to both 4-hydroxy-tamoxifen and ICI 182,780 (fulvestrant, faslodex), however, in the cell line (SUM 149) which also exhibits hyperactivation of NFkB and RhoC overexpression (Pan et al., 2002; van Golen et al., 2000a, 2000b), the re-expressed ERα was not able to restore responses to either anti-estrogen. A possible explanation for this is the hyperactivation of NFkB in these cells which is known to result in anti-estrogen resistance in breast cancer cells (Campbell et al., 2001; Degraffenried et al., 2004; Nakshatri et al., 1997; Riggins et al., 2005). Thus, even though the MAPK repression of ERα mechanism is operative in these cells and can be targeted to allow for re-expression of ERα, these cells have additional cell signaling alterations that allow them to bypass ERα and remain anti-estrogen resistant although now ERα+. Indeed, these cells were very resistant to growth inhibition induced by MAPK inhibition whereas even modest inhibition of NFkB significantly impacted their proliferation. The third cell line type were those cell lines (SUM 102 and SUM 159) in which MAPK inhibition alone could not restore ERα expression and which turned out to exhibit hypermethylation of the ERα promoter. These two cell lines in fact also were representative of the basal genotype of breast cancer as opposed to the luminal genotype. Whether in fact, basal breast cancers are marked by ERα promoter hypermethylation or whether ERα promoter hypermethylation occurs in a subset of basal breast cancers remains to be determined. Understanding these features of basal versus luminal breast cancers is important in terms of understanding the mechanisms of lack of ERα expression. While it is certainly possible that reversible mechanisms such as MAPK mediated down-regulation of ERα are operative in luminal breast cancers and more permanent mechanisms such as hypermethylation of the ERα promoter are operative in basal breast cancers, our data with our primary cultures from ERα-breast tumors would seem to argue against this, at least preliminarily. The number of ERα- luminal breast cancers that are not also erbB-2/Her-2neu overexpressors is not large. And yet, both of the triple negative tumors from which we generated primary cultures exhibited MAPK mediated down-regulation of ERα expression and did not have hypermethylation of the ERα promoter. It would seem too lucky that both of these happened to be representative of the small number of luminal triple negatives that exist. Analyses of the increasing number of primary cultures we have from triple negative breast cancers will help answer this question.

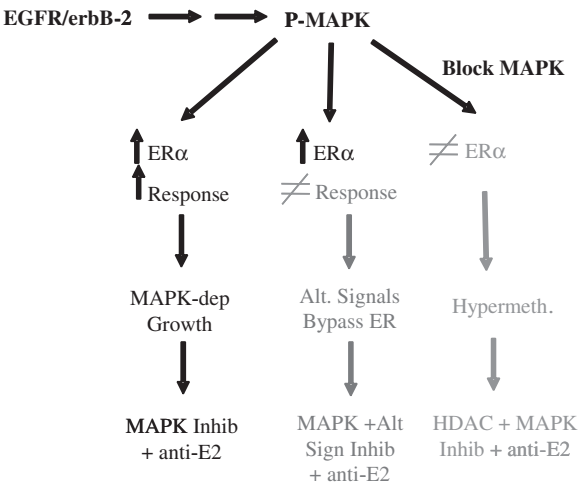

Fig. 3.9 Model of ERα-breast cancer subsets with potential mechanisms and therapies for each

Together these data are suggestive of a number of important possibilities for the treatment of ERα-breast cancer (Fig. 3.9). First, it is clear that in a significant majority of ERα-breast tumors, hyperactivation of MAPK by upstream overexpressed/hyperactive EGFR or c-erbB-2 represses ERα expression and thus can be targeted to allow for re-expression of ERα. This targeting can be at the level of MAPK activity itself or via upstream inhibition of EGFR/erbB-2 signaling. In the subset of ERα-tumors exhibiting hypermethylation of the ERα promoter, such targeting alone is not successful in restoring ERα expression but would most likely be necessary to maintain ERα expression after demethylation of the promoter as these tumors also exhibit high MAPK activity. Importantly, restoration of ERα expression simultaneously restores estrogen/anti-estrogen responses in those ERα-tumors where MAPK signaling seems to be the predominant mediator of proliferation. However, where alternative signaling pathways, such as NFkB, appear to be the predominant proliferation mediators, concomitant inhibition of the alternate signaling pathway would be necessary to allow the restored ERα to mediate anti-estrogen responses. In those tumors exhibiting hypermethylation of the ERα promoter, where it has recently been shown that inhibitors of histone deacetylases (HDACs) is equally effective in relieving the repression of ERα transcription (Zhou et al., 2007), a combination of an HDAC inhibitor and MAPK inhibition may be an effective means of restoring anti-estrogen responses. Finally, these data indicate that ERα status, rather than being solely positive or negative, is a dynamic process strongly impacted by the signaling environment of breast cancer cells.

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Abbreviations

ERα:	estrogen receptor α
DCIS:	ductal carcinoma in situ
ADH:	atypical ductal hyperplasia
EGFR:	epidermal growth factor receptor
MAPK:	mitogen activated protein kinase
c-erbB-2:	epidermal growth factor receptor 2
siRNA:	short interfering RNA
ERK:	extracellular signal regulated kinase
ERE:	estrogen response element
PR:	progesterone receptor
GREB1:	gene regulated by estrogen in breast cancer protein
SDF-1:	stromal-derived factor 1
ELF-4:	E74-like factor 4
ETV5:	ets variant gene 5
RelB:	v-rel reticuloendotheliosis viral oncogene homolog B
VEGF:	vascular endothelial growth factor
PDCD4:	programmed cell death 4
NFkB:	nuclear factor of kappa light polypeptide gene enhancer in B-cells
HDACs:	histone deacetylases

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Chapter 4
The Dark Side of Antihormonal Action in Breast Cancer

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Contents

Abstract Antihormones are of substantial benefit in treating oestrogen receptor-α positive (ER+) breast cancer. However, their anti-tumour effect is limited by emergence of resistance. Our in vitro studies are highlighting a new underlying concept: that antihormones are not passive bystanders but alongside growth inhibitory effects promote adverse compensatory mechanisms within tumour cells. These mechanisms involve drug-induction of signalling elements normally suppressed by oestrogen (E2)-occupied ER̃ While best exemplified by the tyrosine kinases epidermal growth factor receptor and HER2, microarrays reveal the true diversity of the induced signalling kinases, where their potential to promote resistance is exacerbated under paracrine conditions. Such drug-induced events permit initial ER+ breast cancer cell survival, allow development and maintenance of resistance, and also promote gain of invasiveness under conditions of poor intercellular contact. In addition, prolonged antihormonal exposure is associated with epigenetic

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silencing of classical E2-induced tumour suppressors, an event which further contributes to resistance. Based on proof of principle experiments targeting induced signalling events alongside antihormones or restoring E2-induced suppressor genes through DNA methylation inhibitor-containing strategies, it is our belief that continued deciphering of these mechanisms will reveal improved treatments for breast cancer.

Keywords Antihormone · Resistance · Microarray · Compensatory signalling · Tumour suppressor

4.1 Introduction

Antihormones that deplete oestrogen (E2)/oestrogen receptor α (ER) signalling promote worthwhile tumour remissions and significant survival benefits in many ER positive (ER+) patients in the advanced and adjuvant setting. Some of these agents act by competing with E2 for binding to its target receptor in breast cancer cells, resulting in an ER conformational change that blocks one of the two receptor Activation Functions, AF-2, as exemplified by "partial" antioestrogens such as tamoxifen. Others, notably aromatase inhibitors, severely deplete the oestrogenic environment which theoretically should drive the ER into a fully inactive state, potentially equating with improved responses seen with such agents versus tamoxifen. However, the efficacy of all current antihormones is incomplete since there are a proportion of ER+ patients who exhibit an apparent intrinsic resistance, while despite differences in response duration according to antihormone type, acquisition of resistance also remain inevitable for 40% initial responders in the adjuvant setting and almost all patients with advanced metastatic disease (Nicholson and Johnston 2005; Normanno et al. 2005). Unfortunately, resistance can also associate with increased metastatic capacity of breast cancer cells (Hiscox et al. 2004, 2006a) and invariably ultimately equates with poorer outlook clinically.

Many mechanisms have been associated with antihormone resistance in ER+ breast cancer. Of note, based on pre-clinical studies including our own primarily with tamoxifen, changes in the dominant growth factor receptors (e.g. the receptor tyrosine kinases HER2 and the additional erbB family member epidermal growth factor receptor [EGFR]) and their downstream kinases (notably Ras/Raf/mitogen-activated protein kinases [MAPKs] and phosphoinositide 3-kinase [PI3K]/protein kinase B [AKT] signalling) have been substantially implicated in driving resistance (Gee et al. 2005a; Nicholson et al. 2007). Such signalling can be apparent de novo in the tamoxifen refractory state (e.g. associated with HER2 amplification), or can be a feature gained in the relapsed tumour cells (Knowlden et al. 2003). In some instances, these growth factor signalling pathways can harness the ER to promote growth despite presence of antihormones. Indeed, growth factor pathway/ER cross-talk at the nuclear level (including growth factor-driven ER phosphorylation

and coactivator recruitment; Britton et al. 2006; Arpino et al. 2008), as well as non-genomic events at the plasma membrane (Fan et al. 2007), have been cumulatively implicated in promoting agonistic behaviour of tamoxifen in resistance and E2 hypersensitivity in the steroid-deprived environment (Nicholson et al. 2004). In addition, using classical signalling techniques such as Western blotting and immunocytochemistry as applied to our acquired tamoxifen resistant in vitro breast cancer cell model (TAMR), we have been able to identify that a further important growth factor receptor input in driving acquired tamoxifen resistance is the insulin-like growth factor receptor (IGF1R) that acts to facilitate EGFR signalling via activation of the tyrosine kinase (TK) Src kinase (Knowlden et al. 2005). Of note, when extreme, growth factor pathways can also work in an ER independent manner and can even promote ER loss in vitro and clinically in a small proportion of patients (Holloway et al. 2004; Munzone et al. 2006).

Such growth factor-driven mechanisms and their cross-talk with ER are being exploited therapeutically. Targeting of functional ER in recurrent disease through use of further antihormonal measures, such as the "pure" antioestrogen fulvestrant (faslodex ®) which reduces ER level, can be beneficial in breast cancer (Howell 2006; Chia and Gradishar 2008). However, intrinsic and acquired resistance again remain a significant hurdle. Based on promising model data, diverse clinical studies are also exploring the value of anti-erbB pharmacological inhibitors/antibodies and also agents blocking candidate downstream signalling (including MAPK inhibitors, farnesyltransferase inhibitors and mammalian target of rapamycin inhibitors), where the aim is again to treat resistance, and (alongside antihormones) delay this state and restore response (Johnston et al. 2008). However, to date many of these studies have proved relatively disappointing, with therapeutic resistance again a pervading problem (see Chapter 10). For example, in breast cancer while clinical benefit was commonly seen in our acquired resistance study with the anti-EGFR agent gefitinib, this largely comprised disease stabilisation that invariably culminated in disease relapse (Agrawal et al. 2005). Based on model systems, these hurdles were perhaps predictable since, for example, our EGFR+ TAMR cells showed initial growth inhibitory responses to the anti-EGFR agent gefitinib but were ultimately subject to resistance, an event also associated with a further gain in aggressive behaviour (Jones et al. 2004), while one of our EGFR+ fulvestrant resistant models (FASRLT) proved largely de novo resistant to this agent. In addition, EGFR blockade exerted only partial inhibitory effects on invasion of such acquired resistant models (Hiscox et al. 2004). Moreover, breast cancer xenograft studies (Shou et al. 2004) indicated that EGFR targeting alongside antihormones delayed (rather than prevented) development of resistance, although pan-erbB approaches can be more successful in such models (Arpino et al. 2007). Problems of resistance appear to extend to the anti-HER2 agent herceptin. While of improved value in treating breast cancer patients when combined with chemotherapy, responses to herceptin as a single agent only occur in $\sim$30% of HER2+ patients (McKeage and Perry 2002) and again relapse is invariably the ultimate outcome following initial response to various herceptin treatment strategies (Nahta et al. 2006). While there are many reasons likely to contribute

to the relatively disappointing performance of these types of agents clinically, it is likely that there are additional important determinants of endocrine resistance and associated progression in breast cancer that remain to be defined. Indeed, there is emerging data that clinical antihormone resistance is associated with heterogeneous gene expression profiles (Miller et al. 2008), suggesting that resistance involves multiple underlying pathways.

Use of high through-put microarray strategies has potential to be particularly enlightening in determining the breadth of drivers for resistant growth and progression. Our group has embraced Affymetrix (HG-U133A GeneChip) microarray technology as applied to in vitro breast cancer models to focus on identifying deregulated members of the "kinome" and their key regulators in this context. Particular emphasis has initially been placed on the TK category of the kinome (as defined by Manning et al. 2002 and the "KinWeb" resource, *http://kinweb.ceinge.unina.it/*) since these have commonly been implicated in neoplastic development and progression and moreover are being intensively studied as targets for anti-cancer drug development (Vieth et al. 2005). Alongside use of web-based GeneSifter™ profiling and cluster analysis of the expression data, two external collaborations are significantly aiding our array analysis: ALMAC *(http://www.almac-diagnostics.com/)* who are applying sophisticated global pathway and virtual network building algorithms to our microarray data to predict how deregulated signalling genes might interact, and Dr. GR Ball of The Nottingham Trent University School of Biomedical and Natural Sciences who is applying linear regression analyses to the same datasets. These discovery studies are being complemented within the Tenovus Centre, Cardiff by RT-PCR and protein verification, examining siRNA knockdown of the verified targets in relation to breast cancer cell growth and invasion, as well as exploring potential clinical relevance through virtual profiling in clinical breast cancer using the online Oncomine™ cancer transcriptome database, *http://www.oncomine.org* (Rhodes et al. 2004).

The following article presents two key concepts relevant to antihormone resistance, largely unexplored in the context of breast cancer resistance, that have been derived primarily through application of microarrays to our in vitro breast cancer models. Specifically, we have investigated impact of antihormones on the gene expression profile of ER+ MCF-7 cells and also examined their acquired resistant variants (encompassing resistance to tamoxifen, fulvestrant or E2-deprivation), most recently extending our studies to multiple models to more fully-reflect the breadth of ER+ breast cancer sub-types. A common theme of our findings is that antihormonal agents are not merely passive bystanders, but that alongside growth inhibitory effects they exert more sinister activity in that they also influence gene expression and signalling in an undesirable manner (Gee et al. 2006). Importantly, this drug-induced "re-programming" appears to limit the magnitude of initial antihormone response and ultimately promotes and maintains resistant growth, as well as facilitating further features of progression. Based on proof of principle data, it is our belief that continued deciphering of these mechanisms could provide improved treatments for breast cancer in the future.

4.2 Compensatory Pathways Are Induced by Antihormonal Treatment

4.2.1 Oestrogen Repression and the Antihormone-Induced Compensatory Genes EGFR and HER2

Classically, E2 acts to induce expression of genes whose target promoters bear oestrogen response elements (EREs). However, it is transcriptional repression of genes that has been reported to comprise the bulk (70%) of expression changes associated with E2 challenge of ER+ breast cancer models (Frasor et al. 2003, 2004), although the underlying molecular biology of this phenomenon remains poorly-understood (Zubairy and Oesterreich 2005). One mechanism proposed is that the E2/ER complex enters into protein/protein interactions with further transcription factors (e.g. the nuclear factor of kappa light polypeptide gene enhancer in B-cells [NFκB]) leading to repression at diverse response elements (Stein and Yang 1995; Valentine et al. 2000; Kalaitzidis and Gilmore 2005). Competition for, or between, coactivators at E2-repressed gene promoters may also contribute. For example, HER2 has been established as an E2-repressed, antihormone-induced gene in breast cancer models representative of several ER+ sub-types (Bates and Hurst 1997; Newman et al. 2000). E2 repression of HER2 is reported to occur via effects at intron 1 involving competition for the co-activator SRC1 between the E2/ER complex and AP-2 transcription factor (Newman et al. 2000). EGFR is also E2-repressed in multiple ER+ breast cancer models, an event again associated with a negative regulatory element in the first intron (Yarden et al. 2001; Wilson and Chrysogelos 2002). Finally there are data to indicate that the E2/ER complex may recruit repressors to some gene promoters. For example, DEAD box RNA helicase (DP97) corepressor recruitment to the HER2 gene is promoted by E2 (Rajendran et al. 2003), while SMRT and further N-CoR recruitment contributes to ER/Sp transcription factor-mediated E2 repression at GC-rich sites in the vascular endothelial growth factor receptor-2 gene (VEGFR2; Higgins et al. 2008). With regards to regulation of the anti-proliferative gene cyclin G2, the E2/ER complex again recruits N-CoR and, interestingly, histone deacetylases (HDACs), associated with release of RNA polymerase II and transcriptional repression (Stossi et al. 2006). There is emerging data that such repressive events can occur at ERE-bearing promoters in breast cancer cells (Kaipparettu et al. 2008; Ye et al. 2008).

In many instances the repressive effects of the E2/ER complex in ER+ breast cancer cells can be counteracted by antihormones during early response, triggering gene re-expression. Although the consequences of these antihormone-induced events for the tumour cell have not been significantly explored, Frasor et al. (2004) and others have reported that a proportion of the E2-suppressed genes (e.g. cyclin G1) are anti-proliferative/proapoptotic (Shaw et al. 2005; Stossi et al. 2006). Thus it is likely that their expression contributes to anti-tumour effect when induced by antihormones. However, there appears to be further important pharmacological significance of E2 repression. Our emerging experiences in breast cancer model

systems indicate early ER blockade, from 1 week treatment, also induces expression of diverse cell survival/proliferation signalling genes (Gee et al. 2003, 2006). Interestingly, the growth inhibitory effects of antihormones are rarely complete in ER+ models, with some cells evading growth inhibition during the drug responsive phase, culminating in anti-tumour responses of finite duration (McClelland et al. 2001; Gee et al. 2003, 2006). Furthermore, antihormones exert considerable anti-proliferative effects but generally promote only modest cell kill (Gee et al. 2003). In clinical breast cancer, while persisting tumour cells cannot be easily tracked following adjuvant therapy, the growth inhibitory effects of antihormones must nevertheless invariably also be incomplete since relapse can ultimately occur (Kenny et al. 2001). Similarly in the advanced setting, quality of response is variable (and at its extremes may represent a disease stabilisation; Cheung et al. 1997), where subsequent acquisition of resistance is sadly inevitable. Clearly, early "protective" effects must be present during antihormone-treatment that permit emergence of resistance. Our previous in vitro studies in MCF-7 cells monitoring two key E2-repressed genes, EGFR and its favoured heterodimerisation partner HER2, have provided robust proof of principle that signalling genes induced by antihormones such as tamoxifen or fulvestrant have considerable potential to limit initial anti-tumour response of ER+ cells and to maintain a cell population from which resistance subsequently emerges (McClelland et al. 2001; Gee et al. 2003). While initially inhibiting IGF1R, we determined that tamoxifen induced EGFR expression (alongside HER2). This re-programming of the cells maintained residual activity through kinases including MAPK and AKT and their cross-talk with ER, low levels of proliferation and some expression of the pro-survival gene B-cell lymphoma protein 2 (bcl-2). The drug's anti-tumour effects were consequently associated with only partial inhibitory effects on proliferation and only minimal induction of apoptosis, culminating in incomplete growth inhibition (Gee et al. 2003). By three months, the majority of tamoxifen treated cells were EGFR+ and had substantially gained kinase activity. Not surprisingly, tamoxifen resistance emerged at this time, co-incident with restoration of ER activity and hence expression of ER-regulated growth factor ligands, notably insulin-like growth factors and amphiregulin, that completed an EGFR/HER2 autocrine mitogenic loop facilitated by IGF1R (Knowlden et al. 2003, 2005; Britton et al. 2006). While the data remain controversial, some studies indicate there may also be modest increases in various components of EGFR/HER2/kinase signalling at the time of tamoxifen relapse in some breast cancer patients (Gee et al. 1999, 2005a; Gee and Hutcheson 2005; Gutierrez et al. 2005).

4.2.2 Microarrays Reveal the Considerable Diversity of Antihormone-Induced Compensatory Genes

Our application of microarray technology is revealing the surprising breadth of re-programming of cells, far exceeding gain of either EGFR or HER2, that occurs when ER+ breast cancer cells are treated with antihormones. Several studies have

employed microarrays to decipher transcriptional impact of E2 and whether antihormones can reverse these profiles (Inoue et al. 2002; Levenson et al. 2002a,b; Cunliffe et al. 2003; Frasor et al. 2003, 2004; Hodges et al. 2003); however, our microarray studies focusing on determining the spectrum of antihormone-induced events with positive signalling ontology that may limit drug response (and thus could provide future targets alongside antihormones) are, to our knowledge, unique. Our initial studies using smaller format arrays revealed a number of interesting themes among antihormone-induced events that are now being strengthened by high-throughput Affymetrix studies (Gee et al. 2004, 2006; Shaw et al. 2005).

4.2.2.1 Elements that May Facilitate Resistance Can Be Induced by Specific Antihormones or Universally by ER Blockade

We have observed that some signalling genes are induced by antioestrogens more effectively than E2 deprivation (Shaw et al. 2005), as exemplified by Bcl-2-associated athanogene (Bag1). The Bag1 co-chaperone is reported to influence protein refolding, to interact with bcl-2, ER and growth factor receptor signalling, and to promote proteasomal degradation of denatured proteins. As such, it can promote cell survival and limit chemo-/radioresponse (Townsend et al. 2005). Use of the Affymetrix platform has subsequently revealed 15 antihormone-induced TKs (alongside EGFR and HER2; Gee et al. 2006) with undesirable ontology and confirmed that some of these were increased substantially only by antioestrogens, including various Ephrin receptors (a TK class of emerging interest in breast cancer; Fox and Kandpal 2004), and also the target receptor for hepatocyte growth factor (HGF)/scatter factor, Met (Hiscox et al. 2006b). However, other genes such as NFκB1(p105) are universally induced by all antihormones, implying this transcription factor that can lie downstream of several signalling pathways may play a generic role in limiting initial antihormone response. NFκB1 is a member of the Rel family that dimerise to drive transcription of NFκB-dependent genes and where such signalling is E2-repressed at multiple levels (Kalaitzidis and Gilmore 2005). In parallel, we observed increases in nuclear NFκB1 immunostaining (indicative of increased translocation of active p50 NFκB1) during treatment with various antihormones, as further confirmed by DNA binding ELISA assays, while antihormone induction of transcriptional NFκB1 activity was demonstrated using reporter gene construct assays (Shaw et al. 2005). NFκB signalling has been implicated substantially in promoting cell survival/proliferation under conditions of environmental stress and in chemo- and radioresistance (Wu and Kral 2005) and as such is a strong candidate to promote antihormone resistance. In the Affymetrix array studies some TKs were also induced by all the antihormones examined, exemplified by a Src kinase family member, Lyn, that is reported to be glucose stress-activated and linked to imatinib-resistant leukaemia (Wu et al. 2008), hormone-refractory prostate cancers (Goldenberg-Furmanov et al. 2004) and chemoresistant colon tumours (Bates et al. 2001).

4.2.2.2 While Some Antihormone-Induced Events Are Only Transiently Increased During Initial Response, Others Persist into Resistance Adding to the Mechanistic Complexity Underlying this State

Extended array profiling of E2-repressed, antihormone-induced genes into acquired resistance using our MCF-7 sublines resistant to antioestrogens (TAMR and FASRLT cells; McClelland et al. 2001; Knowlden et al. 2003) or to severe E2 deprivation (XMCF-7 cells; Staka et al. 2005) has revealed two cohorts of antihormone-induced genes (Gee et al. 2006). Firstly, there are genes where any cell survival/proliferative effects must be contributory only during the antihormone responsive phase since they are transiently expressed. Secondly, however, some antihormone-induced genes persist into the acquired resistant variants, as exemplified by EGFR and HER2 whose expression (and activity) remains prominent in our TAMR cells (Knowlden et al. 2003). An example of a further TK maintained at high levels in the TAMR cells is Lyn (Gee et al. 2006), where we have recently confirmed an important contribution to proliferation and cell survival through siRNA and pharmacological challenge experiments (R Hendley, personal communication). The transcription factor NFκB1 was also retained at a high expression/activity level in the MCF-7X and FASRLT resistant cells, where interestingly constitutive NFκB activity has been reported in further acquired E2-independent (Pratt et al. 2003) and fulvestrant resistant models (Riggins et al. 2005) as well as in tamoxifen resistant cell lines and tamoxifen relapse clinically (Zhou et al. 2005, 2007).

Extended profiling has revealed that longer-term treatment, concomitant with emergence of resistance, in addition to retaining some of the early-induced elements is also associated with gain of further potentially-adverse signalling genes. We observed that 26 kinases with possible adverse ontology and undesirable Oncomine™ transcriptional profile in clinical breast cancer (encompassing several TKs in addition to EGFR) were deregulated in our aggressive FASRLT model. These included Met, a TK we have previously shown to be able to contribute to invasive behaviour of fulvestrant resistant cells (Hiscox et al. 2006b). Interestingly, some of the deregulated kinases we observed have been reported to predict basal phenotype and also adverse prognosis within luminal breast cancers (Finetti et al. 2008), in keeping with the adverse behaviour that is prominent in the essentially-luminal FASRLT model. In addition to these observations of substantial diversity of kinases associated with acquisition of resistance, our studies showed that molecules that are capable of further enhancing TK signalling are also gained. Of particular note is the adhesion molecule CD44 (increased in both the TAMR and FASRLT models; Harper et al. 2005) that can impact on cancer cell growth and invasion through diverse mechanisms, including a reported ability to enhance signalling from multiple TKs including erbB family members, Met, Src kinase and Lyn (Ponta et al. 2003). Clearly, rather than simple, restricted mitogenic/proinvasive networks, there are many kinases and TKs deregulated in antihormone resistant breast cancer cells coupled with “facilitators” capable of maximising signalling through multiple TKs. Examination of gene lists from further in vitro microarray studies equally indicates diversity alongside erbB receptors and that it is genes normally regulated (positively

or negatively) by ER that are commonly targeted for deregulation in antihormone resistance (Fan et al. 2006), aspects potentially conferring remarkable flexibility on the breast cancer cell when faced with blockade of any one pathway. This finding has obvious significance when considering the clinical shortfalls of targeted signal transduction inhibitors to date.

4.2.2.3 The Adverse Impact of Some Antihormone-Induced Genes on Breast Cancer Cell Behaviour May Only Be Maximised in an Appropriate Cell Context

Antihormone-Induced Invasion is Dependent on Intercellular Contacts

It is reported that E2/ER signalling exerts a protective effect on invasiveness and motility (Platet et al. 2004), exemplified by the low basal migratory behaviour that is a feature of MCF-7 cells (Hiscox et al. 2004, 2006a; Borley et al. 2007), and in general ER+ breast cancers have a better prognosis. The mechanism(s) whereby E2 suppresses invasiveness remains largely unknown, but seems to require integrity of the functional ER domains that are also involved in transcriptional activation of target genes (Platet et al. 2000; Maynadier et al. 2008). As such, E2 may act to stimulate expression of anti-invasive ERE-regulated genes (Platet et al. 2004), but it is also feasible that ER protein-protein interactions and repression events at EREs or further response elements may contribute if such events limit pro-invasive genes (Platet et al. 2000). This has been exemplified by recent studies examining Slug, a gene thought to repress E-cadherin expression leading to an epithelial-mesenchymal transition (EMT), whose expression is E2-repressed via half-ERE sites (Ye et al. 2008).

We (and others) have noted in turn that ER blockade using antioestrogens is associated with a small induction of MCF-7 invasiveness in vitro (Platet et al. 2000, 2004; Hiscox et al. 2006c; Borley et al. 2007). Our array studies have revealed some antioestrogen-induced genes do indeed have a pro-invasive ontology (Shaw et al. 2005; Wu and Kral 2005). These include RhoE (Rho family GTPase 3), an anti-proliferative Rnd family member that (controversially) also promotes actin cytoskeleton remodelling, rounding and migration (Guasch et al. 1998; Riento et al. 2005; Klein et al. 2008), as well as several TKs again including the Src family member Lyn (Suzuki et al. 1998). In addition to genes increased at an expression level, we have shown that Src kinase activity is also enhanced by tamoxifen (Hiscox et al. 2006c,d; Borley et al. 2007) and our preliminary data indicates this phenomenon extends to multiple breast cancer models reflective of ER+ breast cancer sub-types. It is thus feasible that these genes may contribute towards the small inductive effect of invasiveness exhibited in the presence of antioestrogens, and could also play a more substantial pro-invasive role following longer-term antioestrogen challenge when the more aggressive acquired resistant state emerges (Hiscox et al. 2006a,d; Borley et al. 2007). In keeping with this concept, increased expression of these genes is invariably retained in our antioestrogen resistant cell lines, where we have shown through siRNA studies that Lyn contributes to migratory behaviour of TAMR

cells (R Hendley, personal communication), also with a key role for the increased activation of Src kinase (Hiscox et al. 2006d).

However, significant induction of the various pro-migratory genes clearly does not result in a substantially increased invasiveness during the antioestrogen responsive phase. Intriguingly, we have found that the full impact of adverse antioestrogen-induced genes may only be apparent in a cell context where intercellular contacts are compromised. MCF-7 cells have strong cell-cell contacts maintained by E-cadherin (Hiscox et al. 2004, 2006a,c; Borley et al. 2007). However, neutralisation of E-cadherin, which confers only small increases in invasiveness, substantially enhances the ability of antioestrogens to induce MCF-7 invasiveness (Gee et al. 2006; Hiscox et al. 2006c; Borley et al. 2007; see Chapter 8). Such observations could have important implications for antioestrogen use in ER+ tumours with poor intercellular contacts, potentially where E-cadherin expression is lost by genetic or epigenetic mechanisms (Droufakou et al. 2001) as exemplified by lobular cancers, or its contribution towards maintaining cell-cell contacts is dysfunctional (Rakha et al. 2005). In such tumours, antioestrogen-induced events within cells surviving treatment may promote aggressive cellular behaviour apparent at the time of relapse and hence ultimately poorer prognosis. This has recently been reported for lobular cancers, where there is a persistent late relapse rate, and overall outcome can be worse than for their ductal counterparts (Rakha et al. 2008).

Importance of Paracrine Environment

Interestingly, while some of the antihormone-induced TKs revealed using our Affymetrix array studies may directly contribute to breast cancer cell growth, we are amassing evidence that the function of others may only become apparent in a paracrine environment, adding a further layer of complexity to resistance mechanisms that may be of key importance in vivo. Thus, while we have observed the additional erbB receptor HER3 can be antihormone-induced and hence is at low levels in MCF-7 cells (Knowlden et al. 2003), this model (and its resistant sub-lines) fails to produce ligands for the receptor. However, mimicking paracrine ligand availability through exogenous treatment of these cells with heregulin B1 (a ligand that hyperactivates HER3 receptor and its favoured heterodimer HER2) has revealed the considerable potential of this receptor to subvert antioestrogen response (Nicholson et al. 2004), in accordance with its potent ability to promote growth and progression of breast cancer (Tsai et al. 2003). This concept has been reiterated with regards to induction of invasive behaviour through studies performed in our model chronically-exposed to fulvestrant which has increased expression of the TK Met (Hiscox et al. 2006b). Activity of this receptor and invasive behaviour can be substantially further enhanced in FASRLT cells by exogenous HGF, a factor not produced by FASRLT cells but fibroblast-derived in a paracrine environment (Hiscox et al. 2006b). Clearly the problem of induced genes is likely to be exacerbated by the in vivo milieu since clinical breast tumours are invariably exposed to high levels of diverse growth factor ligands arising from paracrine and endocrine means (Dunn et al. 2004;

Hiscox et al. 2006b; Hutcheson et al. 2007) providing further growth escape and pro-invasive routes. We additionally noted that our antioestrogen resistant models expressed particular pro-angiogenic genes, for example VEGF (a growth factor that has previously been shown to be induced during chemoendocrine therapy in breast cancer cells; Fersis et al. 2004). This aspect could further contribute to tumour growth and progression, albeit again within an in vivo environment (see Chapter 8).

4.3 Long-Term Use of Antihormones Also Suppresses Expression of Tumour Suppressor/Pro-Apoptotic Genes

In addition to activation of oncogenes, genetic events (loss of heterozygosity and mutation) that inactivate tumour suppressors such as p53 contribute to breast cancer development and progression. However, epigenetic alterations (again including those targeting tumour suppressors) have also been implicated in breast neoplasia where agents to reverse these alterations are of current interest in clinical cancer trials (Jones and Laird 1999; Widschwendter and Jones 2002; Miyamoto and Ushijima 2005). Epigenetic change comprises heritable alterations that impact on chromatin organisation rather than DNA sequence, where modification in histones, chromatin-related proteins and DNA hypermethylation of promoter CpG islands (catalysed by DNA methyl transferases) are known to contribute to the complexities of epigenetic silencing. CpG hypermethylation may inhibit transcription by interfering with recruitment/function of basal transcription factors and co-regulators, and can also initiate recruitment of methyl-CpG binding domain proteins to silence gene expression. Interestingly, lifetime exposure to oestrogens has been linked substantially with increased risk of breast cancer and there is emerging literature to indicate that gene-suppressive effects of E2 at tumour suppressors (e.g. runt-related transcription factor 3, RUNX3, Cheng et al. 2008) may, in some instances, culminate in their epigenetic silencing. E2 can induce repressive histone modifications (e.g. dimethylated H3K9) during transient gene suppression which feasibly may render genes more vulnerable to long-term silencing during breast cancer development (Cheng et al. 2008). Moreover, studies of several E2-repressed genes, including the tumour suppressor gene cyclin G1, indicate that alongside corepressors such as N-CoR the E2/ER complex can recruit HDACs to gene promoters, creating a repressive chromatin conformation that inhibits expression (Stossi et al. 2006). It is feasible that part of the protective effects of antihormones described for breast cancer development may involve interference with such E2-driven epigenetic events. However, evidence from studies of Slug, an E2-repressed gene with pro-invasive ontology, indicates adverse signalling genes can equally be subject to E2-driven silencing (Ye et al. 2008) and clearly interference with such events could prove undesirable in the context of cancer development and progression. Indeed, hypomethylation is known to lead to genomic instability and development of tumours (Miyamoto and Ushijima 2005). There is also evidence from MCF-7 studies that antihormones

are able to ultimately induce hypomethylation at a significant cohort of promoters, including potential growth inducing genes, and that this event is concomitant with antihormone resistance (Fan et al. 2006). Furthermore, application of the DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine (5AZA) was able to accelerate emergence of an EGFR+ tamoxifen resistant phenotype in ZR75-1 breast cancer cells (Van Agthoven et al. 1994), while it has been shown that it is possible to block the normal E2 suppression of some adverse genes via treating with HDAC or DNA methylation inhibitors (Van Agthoven et al. 1994; Ye et al. 2008).

However, it should not be forgotten that through its genomic mechanism of action E2 classically acts to induce expression of genes bearing EREs, with antioestrogens in turn interfering with this event. Conformational change induced by tamoxifen binding to ER provides docking sites for corepressors such as NCoR (Lavinsky et al. 1998), SMRT, REA and SAFB1, which in turn can recruit HDACs to ERE-bearing genes. Literature indicates that in suppressing E2-stimulated genes, ER signalling disruption has the capacity in the long-term to completely silence their transcription through hypermethylation of CpG islands in their promoters (Leu et al. 2004). Proof of principle studies describe impact of ER siRNA on the ER-regulated genes progesterone receptor (PgR) and trefoil factor 1 (pS2), where initial repressive chromatin modifications with longer-term progressive accumulation of DNA methylation at the EREs of the promoters have been noted (Leu et al. 2004). Their expression could be restored by treatment with a methylation inhibitor and subsequent E2 treatment. Badia et al. (2000) identified that long-term tamoxifen treatment also has potential to modify chromatin structure and silence ER target genes such as PgR, although methylation was not implicated. Their studies subsequently revealed there were aspects to long-term tamoxifen-mediated silencing that were additional to HDAC recruitment (Demirpence et al. 2002) and that tamoxifen could recruit Heterochromatin Protein 1, which was able to silence an oestrogen-regulated luciferase transgene (Vit-tk-luciferase; Oliva et al. 2005).

We have explored the impact of long-term blockade of ER on expression of E2-promoted genes during chronic exposure to antioestrogens tamoxifen or fulvestrant to begin to address if there is a relationship between gene silencing and resistance (Stone et al. 2008). These studies have highlighted that both PgR and pS2 are depleted relative to parental MCF-7 cells in resistant cells, where their expression is not restored by up to 6mo antihormone withdrawal. Studies of pS2 in TAMR cells revealed increased levels of promoter methylation as detected using “MethylLight”, a fluorescence-based real-time PCR assay (collaboration with Dr. HM Fiegl, Medical University of Innsbruck & Dr. M Widschwendter; University College London). Treatment of TAMR cells with the DNA methylation inhibitor 5AZA was able to restore E2-inducible expression of PgR and pS2. These data indicate that epigenetic silencing of classically E2-regulated genes can arise during long-term tamoxifen challenge and that this is apparent in the emerging resistant state. Gene hypermethylation events have also been reported in further antihormone resistant models, although their frequency is somewhat less than hypomethylation (perhaps revealing hypermethylation at particular genes during antihormone treatment can be growth inhibitory (Fan et al. 2006).

Interestingly, our studies also reveal a proportion of the E2-regulated genes silenced by long-term antioestrogen treatment may normally be tumour suppressive or pro-apoptotic in function. We were able to show that in vitro exposure of MCF-7 cells to tamoxifen or fulvestrant for prolonged periods (> 2 years) was associated with promotion of an irreversible aspect to the resultant resistant growth, suggesting an epigenetic contribution (Stone et al. 2008). This was clearly independent of any EGFR-driven mitogenic input, since the elevated level of this receptor that is apparent in TAMR cells was, in contrast, reversible on antihormone withdrawal. Moreover, while oestradiol was mitogenic and 5AZA of little effect as a single agent in cells previously exposed long-term to tamoxifen, physiological doses of E2 plus 5AZA co-treatment was profoundly growth inhibitory in a manner that was clearly ER-regulated since it was tamoxifen reversible (Stone et al. 2008). E2 + 5AZA treatment was not growth inhibitory to the parental MCF-7 cell line, and so the epigenetic contribution in resistance must be a direct consequence of long-term antioestrogen exposure. Initial Affymetrix profiling of cells previously exposed long-term to tamoxifen has revealed a cohort of potential growth suppressive/pro-apoptotic genes that are switched off during the development of resistance and re-expressed by E2 + 5AZA co treatment (Stone et al. 2008). These include RASAL-1 (RAS protein activator like-1), a GTPase-activating protein (GAP) which is reported to normally terminate small GTPase signalling (potentially downstream of receptor TKs) by inducing GTP hydrolysis (Bos et al. 2007). Silencing of this GAP would be expected to facilitate growth factor signalling through small GTPases and thereby potentially could contribute to resistant growth. We are currently embarking on PCR verification and promoter methylation studies for the various genes, and will then examine if their knockdown accelerates emergence of antioestrogen resistance, and in turn if their overexpression restores growth control in resistant states. Two previous studies are supportive of the concept of antihormonal silencing of potential tumour suppressors. SAGE studies revealed that expression of CtIP (or retinoblastoma binding protein 8) was decreased in acquired tamoxifen resistant models, where its knockdown in MCF-7 promoted tamoxifen resistance, and induction restored response (Wu et al. 2007). A study by Treeck et al. (2004) has also reported that long-term tamoxifen treatment of MCF-7 cells decreased levels of several pro-apoptotic genes and impaired subsequent apoptotic response to etoposide treatment. However, no genes from either study overlap with our own microarray findings. In total, alongside antihormone-induced compensatory signalling, it is our belief that silencing of various tumour suppressors could play a significant role in limiting growth inhibitory effects of long-term tamoxifen treatment to promote resistance and progression.

4.4 Therapeutic Implications

The above described studies have shown that the effects of antihormones in ER+ breast cancer cells are not just limited to anti-proliferative events since such agents also appear to be potent inducers of adverse signalling kinases and their regulatory

networks (exemplified by EGFR/HER2; Gee et al. 2003). Through autocrine/paracrine mechanisms, the drug-induced elements are likely to sustain residual growth during treatment and promote (and in some instances maintain) resistance (Gee et al. 2003, 2006; Knowlden et al. 2003; Shaw et al. 2005), also encouraging invasive behaviour during treatment in certain contexts (Hiscox et al. 2006b; Borley et al. 2007). Some induced mechanisms may be unique to particular antihormones, others appear shared between agents. Our findings have considerable therapeutic implications: blockade of induced signalling genes alongside antihormones would be predicted to promote superior anti-tumour response and subvert gain of resistance and progression. Our proof of principle in vitro studies targeting induced EGFR alongside antioestrogens are supportive that such strategies can achieve previously-unobtainable levels of suppression of proliferation coupled with substantial induction of apoptosis (Gee et al. 2003, 2006; Nicholson et al. 2005), culminating in a markedly improved anti-tumour effect, substantial delay and, in some instances, prevention of resistance in vitro. There are also supportive in vivo model data for the potential of anti-EGFR or anti-HER2 plus antihormone strategies (Shou et al. 2004).

Interestingly, however, in many instances the induced events appear distinct from any HER2/EGFR input (Shaw et al. 2005; Gee et al. 2006) and hence continued exploration of the breadth of drug-induced signalling mechanisms could provide superior therapeutic approaches to improve antihormone response and subvert resistance, as well as indicating multi-gene signatures for antihormonal response/failure and adverse prognosis. By example, we have observed that improved growth inhibitory effects were achieved either using a selective NfKB1 siRNA or using the IκBα/IκBβ inhibitor parthenolide in the presence of the antioestrogen fulvestrant where such signalling is at least partially independent of EGFR pathway (Shaw et al. 2005; Gee et al. 2006). Among the kinases, we have found that inhibition of Src alongside tamoxifen also appears to delay resistance and furthermore reduces antioestrogen-induced invasive behaviour again in a largely EGFR independent manner (Hiscox et al. 2006c,d, 2007; Borley et al. 2007). Emerging clinical experience with EGFR and HER2 blockade indicates inhibiting any one kinase (alone or alongside antihormones) may exert only limited impact (Johnston et al. 2008), and hence it could prove more successful to target shared signalling "nodes" downstream of diverse antihormone-induced elements, or alternatively consider pan-inhibition, alongside antihormonal measures (Gee et al. 2005b; Powers and Workman 2006). Finally, blockade of the epigenetic mechanisms promoted by antihormones in order to restore E2-regulated tumour suppressor gene expression could provide further new avenues to treat established resistance (e.g. using E2 treatment together with a DNA methylase inhibitor; Stone et al. 2008), or perhaps delay its emergence when combined early with antihormones. Interestingly, chromatin remodelling drugs (e.g. HDAC or methylation inhibitors) are of clinical interest in cancer although many researchers remain cautious regarding the potential broader genomic impact of such agents (Miyamoto and Ushijima 2005). Restoration of tumour suppressors on an individual basis could prove more desirable if this can ultimately be achieved through alternative strategies. In total, however, our findings appear

to advocate evaluating epigenetic intervention, alongside inhibitory strategies for antihormone-induced compensatory growth factor/kinase signalling, if we are to maximally hinder emergence of antihormone resistance.

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Abbreviations

E2:	oestrogen
ER:	oestrogen receptor
ER+:	oestrogen receptor positive
MAPK:	mitogen-activated protein kinase
PI3K:	phosphoinositide 3-kinase
AKT:	protein kinase B
EGFR:	epidermal growth factor receptor
IGF1R:	insulin-like growth factor receptor
TK:	tyrosine kinase
ERE:	oestrogen response element
NFκB:	nuclear factor of kappa light polypeptide gene enhancer in B-cells
VEGFR2:	vascular endothelial growth factor receptor-2 gene
HDAC:	histone deacetylase
Bcl-2:	B-cell lymphoma protein 2
Bag1:	bcl-2-associated athanogene
HGF:	hepatocyte growth factor/scatter factor
Met:	hepatocyte growth factor/scatter factor receptor
RhoE:	Rho family GTPase 3
RUNX3:	runt-related transcription factor 3
5AZA:	5-aza-2′-deoxycytidine
PgR:	progesterone receptor
pS2:	trefoil factor 1
RASAL-1:	RAS protein activator like-1
GAP:	GTPase-activating protein

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Chapter 5
Influence of the Tumour Microenvironment

Rosemary A. Walker

Contents

Abstract In normal tissues the stromal microenvironment plays a dominant role in controlling tissue function. In breast carcinoma the importance of the tumour microenvironment in tumour initiation, proliferation and metastasis is becoming increasingly evident. The microenvironment comprises fibroblasts, macrophages/lymphocytes and extracellular matrix. Tumour associated fibroblasts exhibit different genetic and epigenetic characteristics compared to normal, which may or may not be due to the influence of the tumour cells. Expression of Insulin Growth Factors, Hepatocyte Growth Factor and Interleukin 6 by stromal cells, plus other factors, may influence development of endocrine resistance or the behaviour of resistant cells. Understanding cross talk between tumour and stromal cells is critical. There is a need for relevant in vitro and in vivo models.

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5.1 Introduction

In normal breast interactions between the luminal epithelial cells, the myoepithelial cells, the basement membrane and the surrounding stoma are critical for the regulation of differentiation and cell growth (Howlett and Bissell 1993). There are many signalling pathways involved in maintaining normal differentiation which include chemokines and cytokines, growth factors, steroid hormones, integrins and adhesion molecules (Hansen and Bissell 2000). Apart from luminal epithelial cell-cell adhesion the factors involved are from, or interact with, the surrounding stromal microenvironment. Therefore, the surrounding stromal microenvironment plays a dominant role in controlling tissue function.

In view of its role in normal breast it would not be surprising that alterations in the nature and dynamics of the stromal microenvironment would be of major importance in breast malignancy. Dvorak (1986) considered tumour stroma to resemble wound healing except that physiological controls were not maintained. Previously tumour characteristics relating to the stroma were used to categorise breast cancers – Scirrhous carcinomas (Willis 1967). It is only more recently that studies have begun to address the importance of the microenvironment in tumour initiation, progression and metastasis (Liotta and Kohn 2001; De Wever and Mareel 2003; Weaver and Gilbert 2004; Barcellos-Hoff and Medina 2005; Hu and Polyak 2007). There is less understanding of its role in therapeutic responses. This chapter will discuss the different components of the tumour microenvironment, their roles in breast cancer behaviour and their possible roles in endocrine resistance.

5.2 Tumour Microenvironment

The tumour microenvironment comprises fibroblasts, macrophages/lymphocytes, endothelial cells, new vessels and extracellular matrix/stromal proteins in different combinations. Breast cancers vary in the extent of stromal reaction and infiltrate by the fibroblasts, macrophages and lymphocytes. Estrogen Receptor (ER) positive breast cancers are more likely to have stroma with elastosis (Masters et al. 1978), whereas ER negative, medullary-like/BRCA 1 cancers have little stroma with a prominent lymphocytic infiltrate (Lakhani et al. 2002).

5.2.1 Fibroblasts

These are one of the main cellular components of the stroma. Tumour associated fibroblasts (TAFs) undergo activation to become myofibroblasts. They secrete a range

Table 5.1 Factors and proteins secreted by tumour associated fibroblasts

Extracellular Matrix	Fibronectin Tenascin C Collagen Hyaluronan Migration Stimulating Factor
Metalloproteinases	e.g. MMPs 1, 2, 3, 11, 12, 13
Growth Factors	Transforming growth factor beta (TGFβ) Vascular endothelial growth factor (VEGF) Basic fibroblast growth factor (bFGF) Hepatocyte growth factor (HGF) Insulin growth factors I and II (IGF-1/11) Stroma derived factor – 1 (SDF-1)
Cytokines	Interleukin-6 (IL-6) Tumour Necrosis Factor Alpha (TFFα)

of growth factors, extracellular matrix proteins and metalloproteinases (Table 5.1), which have been implicated in tumour promotion, growth, invasion and metastasis. Paracrine mechanisms are key to these processes, with factors secreted by TAFs binding to relevant receptors on tumour cells and activating signalling pathways. In vitro, inclusion of fibroblast conditioned medium with breast cancer cells injected into nude mice enhanced tumour growth (Noel et al. 1993). There is evidence that TAFs have distinctive gene expression patterns that differentiate them from normal breast fibroblasts, and which support tumour growth and invasion (Chang et al. 2004; Singer et al. 2007). Although these differences may relate to tumour-derived signals, both genetic and epigenetic alterations have been identified in stromal cells, concurrent and independent of tumour cells (Moinfar et al. 2000; Fukino et al. 2004, 2007; Hu et al. 2005). Hu et al. (2005) identified DNA methylation changes in TAFs supporting the hypothesis that phenotypic changes are at least in part the result of epigenetic modifications.

The dynamic interaction that can exist between tumour epithelial cells and stromal fibroblasts has been demonstrated in a mouse model system in which transforming growth factor (TGF) beta type II receptor was specifically deleted in fibroblasts. The blocking of TGFβ signalling in fibroblasts promoted the growth and invasion of the cancer cells by the upregulation of TGFα, Macrophage Stimulating Protein (MSP) and Hepatocyte Growth Factor (HGF) pathways (Cheng et al. 2005).

5.2.2 Macrophages/Lymphocytes

There are differences in the populations of macrophages and lymphocytes between normal breast and breast cancers (Zuk and Walker 1988; Bhan and DesMarais 1983), and certain types of breast cancers e.g. medullary/medullary-like are associated with a prominent lymphocytic infiltrate (Jacquemier et al. 2005).

Table 5.2 Factors secreted by tumour associated macrophages

Growth factors	Transforming growth factor beta (TGFβ)
Cytokines	CCL2, CXCL8 Interleukins (IL-1, IL-6, IL-10)
Other components	Proteases (MMPs) Angiogenic mediators Interleukins – IL-1, IL-6, IL-10

Macrophages secrete many cytokines and other proteins (Table 5.2) that can promote tumour growth and invasion. Tumour necrosis factor alpha (TNF-α), has cytotoxic and apoptotic activities but expression in breast cancer relates to metastasis (Miles et al. 1994). Its tumour-promoting functions may relate to its ability to promote Metalloproteinase expression and angiogenesis (Balkwill 2002). TNF-α (along with Interleukin-6, see Section 8.3.3) can also regulate oestrogen synthesis with in breast by increasing the activity of estradiol 17β hydrosteroid dehydrogenase and estrone sulfatase (Purohit and Newman 2002).

Tumour cells can secrete monocyte chemoattractants (CCL5 and CCL2) which induce monocyte infiltration. The resulting tumour-associated macrophages secrete mediators such as TNF-α which then promotes expression of tumour-promoting factors by the cancer cells, so illustrating the complexity of the tumour-stromal microenvironment (Ben Baruch 2003).

5.2.3 Extracellular Matrix

The extracellular matrix (ECM) provides a scaffold for epithelial cells in tissues and has a central role in controlling differentiation, growth and migration (Lee and Streuli 1999). The ECM of breast cancers differs from that of normal breast (Ramov-Jenson et al. 1996). Fibronectin expression is increased and there are changes in the isoform profile with upregulation of the ED-A and ED-B domains (Kaczmarek et al. 1994; Castellain et al. 1994). In vitro, fibronectin can induce ER alpha-mediated transcription and reduce cell migration of MCF-7 cells. AF-1 is required for transcription, and there is co-incident c-src activation (Sisci et al. 2004). Migration-stimulating factor (MSP) is a truncated isoform of fibronectin that is expressed by TAFs and also cancer cells, and has potent mitogenic effects (Schor et al. 2003).

Laminin can inhibit oestrogen induced proliferation of MCF-7 cells both in vitro and in vivo, and can inhibit anti-oestrogen responsiveness (Haslam and Woodward 2003). Tenascin-C is a multifunctional protein that is upregulated in the stroma of breast cancer. High expression has been related to more aggressive features of breast cancers (Jahkola et al. 1998). As with many ECM proteins diversity is generated through expression of alternatively spliced isoforms which introduce functionally relevant domains into the mature protein (Jones and Jones 2000). We have detected specific changes in TN-C isoform profiles in breast cancers with induction of two isoforms not found in normal breast (Adams et al. 2002). These isoforms appear to

specifically promote breast cancer growth and invasion (manuscript in preparation). A further member of the Tenascin family, Tenascin-W, has recently been shown to be upregulated in low grade breast cancers (Degan et al. 2007).

A recent array study has identified four different extracellular matrix signatures which identify breast cancer subgroups with different clinical outcomes (Bergamaschi et al. 2008). ECM1 group had a low level of ER positive and luminal A cancers but a higher level of basal-like, triple negative cancers, whereas ECM4 group was the converse. ECM2 and 3 had similar numbers of ER positive cancers but there were more luminal B and HER2 positive cases in the former. No data are given about response to endocrine therapy. Analysis of further cases with information about response would be of interest to determine whether the ECM characteristics can predict for endocrine resistance.

5.3 Endocrine Resistance – Potential Factors From the Tumour Microenvironment

The previous section summarised the many components and complexities of the tumour microenvironment. A selected number of possible factors that could play a role in endocrine resistance are discussed there. The most important feature to take into consideration with these and any other factors from the tumour microenvironment is that there is cross talk between the cancer cells and the stroma. Factors from stromal cells and the stroma can modify signalling within the cancer cells but this is dependent on the relevant receptors being present, and conversely tumour cells can secrete factors that can modify expression of stromal cells.

5.3.1 Insulin Growth Factors

Insulin growth factors (IGF) I and II are potent mitogens for breast cancer cells in vitro (Karey and Sirbasku 1988; Osborne et al. 1989), and in combination with oestradiol are synergistic in their effects on growth of MCF-7 cells (Stewart et al. 1990; Thorsen et al. 1992). Their growth stimulatory effects can be blocked both in vitro and in vivo by a specific antibody to Insulin Growth Factor Receptor (IGFR) I (Arteaga et al. 1989; Cullen et al. 1990) suggesting that stimulation is via the type I and not the type II IGFR.

IGFs can prime activation of several kinases that can phosphorylate ER and initiate oestrogen response element mediated gene expression (Yee and Lee 2000). The anti oestrogen tamoxifen can inhibit IGFI mediated proliferation in ER positive breast cancer cells (Guvakova and Surmacz 1997). Using both primary cultures of fibroblasts from benign and malignant breast and in situ hybridisation of benign and malignant breast tissues IGF1 was found to be expressed in fibroblasts of normal/benign breast but rarely in fibroblasts from cancers, whereas IFGII was expressed by TAFs and not normal fibroblasts (Cullen et al. 1991; Paik 1992; Ellis

et al. 1994; Singer et al. 1995). There is therefore a switch to expression of the fetal type IGFII by fibroblasts associated with breast cancers.

There is increasing evidence of a role of IGFR1 signalling in tamoxifen resistance (Wiseman et al. 1993; Jones et al. 2004; Knowlden et al. 2005), and of cross talk with epidermal growth factor receptor (EGFR) which is also implicated in hormone resistance. In in vitro models of tamoxifen resistance exogenous IFGII promoted IGFRI and EGFR phosphorylation and increased cell proliferation (Knowlden et al. 2005). There was increased mRNA expression of IGFII in the resistant cells, suggesting that autocrine mechanisms were involved. Co-culture experiments of TAFs and wild type/resistant cells would be required to determine whether the altered IGF profile found in TAFs has a role in the development and/or maintenance of resistance.

5.3.2 Hepatocyte Growth Factor

Hepatocyte Growth Factor (HGF)/Scatter factor (SF) is a multifunctional cytokine that is produced by stromal cells (Jiang and Hiscox 1997). It acts in a paracrine fashion and activates the c-Met receptor protein. This promotes loss of cell-cell adhesion and enhanced cell migration. C-Met expression has been demonstrated in breast cancers and associated with poorer outcome (Lee et al. 2005; Leyngel et al. 2005).

Recent data on Fulvestrant (pure anti-oestrogen) resistant breast cancer cells shows that they have increased motility and invasive capacity (Hiscox et al. 2006). Microarray comparisons found enhanced expression of c-Met. Stimulation by HGF/SF secreting fibroblasts enhanced their aggressive phenotype which was suppressed by neutralisation/knockdown of c-Met. The effects on invasion was seen with exogenous HGF, fibroblast conditioned medium or co-culture with fibroblast cells. This suggests that breast cancer TAFs could enhance the metastatic advantage of cancers that develop Fulvestrant resistance.

5.3.3 Interleukin-6

Interleukin-6 (IL-6) is a pleiotropic cytokine that is produced by fibroblasts, macrophages and lymphocytes. IL-6 is important in the regulation of local oestrogen biosynthesis in breast tissues by activating enzymes involved in oestrogen synthesis: aromatase, oestradiol 17β-hydroxysteroid dehydrogenase and estrone sulfatase (Speirs et al. 1993; Duncan et al. 1994; Singh et al. 1995; Newman et al. 2000). In vitro, IL-6 can activate ERα in primary breast cancer cells (Speirs et al. 2000). Prostaglandin E2 (PGE2) can have similar stimulatory effects on oestrogen synthesis and also regulates IL-6 production in fibroblasts (Singh et al. 1999; Zhang et al. 1988).

The significance of IL-6 in breast cancer is not clear (Knüpfer and PreiB 2007) probably due to its multiple variable effects. The in vitro data, its ability to regulate

local oestrogen synthesis and its expression in stromal cells of breast cancers suggest that further studies to investigate whether it has a role in endocrine resistance as indicated.

5.4 Model Systems

Interactions between cancer cells and the tumour microenvironment are dynamic, involving cross talk. Analysing the effect of stromal cells on cancer cells can be done in several ways: culture with conditioned medium from stromal cells; two dimensional co-culture of stromal cells and cancer cells; three dimensional model of stromal cells and cancer cells within a stroma; in vivo animal models. Co-culture experiments can provide useful data but in order to recapitulate what is happening in a human breast cancer a three dimensional model (Nelson and Bissell 2005) or in vivo model is required. The following describes two recent studies that have used models to assess the microenvironment and endocrine resistance, plus some preliminary data (Hiscox and Walker, unpublished data).

5.4.1 Effect of Fibroblast Conditioned Medium on Cell Migration of Resistance Cells

In order to extend studies of the effect of stromal cells on migration of Fulvestrant resistant MCF-7 cells (Hiscox et al. 2006) wild type MCF-7, tamoxifen resistant MCF-7 and Fulvestrant resistant MCF-7 were cultured with fibroblast conditioned medium from four separate donors. These were all from fibroblasts isolated from reduction mammoplasties from women 18–45 yrs (median 26 yrs) after two to four passages. Figure 5.1 shows that all conditioned media result in increased migration of the resistant cells compared to wild type, with effects being greater on tamoxifen resistant cells. Since there is evidence of increased c-Met in Fulvestrant resistant cells then analysis of this in the tamoxifen resistant cells needs to be undertaken. Comparison with conditioned medium from TAFs will be of interest in view of the differences identified in fibroblasts from tumour and normal breast.

5.4.2 Three Dimensional Co-Culture of Breast Tumour Fibroblasts and Tamoxifen Sensitive and Resistant Cells

Shekvar et al. (2007) co-cultured fibroblasts from ER and Progesterone receptor (PgR) negative and ER and PgR positive cancers with premalignant (EIII8) and cancer (MCF-7) tamoxifen sensitive cells and EIII8 tamoxifen resistant cells within matrigel as ECM. They found that EIII8 sensitive cells became resistant when cultured with fibroblasts from ER and PgR negative cancers but that this was not mediated by EGFR or IGFR1. All cells exhibited an altered epithelial morphology on

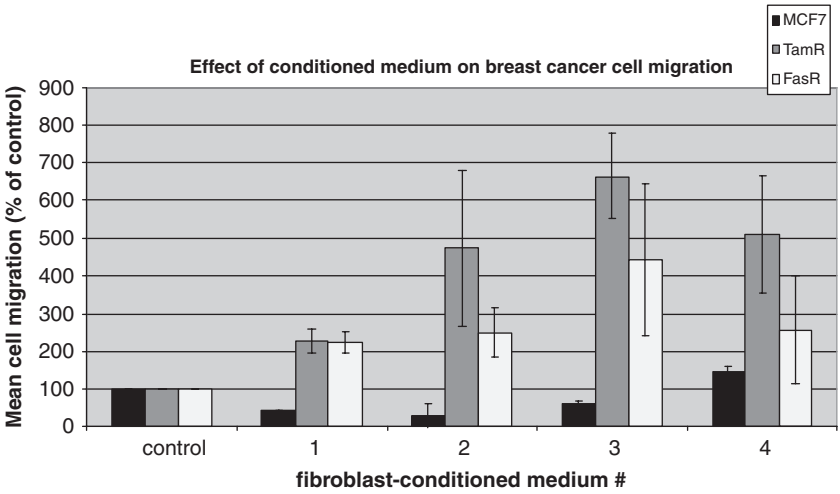

Fig. 5.1 Effect of conditioned medium from fibroblasts isolated from reduction mammoplasties on the migration of wild type, tamoxifen-resistant and faslodex-resistant MCF-7 cells

interaction with fibroblasts. This study consolidates data that there are differences between TAFs from different cancer types and demonstrates that additional changes can be identified from the use of three dimensional culture.

5.4.3 Model of ER Positive Breast Cancer Lymph Node Metastasis

Comparison of in vitro and in vivo models has shown that oestradiol regulates different genes in human breast tumour xenografts in comparison to the same cells in culture, suggesting that the microenvironment can influence oestradiol-dependent gene expression (Harvell et al. 2006). To extend this study Harrell et al. (2007) compared ER positive MCF-7 tumours in nude mice and the corresponding lymph node metastasis in relation to oestrogen withdrawal. Gene expression profiling showed that there were genes that were regulated by the tissue microenvironment, by hormones, or by both. Of interest there were genes that were regulated by oestradiol in the primary tumour but lost oestradiol sensitivity or were regulated in the opposite direction in the nodal metastasis. The authors propose that the lymph node microenvironment can alter oestradiol signalling and may contribute to anti-oestrogen resistance.

This is a good example of an in vivo model. What needs to be developed are systems for studying the interactions of the tumour cells, the tumour stroma and surrounding tissue microenvironment on the development and maintenance of endocrine resistance in human breast carcinoma and on the behaviour of resistant cells.

5.5 Conclusions

The tumour microenvironment is not just a bystander but has roles in tumour initiation, progression and metastasis. Tumour associated fibroblasts exhibit differences from normal breast fibroblasts and can secrete a wide range of growth factors, cytokines, proteases and extracellular matrix proteins. Tumour associated macrophages also secrete factors that promote tumour growth and invasion. The extracellular matrix also differs from normal and likewise has tumour promoting effects.

An important factor in analysing the effect of the microenvironment is that there is cross talk between tumour cells and stromal cells, which is dependent on relevant receptors being present on different cell populations. There are several growth factors and cytokines secreted by stromal cells that could play roles in initiating or maintaining endocrine resistance and modify the behaviour of resistant cells. In order to examine these further there is a need for more complex three dimensional in vitro and in vivo models.

Abbreviations

IGF:	Insulin growth factors
HGF:	Hepatocyte growth factor
TAF:	Tumour associated fibroblast
TGFβ:	transforming growth factor beta
MSP:	Macrophage stimulating protein
TNFα:	Tumour necrosis factor alpha
ECM:	extracellular matrix
IGF1R:	Insulin Growth Factor Receptor
IL-6:	Interleukin-6

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Chapter 6
Are Stem-Like Cells Responsible for Resistance to Therapy in Breast Cancer?

Ciara S. O'Brien, Gillian Farnie, Sacha J. Howell and Robert B. Clarke

Contents

Abstract There is increasing evidence suggesting that some tumours originate from a stem cell population. Such observations appear to support the hypothesis that tumours can be generated and maintained by a small subset of undifferentiated cells able to self renew and differentiate into the bulk tumour population. Recently, cells with cancer stem cell-like properties have been identified within breast cancer tissues suggesting that a proportion of breast cancers may originate from such progenitors. Moreover, the intrinsic resistance of cancer stem cells (CSCs) to a range of chemotherapies suggests that their presence in breast cancer may also play a significant role in the development of an endocrine-resistant state. Future clarification of the role that CSCs play in such tumours, particularly in the context of therapeutic resistance, may lead to new treatment strategies for breast cancer where targeting of the CSCs specifically could lead to better and more sustained responses.

Keywords Breast · Cancer stem cells · Oestrogen receptor · Endocrine therapy · Resistance

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6.1 Introduction

Stem cells are an attractive candidate for the origin of cancer. Similarities between stem cells and cancer cells include their ability to self renew and differentiate, mechanisms which are highly controlled and regulated in normal tissue stem cells (Reya et al., 2001). However, in cancer cells these mechanisms may be dysregulated by aberrant gene expression. The long life span of a normal tissue stem cell may promote the accumulation of mutations and epigenetic changes in highly regulated pathways to promote increasing malignancy within cells. Leukaemic stem cells have a surface-marker phenotype that is similar to normal haematopoietic stem cells supporting the idea that they arise from the stem cell population (Bonnet and Dick, 1997). However, early or late progenitors could also serve as targets for transforming events but the cells would need to acquire mutations both to promote malignancy and to enable them to undergo self-renewal. In this review, we aim to introduce the concept of cancer stem-like cells (CSCs) that are responsible for tumorigenesis, discuss their likely contribution to resistance to breast cancer therapy, and the potential for targetting CSCs and re-sensitising them to treatment.

6.2 Cancer Stem-Like Cells (CSCs) in Breast Tumours

There is now a large body of evidence showing that leukaemia originates from a rare leukaemic or cancer stem-like cell (CSC). The first evidence for CSCs described a small but variable proportion of human acute myeloid leukaemia (AML) cells which could be identified and purified with cell surface markers $CD34^{+}CD38^{-}$. These cells were found to be the only cells capable of transferring AML from human patients to NOD/SCID mice, providing evidence that not all AML cells have in vivo clonogenic capacity and only the small subset of CSCs was capable of regenerating the cancer (Bonnet and Dick, 1997). Many groups have extrapolated the CSC hypothesis from the haematopoietic system to solid cancers and although the evidence for CSCs in solid cancers is in its infancy compared to the haematopoietic field, the evidence is growing rapidly. Cells with CSC characteristics from human brain tumours (glioblastomas) were first isolated by Peter Dirk's group in Toronto using clonogenic sphere culture technique to produce so-called neurospheres (NS) (Singh et al., 2003; Singh et al., 2004). These NS cells are highly enriched for cell surface marker CD133 and nestin (a neural stem cell marker), have a marked capacity for proliferation, self-renewal, and are capable of in vitro differentiation into phenotypes identical to the tumour in situ. CSC populations have also been found in prostate, colon and breast cancers (Al-Hajj et al., 2003; Collins et al., 2005; O'Brien et al., 2007; Ricci-Vitiani et al., 2007). For example, John Dick's group in Toronto reported a xenograft model using subrenal implantation of human colon cancer cell suspensions into pre-irradiated NOD/SCID mice (O'Brien et al., 2007). They demonstrated that 17 out of 17 primary colon cancer samples formed tumours which resembled the original tumour from which it was derived. These tumours could be passaged and would re-form tumours in secondary and tertiary

recipients. Fractionation of colon cancer cells based on CD133 expression revealed that the proportion of $CD133^+$ cells ranged from 1.8 to 24.5%. After implantation into NOD/SCID mice, only one out of 47 mice injected with the $CD133^-$ population formed a tumour compared to 45 out of 49 when implanted with $CD133^+$ cells, suggesting that $CD133^+$ is a CSC marker. Limiting dilution experiments determined that one in every 262 $CD133^+$ colon cancer cells was capable of re-initiating a tumour. In parallel, Ruggero de Maria's group in Italy took a similar approach sorting for $CD133^+$ primary colon cancer cells and demonstrated enrichment for cells that give rise to subcutaneous tumours in SCID mice (Ricci-Vitiani et al., 2007). An in vitro culture system was also used to grow colon cancer cells as colon spheres similar to NS which allows the cells to grow in vitro in an undifferentiated state. Colon spheres were enriched for $CD133^+$CSCs and were capable of growing tumours in mice whereas differentiated $CD133^-$ colon cancer cells were not tumorigenic. Both studies support the CSC hypothesis that suggests that tumours are generated and maintained by a small subset of undifferentiated cells able to self renew and differentiate into the bulk tumour population.

In the breast, Michael Clarke's group was the first to identify a subpopulation of human breast cancer cells which initiated tumours in immune-deficient NOD/SCID mice (Al-Hajj et al., 2003). They reported using a set of cell surface markers to sort cells with an increased tumorogenic capacity. Cells which were $CD44^+$, $CD24^{lo}$, ESA^+ and $lineage^-$ (cells lacking markers CD2, CD3, CD10, CD16, CD18, CD31,CD64 and CD140b), isolated from one primary breast cancer and eight metastases were able to form heterogeneous tumours 8 out of 9 times. The tumours contained not only the $CD44^+$, $CD24^{lo}$, ESA^+ and $lineage^-$ tumour initiating cells but also the phenotypically diverse non-tumorigenic cells that comprise the bulk of tumours. As few as 200 $CD44^+/CD24^{lo}/ESA^+/lineage^-$ cells transplanted into NOD/SCID mice could form tumours with 100% efficiency, while no tumours formed using 200 cells from the $CD44^-/CD24^+/ESA^-$ cell population. A subsequent study by Maria Daidone's group in Milan, Italy carried out on 16 breast lesions (13 primary invasive carcinomas, 1 recurrent carcinoma, and 2 fibroadenomas) using the sphere culture technique resulted in the production of 3 long term primary cultures which had self renewing capacity and could differentiate into the different breast lineages (Ponti et al., 2005). Almost all sphere derived cells were found to be $CD44^+/CD24^{lo}$, however cells with self renewal capacity only accounted for 10–20% of the total cell number, showing that only a sub group within the $CD44^+/CD24^{lo}$ sorted cells had self renewal capacity. This is consistent with only 1 in 200 cells being capable of initiating a tumour in the previous study. Tumour initiating capacity was measured in a long term sphere culture of the MCF7 breast cancer cell line, termed MCF-S. $CD44^+/CD24^{lo}$ cells from parental MCF7s were implanted into the mammary fat pad of SCID mice, and only gave rise to tumours when at least 1 million cells were implanted. However, $CD44^+/CD24^{lo}$ MCF-S cells gave rise to tumours with smaller numbers of cells (10^5, 10^4 and 10^3) with at least a 60% success rate. Thus both the mammosphere culture system and the cell surface marker selection enriched for tumour initiating cells in this study.

These data indicate that sorting for a $CD44^+/CD24^{lo}$ population enriches for tumour initiating cells but highlights the need for additional markers to further

enrich the de facto CSC. One such marker is aldehyde dehydrogenase (ADH), the cellular activity of which can be demonstrated using the fluorescent substrate Aldefluor and flow cytometric analysis (Storms et al., 1999). Using primary human breast cancer samples cultivated as xenografts prior to disaggregation and sorting, Gabriela Dontu's group from Michigan demonstrated that only Aldefluor-positive cells could generate tumours in NOD/SCID mice. When combined with FACS analysis for CD44/24/lin the Aldefluor^{+}/44^{+}/24^{lo}/lin^{-} population of cancer cells could reliably form tumours with as few as 20 cells in the innoculum, whereas 50,000 Aldefluor^{-}/CD44^{+}/24^{lo}/lin^{-} cells failed to form tumours (Ginestier et al., 2007). Importantly, in the same paper, ADH activity was also shown to identify a stem/progenitor population in the normal human mammary gland. Hoechst dye exclusion has also been used to isolate a side population (SP) in normal human breast samples and MCF7 cells (0.2%). The normal SP was enriched for bipotent progenitors and the SP in MCF-7 cells had a greater tumorigenic capacity than the non-SP fraction, when determined by tumour production subcutaneously in NOD/SCID mice (Clarke et al., 2005b; Clayton et al., 2004; Patrawala et al., 2005).

6.3 The Intrinsic Resistance of CSCs to Chemo- and Radiotherapy

Conventional chemotherapy (CT) and radiotherapy (RT) are effective in preventing systemic and local recurrence respectively ((EBCTCG), 2005; Clarke et al., 2005a). Although CT is initially effective in killing cancer cells and controlling tumour growth, all patients with metastatic breast cancer (MBC) and 25% of those with early disease will relapse over time despite the initial response seen in MBC. Likewise, RT reduces the local recurrence rate although a proportion of tumours relapse despite therapy. There are at least two possible explanations for these observations. The first is that all cancer cells acquire resistance to therapy, resulting in decreased overall tumor sensitivity with time. In this case, residual tumors after treatment would then be expected to show no change in the relative proportion of CSCs with tumorigenic properties. The second possibility is that a rare sub-population of CSCs with tumorigenic potential is intrinsically resistant to therapy and residual tumors would then show an increase in tumorigenic CSCs after treatment. Analogous with the propensity of dandelion roots to regenerate the plant above ground following cutting the lawn, re-growth of tumors from an intrinsically resistant subpopulation has been termed "the dandelion hypothesis". This second explanation proposes that only the bulk of the tumour cells are killed whereas CSCs survive treatment and are able to initiate a new tumour at a later time. Characteristics inherent to CSCs could be responsible for their survival during therapy, for example CSCs have the ability to self-renew, can survive anoikis, have a high tumorigenic capacity, efflux toxins efficiently and survive in hypoxic conditions (Farnie et al., 2007; Keith and Simon, 2007; Patrawala et al., 2005; Ricci-Vitiani et al., 2007).

The SP population in hyperplastic tissue removed from mouse mammary tumours virus (MMTV) driven Wnt-1 transgenic mice was >2-fold increased compared to matched background controls. In the same study, radiation selectively enriched mammary gland epithelial progenitors in the MMTV-Wnt1 transgenic compared to non-transgenic control mice (Woodward et al., 2007). Another study reports that MCF7 and MDA-MB-231 breast cancer cells grown as mammospheres were found to be more radioresistant than cells grown in monolayer when subsequently plated in clonogenic assays, suggesting that breast CSCs are radioresistant (Phillips et al., 2006). In glioblastomas, it has been demonstrated that $CD133^+$ CSCs are more radioresistant than the $CD133^-$ non-CSCs since ionising radiation increased the proportion of $CD133^+$ cells in primary human glioblastoma specimens (Bao et al., 2006a). The $CD133^+$ population preferentially activated the CHK1 and 2 DNA damage response genes, and consequently repaired radiation-induced DNA damage more effectively than $CD133^-$ cells. This suggests that $CD133^+$ CSCs would be the source of tumour regrowth in patients after radiation. Use of a specific Chk inhibitor reversed this radioresistance both in vitro and in vivo, indicating that targeting DNA damage check points may overcome this resistance mechanism and improve tumour control using radiation therapy.

The intrinsic therapy resistance of CSCs in human breast tumour cell lines has recently been tested in vitro (Fillmore and Kuperwasser, 2008). Three breast cancer cell lines (SUM159, SUM1315 and MDA-MB-231) were treated for 6 days with either Paclitaxel (Taxol) or 5-Fluorouracil (5FU). Of the surviving cells, the $CD44^+/CD24^{lo}/ESA^+$ CSCs were enriched 5–30 fold compared to control cultures. This suggests that the $CD44^+/CD24^{lo}/ESA^+$ CSCs within the breast cancer cell lines have increased resistance to chemotherapy and may be the cause of tumour recurrence after treatment. A recent study of primary breast tumors in vivo used neoadjuvant treatment, ie. administering chemotherapy before surgery and removed biopsies before and after treatment to compare the numbers of CSCs relative to non-CSCs (Li et al., 2008). Using two common breast cancer therapies, docetaxel or doxorubicin combined with cyclophosphamide, it was demonstrated that several characteristics of CSCs were more prevalent after therapy, suggesting their preferential survival. The proportion of $CD44^+/CD24^{lo}$ cells increased by 2–3 fold and the number of cells able to form MS in in vitro culture was similarly greater. Before tumour treatment, a fourth of samples formed a tumour in SCID/Beige mice, whereas after treatment, half of samples had tumorigenic capacity. Together, these findings suggest that these established breast cancer treatments preferentially kill the non-CSCs compared to the CSCs (Li et al., 2008).

6.4 Are Breast CSCs Resistant to Endocrine Therapy?

Estrogen deprivation is a powerful treatment for breast cancers that express the estrogen receptor-α (ER). However, despite initial response to endocrine therapy, 25% of patients with early breast cancer and all patients with metastatic disease will eventually relapse (Howell and Wardley, 2005).

In normal mouse mammary epithelium, the multipotent stem cell is ER^-negative when isolated by expression pattern of $CD24^{lo}$ and $CD29^+$ (Asselin-Labat et al., 2006). Less than 0.01% of this stem cell population expressed ER compared to 37% of $CD29^{lo}/CD24^+$ luminal cells. In a separate study, the mouse mammary gland luminal compartment was further defined by expression of Sca1, CD133, CD24 and ER (Sleeman et al., 2007). The ER-expressing $CD133^+/Sca1^+/CD24^{hi}$ cells had low proliferative capacity whilst the milk-protein producing $CD133^-/Sca1^-/CD24^{hi}$ cell population had absent ER expression and increased proliferative capacity. The greatest in vivo clonogenic capacity was seen in the ER-negative/$CD24^{lo}$ basal cell population. Therefore in the normal murine mammary gland it could be surmised that ER expression is a marker of a differentiated luminal phenotype with a limited capacity to self renew. Notably, in the normal mouse and the human breast ER-expressing cells do not appear to divide but are often in close vicinity to dividing cells (Clarke et al., 1997; Seagroves et al., 2000).

Unsupervised gene-expression profiling of breast cancer has demonstrated at least 5 molecular subtypes; basal, erbB2, Luminal B, Luminal A and normal-like (Perou et al., 2000; Sorlie et al., 2001). These subtypes may represent a differentiation spectrum comparable to the developmental hierachy of the breast, with poorly differentiated ER-negative basal type at one extreme to well differentiated Luminal A type at the other. As such, the cancer cell of origin of each of these subtypes may represent a different stage of the developmental hierarchy (Sims et al., 2007).

In contrast to the normal breast epithelium, actively dividing ER^+ cells are prominent in breast hyperplasia and breast cancers and expression of the ER is a key determinant of sensitivity to endocrine therapy (Clarke et al., 1997; Shoker et al., 1999). The levels of ER and progesterone receptor (PR) expression are predictive of treatment response rates and distinguish Luminal A tumors, which are highly ER^+ and PR^+, from luminal B tumours which have lower ER expression, do not express PR and coexpress other growth factor receptors (Sorlie et al., 2003). One possible mechanism of resistance to ER targeted endocrine therapy is the presence of an ER^- and treatment resistant CSC population, with the capacity to differentiate and produce treatment sensitive committed ER^+ luminal cancer cells. This would leave behind a resistant population of $ER^{-/lo}$ progenitor-like cells to seed relapse and local metastases. Loss of ER expression from primary to metastatic lesions on relapse has been well described but the concept of the endocrine-resistant CSC remains unproven.

An alternative to ER loss as a mechanism for endocrine resistance has been reported where there is continued expression of ER or the re-activation of the ER pathway and ER-regulated genes after prolonged endocrine therapy leading to drug resistance. For example, in the presence of tamoxifen, ER pathway signalling can still be activated in an oestrogen-independent manner by phosphorylation of the activation function 1 (AF1) domain of the receptor, mediated by growth factor receptor signals and augmented by the action of cancer-associated fibroblasts (Shekhar et al., 2007). Significantly, tamoxifen has both agonist and antagonist properties on ER signalling pathways. The AIB1 gene which is amplified in 5–10% of breast cancers and whose protein product is over-expressed in 50% of breast cancers appears to

promote the agonist properties of tamoxifen but leads to a worse prognosis only in tumours coexpressing erbB2 (Osborne et al., 2003). The increased clonogenicity of primary tumours expressing erbB2 and its increased expression in tamoxifen resistant cells highlights a potential link between this receptor family, the CSC and endocrine therapy resistance.

In human cell line models of acquired tamoxifen and fulvestrant resistance, oestrogen receptor expression becomes progressively down regulated as resistance develops. In parallel, the expression of the erbB2^{+} receptor increases as ER expression diminishes (McClelland et al., 2001; Warri et al., 1991), but can be prevented by inhibition of the EGFR/erbB2 signalling pathway (Arpino et al., 2007). The acquisition of enhanced EGFR/erbB2 pathway signalling with tamoxifen resistance potentially results from selection for a more stem-like phenotype (Farnie et al., 2007; Li et al., 2008). These mechanisms for resistance to endocrine therapy may reflect primitive developmental characteristics or enrichment of CSCs within the cancer leading to resistance to endocrine therapy. However, this hypothesis will require testing, potentially by using a neoadjuvant therapy protocol where tumour samples are collected before and after treatment, similar to the methods used by Li et al. (2008) to analyse CSC enrichment after chemotherapy (Li et al., 2008).

6.5 The Effects of Targeted Therapies on CSCs

It was shown using the MS culture technique in pre-invasive breast cancer that there is dependence for MS colony formation on the epidermal growth factor (EGF) family and Notch receptor signaling pathways (Farnie et al., 2007). This suggests that breast CSCs might be dependent on these pathways. In support of this hypothesis, breast tumours with over-expression of the EGF-related erbB2 receptor (erbB2^{+}) produced a greater number of MS compared to erbB2-negative tumours. In the recent study by Jenny Chang's group in Houston, erbB2^{+} tumours were selected for neoadjuvant treatment with a dual inhibitor of EGF and erbB2 receptor tyrosine kinases called lapatinib, and biopsies were removed for analysis before and after six weeks of therapy (Li et al., 2008). In contrast to treatment with standard chemotherapies, the lapatinib-treated tumours showed no increase in CD44^{+}/CD24^{lo} cells or the proportion of MS-forming cells, suggesting that breast CSCs and non-CSCs are equally dependent on the EGF/erbB2 receptor for their growth and survival.

These findings suggest that breast CSCs may share characteristics with other tumour types. For example, in esophageal carcinoma, Hedgehog (Hh) signalling correlates with therapeutic resistance and Hh inhibition decreased clonogenic survival of cancer cells (Sims-Mourtada et al., 2007; Sims-Mourtada et al., 2006). Similarly, in a recent paper describing a CD133^{+} colon CSC population, interleukin-4 (IL-4) was demonstrated to protect CSCs from apoptosis, and inhibition of IL-4 re-sensitised colon cancer cells to treatment (Todaro et al., 2007).

Another emerging target that is likely to impact on CSCs is antiangiogenic therapy of tumours since evidence is accumulating that both tissue stem cells and CSCs

preferentially associate with blood vessels. For example, in the normal brain, nestin positive precursor and stem cells associate with the capillary vasculature, and in oligodendrogliomas and glioblastomas, there is a direct correlation between nestin positive CSCs and microvessel density (MVD) (Calabrese et al., 2007). This study also reported that the CSCs preferentially associated with the $CD34^+$ capillaries in vivo (in tumour sections) and endothelial vascular tubes in a Matrigel™ culture assay in vitro compared with non-CSCs. In a prior report, this association was shown to be due to CSC secretion of vascular endothelial growth factor (VEGF), which directly stimulates endothelial cell growth (Bao et al., 2006b). Co-transplantation of $CD133^+$/$nestin^+$ CSCs with primary human endothelial cells (PHECs) into nude mice resulted in markedly reduced tumour latency and increased tumour growth compared with CSC injection alone. Antiangiogenic therapy with bevacizumab, a VEGF neutralising monoclonal antibody, resulted in marked reduction in MVD, the proportion of CSCs and neurosphere forming capacity, without impacting on overall tumour cell proliferation, apoptosis or necrosis. It is concluded that "antiangiogenic drugs arrest tumour growth, at least in part, by disrupting a vascular niche microenvironment that is critical for the maintenance of CSCs".

The vascular niche may protect CSCs from therapeutic insults such as chemotherapy or radiotherapy and combinations of such modalities with antiangiogenic agents have been shown to improve outcomes in multiple malignancies including breast cancer (Miller et al., 2007). The role of such combinations in preferentially targeting the vascular niche and thus the CSC has been investigated in an in vivo model of glioma in which co-treatment with the maximum tolerated dose (MTD) of cyclophosphamide and the VEGF receptor 2 (VEGFR2) antagonist DC101 reduced CSC number, assessed by primary and secondary neurosphere forming capacity in vitro, whereas either treatment alone had no inhibitory effect (Folkins et al., 2007). Similarly, in an in vivo model utilising transgenic sphingomyelinase$^{-/-}$ mice, known to possess inherently radiation resistant endothelial cells, Garcia-Barros et al demonstrated the protective effect of such an endothelium on the radiosensitivity of fibrosarcoma and melanoma cell lines xenografted into transgenic versus wild type hosts (Garcia-Barros et al., 2003). In another tumour type, combination of the VEGFR2 antagonist DC101 with irradiation of small cell lung carcinoma xenografts significantly improved long term tumour control. In particular, there was a greater single agent activity in tumours recurring after irradiation compared with treatment naïve tumours, suggesting that the radiation-resistant population of cells may be more dependent upon the vascular niche (Kozin et al., 2007).

In breast cancer, combinations of irradiation and two antiangiogenics have been shown to improve local control rates in an in vivo model utilising the SCK mouse mammary tumour cell line (Dings et al., 2007). However, the authors hypothesised that this synergism was secondary to the demonstrated vessel normalisation and improved tumour oxygenation but their demonstration of reduced MVD with combination therapy, and a reduction in tumour associated neoangiogenesis, suggests that an inhibitory effect on mammary CSCs. Clearly, evidence for a vascular niche for the breast CSC is currently sparse and requires investigation since it could represent an excellent target for therapy in CT, RT or endocrine-resistant disease.

6.6 Potential of Differentiation Treatment to Re-Sensitise CSCs to Therapy

The ability to re-sensitise CSC's to DNA damage or deplete the CSC population before CT, RT or endocrine therapy may decrease recurrence and increase survival of patients significantly. An alternative approach would be a therapy inducing the CSCs to differentiate. Differentiated tumour cells would lack the inherent mechanisms of CSCs needed to survive RT and CT. For example, acute promyelocytic leukaemia has become a curable disease through all-trans retinoic acid-based differentiation induction therapy followed by chemotherapy (Ohno et al., 2003). Depletion of $CD133^+$ CSCs in brain tumours has been induced by differentiation using either Notch inhibition or treatment with bone morphogenetic proteins (BMPs) (Fan et al., 2006; Piccirillo et al., 2006). Both Notch inhibition and BMPs reduced $CD133^+$ expression, increased neural markers of differentiation (GFAP) and stopped tumour growth in NOD/SCID mice. Similarly, Hh inhibition in glioblasoma increased differentiation marker GFAP, decreased neural stem cell marker nestin and cells were no longer able to form tumours in athymic mice (Bar et al., 2007). In the breast, administration of retinoids or histone deacetylases (HDACs) is known to have differentiating effects in normal mammary epithelium but their ability to induce CSC differentiation and re-sensitise breast cancer to CT, RT or endocrine therapy is unknown (Guzman et al., 1999; Liby et al., 2007; Wu et al.,2006).

6.7 Conclusions

In this review, we have summarised current evidence supporting a role for CSCs being at the root of therapeutic resistance in breast cancer. The available data suggest that CSCs may possess mechanisms that enable them to evade current therapeutic modalities. In particular, there is good evidence from several solid tumour types that CSCs preferentially evade CT and RT compared to their more differentiated progeny. The mechanisms for this CSC resistance may be diverse but have been suggested to include differences in drug efflux, survival and DNA damage response and repair. Evidence in breast cancer is limited but suggests that CSCs are resistant in vivo to at least two forms of CT. However, in contrast, the CSCs were demonstrated to be sensitive to therapy targeted to the EGFR/HER2 receptors that are reported to regulate CSC self-renewal. There is also evidence in other tumor types that the vasculature could provide a niche for CSCs and therefore be an excellent target for CSC therapy in resistant disease but this requires further substantiation in breast cancer. Little is known regarding CSC resistance to endocrine therapy but several different underlying mechanisms have been described including activation of pathways known to regulate CSCs. Thus, investigation of breast CSCs and response to endocrine therapy is merited and in the future could lead to better and more sustained responses in ER^+ breast

cancers. Finally, more knowledge about the mechanisms of resistance to therapy could lead to combination therapies. In particular, the potential for differentiation therapy should be further investigated since resistance to treatment in CSCs may be overcome by their enforced maturation into treatment-sensitive non-CSCs.

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Abbreviations

CSC:	cancer stem-like cell
AML:	acute myeloid leukaemia
NOD/SCID:	non-obese diabetic/severe combined immune-deficient
NS:	neurosphere
ADH:	aldehyde dehydrogenase
CT:	chemotherapy
RT:	radiotherapy
MMTV:	mouse mammary tumour virus
MS:	mammosphere
ER:	oestrogen receptor-alpha
PR:	progesterone receptor
AF1:	activation function 1
EGF:	epidermal growth factor
IL4:	interleukin 4
Hh:	hedgehog
VEGF:	vascular endothelial growth factor
PHECs:	primary human endothelial cells
MVD:	microvessel density
HDACs:	histone deacetylases

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Chapter 7
HSP90 Inhibition as an Anticancer Strategy: Novel Approaches and Future Directions

Marissa V. Powers and Paul Workman

Contents

Abstract Heat shock protein 90 is an ATP-dependent molecular chaperone involved in the maturation and stabilisation of a wide-range of proteins in both the presence and absence of cellular stress. Within the ever expanding list of HSP90 client proteins is a broad range of *bona fide* oncoproteins. This has thrust HSP90 into the spotlight as an exciting anticancer drug target. Several natural product and semi-synthetic derivatives have been described which inhibit the activity of HSP90 by preventing the association of the N-terminal domain with ATP. This approach is exemplified by 17-AAG which is the first-in-class HSP90 inhibitor to complete phase I clinical trial and provide proof-of-concept for this approach with the observation of responses in patients with malignant melanoma, multiple myeloma, prostate and breast carcinoma. Research is now focused on the design of more potent and drug-like synthetic small-molecule inhibitors. This article provides a personal

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perspective of the advances made in the development of novel HSP90 inhibitors with particular emphasis on work from our own laboratory. We will also review alternative approaches to inhibit HSP90 which are currently being evaluated. These include selectively inhibiting particular HSP90 isoforms, blocking co-chaperone interactions, designing substrate mimetics and modulating the post-translational modifications of HSP90.

Keywords 17-AAG · Heat shock protein inhibitors · HSP90 · siRNA

7.1 The Therapeutic Potential of HSP90 Inhibition

Heat shock protein 90 (HSP90) is a highly abundant molecular chaperone induced in response to stress to prevent the misfolding and aggregation of unfolded proteins (Young et al., 2004). When this is not possible, HSP90 directs irreparably damaged proteins for degradation via the ubiquitin-proteasome pathway (Connell et al., 2001; Demand et al., 2001). Furthermore, in the absence of stress HSP90 is important in managing the conformation, localisation and functional maturation of a wide range of so-called 'client proteins'. These include steroid hormone receptors, receptor tyrosine kinases and other proteins involved in a variety of cellular processes (Wegele et al., 2004; Sreedhar et al., 2004b).

The function of HSP90 is critically linked to the cycle of N-terminal ATP/ADP exchange and ATP hydrolysis which is controlled by a conformational change in its structure (reviewed in Pearl et al., 2008). HSP90 exists as a dimer with each monomer constitutively associated with its partner via the C-terminal domain. In the ADP-bound state the N-terminal ATP binding domains are not closely associated and the chaperone is considered to be in an immature, inactive, 'open' state (Fig. 7.1). However, binding of ATP induces a conformational change in the chaperone to bring the N-terminal domains into close proximity with one another in a 'closed' conformation (Prodromou et al., 2000). This process has been referred to as a 'molecular clamp' mechanism (Prodromou et al., 2000). The rate of ATP hydrolysis by HSP90 is closely linked to the association of a number of co-chaperone proteins including HSP70, HOP, CDC37, p23 and AHA1 which act in concert with HSP90 to fine tune its activity (Fig. 7.1). Description of the function of each of these accessory proteins is beyond the scope of this article and the reader is referred to more specific reviews (Riggs et al., 2004; Pearl et al., 2008).

Because of the wide range of cellular processes with which HSP90 is associated, this molecular chaperone may not initially appear as an obvious target for therapeutic intervention. However, HSP90 has been implicated in oncogenesis and malignant progression due to its overexpression in many cancers and its association with poor prognosis (Jameel et al., 1992; Gress et al., 1994; Sreedhar et al., 2004a; Pick et al., 2007; Gallegos Ruiz et al., 2008). Furthermore, within the ever expanding list of substrates known to associate with HSP90 (see http://www.picard.ch/downloads/Hsp90interactors.pdf) there is a plethora of *bona fide* oncoproteins, including kinases

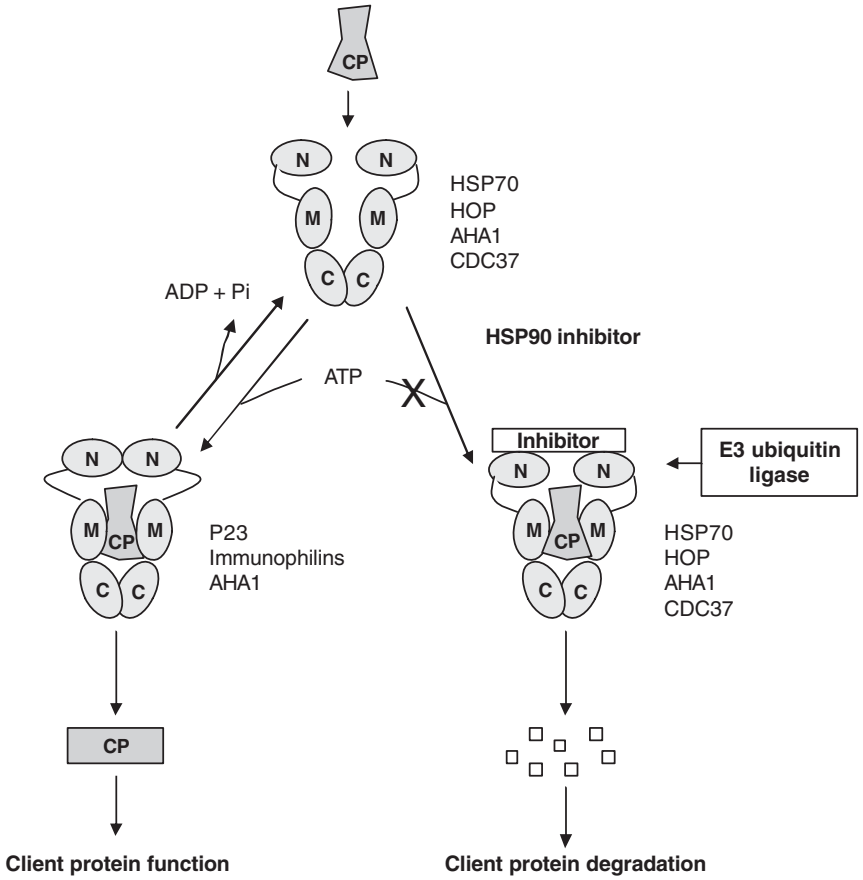

Fig. 7.1 Schematic of the proposed chaperone cycle for HSP90 showing the conformational changes associated with the activity of HSP90. Substrates are initially recruited to HSP90 by HSP70. During this phase HSP90 adopts an open conformation and also associates with the co-chaperones HOP, AHA1 and CDC37 to form the immature complex. Upon ATP binding a conformational change is induced which results in the transient dimerisation of the N-terminal ATP-binding domains and exchange of co-chaperones. ATP hydrolysis is coupled with substrate maturation and release. In the presence of an inhibitor, ATP is unable to bind and client proteins are retained within the immature complex. An E3 ubiquitin ligase is recruited resulting in client protein ubiquitination and degradation via the proteasome

such as BRAF, CRAF, AKT/PKB, ERBB2 and EGFR, together with oestrogen and androgen receptors, mutant p53, HIF1α and telomerase hTERT, all of which are involved in the six hallmarks cancer (Hanahan and Weinberg, 2000). HSP90 inhibition leads to the recruitment of an E3 ubiquitin ligase which ubiquitinates the associated client proteins resulting in their degradation via the ubiquitin-proteasome pathway (Connell et al., 2001; Demand et al., 2001). Therefore, inhibition of HSP90 function offers the opportunity to degrade a large number of oncogenic client proteins

and hence to simultaneously antagonise all of the hallmarks traits of malignancy, including uncontrolled proliferation, avoidance of apoptosis, immortalisation, invasion, angiogenesis and metastasis (Hanahan and Weinberg, 2000). This combinatorial attack on multiple oncogenic pathways should also reduce the opportunity for resistance developing to HSP90 inhibition when compared to more conventional therapies.

The ubiquitous involvement of HSP90 in regulating multiple cellular functions led to initial concerns about potential toxicity. However, there are several reasons why therapeutic selectivity for cancer versus healthy cells may be expected. First, increased expression of HSP90 has been widely reported in a range of human malignancies (reviewed in Sreedhar et al., 2004a). This could be a consequence of the stressful microenvironment of the solid tumour which may possibly increase the cancer cell's dependence on molecular chaperones. Consistent with this is the observation that HSP90 extracted from healthy cells exists in an uncomplexed, inactive state, whereas HSP90 from tumour cells is present in a large multi-chaperone complex which is more sensitive to inhibition (Kamal et al., 2003). Secondly, cancer cells become 'addicted' to survival pathways which dictate malignancy. Therefore, cancer cells are much more sensitive to the depletion of critical oncoproteins that drive these pathways than normal cells. Finally, oncoproteins which are involved in maintaining malignancy are often expressed in mutated, activated forms that have a greater dependence on HSP90 activity than their normal counterparts. An example of this is mutant BRAF which we and others have shown to be reliant on HSP90 function for folding and stability and to be much more sensitive to degradation following 17-AAG treatment than the wild type form (da Rocha Dias et al., 2005; Grbovic et al., 2006).

In this review, we provide an update of our previous studies (Powers and Workman, 2006) and describe our recent work aimed at developing inhibitors of the HSP90 molecular chaperone family and at understanding the consequences of inhibition in both the preclinical and clinical setting. We will discuss some of the latest developments with HSP90 N-terminal ATP site inhibitors. We will also describe novel approaches which are being evaluated to block HSP90 molecular chaperone function. These include selective inhibition of particular HSP90 isoforms, modulation of co-chaperone protein interactions, design of substrate mimetics and alteration of the post-translational modifications of HSP90. Once again examples will be taken mainly from the work in our own laboratory.

7.2 HSP90 ATP Site Inhibitors: Natural Products and Semi-Synthetic Derivatives

The first HSP90 inhibitors to be described were natural products which included radicicol and the benzoquinone ansamycin, geldanamycin (Fig. 7.2). Both bind to the N-terminal nucleotide binding domain of HSP90 and block the ATPase-coupled chaperone cycle leading to client protein degradation by the proteasome (Schulte

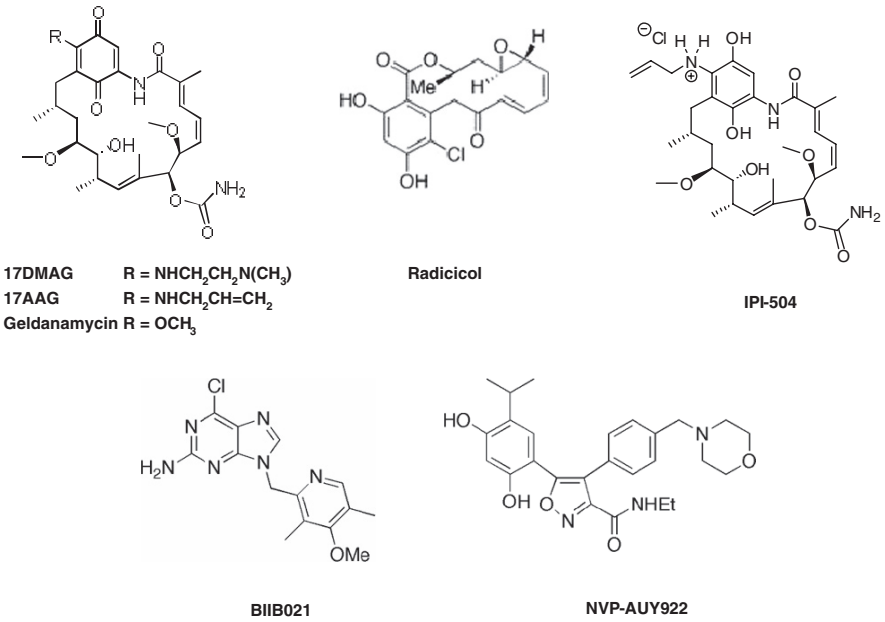

Fig. 7.2 Examples of different classes of HSP90 inhibitors

et al., 1998; Smith et al., 1995; Roe et al., 1999). However, whilst both natural products played a critical role in elucidating the biology of the HSP90 chaperone cycle and the consequences of its inhibition, neither was suitable for clinical development. Radicicol displayed little in vivo activity in animal models due to its chemical reactivity and instability (Soga et al., 1999) whereas development of geldanamycin was restricted by unacceptable levels of toxicity (Supko et al., 1995). However, a semi-synthetic analogue of geldanamycin, 17-AAG (17-allylamino-17-demethoxygeldanamycin; tanespimycin; Fig. 7.2), is better tolerated and exhibits a higher therapeutic index than its parent compound. Phase I clinical studies with 17-AAG carried out by ourselves (Banerji et al., 2005a) and others (e.g. Goetz et al., 2005; Grem et al., 2005) have provided the first convincing proof-of-concept for HSP90 inhibition in human patients. This was demonstrated by depletion of client proteins and induction of HSP70 expression (Banerji et al., 2005a; Goetz et al., 2005; Grem et al., 2005) which collectively represent the validated molecular signature of HSP90 inhibition (Banerji et al., 2005b). Early signs of therapeutic activity were seen in melanoma (Banerji et al., 2005a), breast and prostate cancers (see below and Pacey et al., 2006; Solit and Rosen, 2006; Modi et al., 2007).

We have recently extended our preclinical studies (da Rocha Dias et al., 2005) to investigate possible mechanisms underlying the clinical response to 17-AAG in patients with malignant melanoma observed during our own phase I trial. BRAF and NRAS mutations are extremely common in melanoma with most patients having either BRAF or NRAS mutations but not both (Davies et al., 2002; Edlundh-Rose

et al., 2006; Goel et al., 2006; Reifenberger et al., 2004). As mentioned earlier, mutated BRAF has a much greater dependence on HSP90 function than the wild type counterpart, making it necessary to understand the relationship between BRAF and NRAS mutation status and the response of patients with melanoma to 17-AAG (Banerji et al., 2008a). In collaborative studies with Professor Richard Marais, we have investigated the effect of the NRAS and BRAF mutation status in six melanoma patients, all of whom had been treated with pharmacologically active doses of 17-AAG (Banerji et al., 2008a). One patient with disease stabilisation for 49 months had a ^{G13D}NRAS mutation and ^{WT}BRAF whereas a second patient who had stable disease for 15 months had a ^{V600E}BRAF mutation and ^{WT}NRAS. Patients who had melanomas with ^{WT}BRAF/^{WT}NRAS all progressed within 1 to 1.5 months while receiving 17-AAG (Banerji et al., 2008a). These novel observations, though based on small patient numbers and therefore very preliminary, suggest that BRAF and NRAS mutation status should be considered during future phase II clinical trials of HSP90 inhibitors in melanoma (Banerji et al., 2008a).

In addition to the encouraging results being produced from the clinical trials using 17-AAG as a single agent, evidence of activity with 17-AAG has also been reported in combination with trastuzumab in trastuzumab-refractory ERBB2-positive breast cancer (Modi et al., 2007), with the proteasome inhibitor bortezomib (Mimnaugh et al., 2004) which may offer a therapeutic strategy for the treatment of multiple myeloma, and with cytotoxics such as paclitaxel as an effective therapy in lung adenocarcinoma patients (Sawai et al., 2008). The combination of 17-AAG with cytotoxic agents such as paclitaxel, cisplatin and oxalipatin (Munster et al., 2001; Rakitina et al., 2003; Vasilevskaya et al., 2003; Vasilevskaya et al., 2004), tyrosine kinase inhibitors like imatinib (Radujkovic et al., 2005) and radiation treatment (Enmon et al., 2003; Bisht et al., 2003) have been studied with positive results being observed. In collaborative studies with Professor Ann Jackman and colleagues, we have demonstrated a beneficial interaction between 17-AAG and paclitaxel in those human ovarian cancer cell lines that have PI3 kinase pathway activation (Sain et al., 2006) and have also recently reported, using both in vitro cell culture and in vivo human tumour xenograft models, a therapeutic benefit for the combination of 17-AAG with carboplatin for the treatment of human ovarian cancer (Banerji et al., 2008b).

Although no HSP90 inhibitor has yet been approved for cancer treatment, the early clinical results with 17-AAG have begun to validate the potential of inhibiting HSP90 as a therapeutic approach to treat cancer. However, 17-AAG is not without its limitations, which include hepatotoxicity which may be caused by the redox active benzoquinone moiety, poor solubility necessitating the use of cumbersome formulations, and variable reduction by the polymorphic oxidoreductase enzyme NQO1/DT-diaphorase to the more potent hydroquinone form (Kelland et al., 1999). In addition, 17-AAG demonstrates reduced activity in the presence of P-glycoprotein (Kelland et al., 1999) and is metabolised by the polymorphic cytochrome P450 CYP3A4 (Egorin et al., 1998) giving rise to the potential for variable pharmacokinetics and drug-drug interactions. A number of these issues have been circumvented by the clinical introduction of more soluble derivatives such

as 17-DMAG (17-dimethylaminoethylamino-17-demethoxygeldanamycin; alvespimycin; Fig. 7.2) and IPI-504 (17-allylamino-17-demethoxygeldanamycin hydroquinone hydrochloride; retaspimycin Fig. 7.2) which is the more potent hydroquinone form of 17-AAG (Sydor et al., 2006).

Macbecin is another example of a benzoquinone ansamycin which has antitumour activity. This natural product has been shown recently to inhibit HSP90 function by binding to the N-terminal ATPase domain of HSP90 with a higher affinity than geldanamycin (Martin et al., 2008). Reflecting this, macbecin inhibits the ATPase activity of HSP90 with greater potency than geldanamycin to induce depletion of HSP90 client proteins including CRAF and ERBB2 and growth arrest of prostate cancer cells in vitro and in vivo using tumour xenograft models (Martin et al., 2008). Structural studies, comparing macbecin to geldanamycn, revealed significant differences in HSP90 binding characteristics (Martin et al., 2008). These differences offer the opportunity to develop novel HSP90 inhibitors using a proven structural scaffold.

A number of radicicol analogues have been developed which include in particular oxime derivatives (KF25706 and KF58333) that retain the capacity to inhibit HSP90 function but also demonstrate therapeutic activity in human tumour xenograft models (Soga et al., 1999; Soga et al., 2001). However, these have not yet progressed into clinical evaluation possibly due to reported toxicity to the eye in animals (Janin, 2005).

7.3 Synthetic Small-Molecule HSP90 Inhibitors

Natural products are intrinsically complex and inherently offer the potential for off-target effects. In addition, their synthesis is not easily adaptable to the processes required in a drug development programme. As a consequence, focus has shifted to the development of lower molecular mass inhibitors of HSP90. The first class to be described was based on a purine scaffold (reviewed in detail in Chiosis, 2006) designed to mimic the unusual 'C-shape' adopted by ADP when bound to the N-terminal domain nucleotide binding site of HSP90 (Prodromou et al., 1997; Stebbins et al., 1997). In collaboration with Vernalis, we reported X-ray co-crystal structures for the lead purine inhibitor PU3 which showed that this agent did indeed mimic ADP (Wright et al., 2004). However, the compound also induced an unexpected conformational change in the ATP-binding site to open up a novel lipophilic pocket (Wright et al., 2004). More potent and soluble analogues with activity in human tumour xenografts have been generated (He et al., 2006; Kasibhatla et al., 2007) including the optimised purine-base drug BIIB021 (Fig. 7.2) which has recently entered clinical trials.

High-throughput screening was used in our own studies to identify a novel group of water soluble HSP90 inhibitors containing a pyrazole unit, a benzodioxan core and a resorcinol ring which is the binding mode anchor of this family (Cheung et al., 2005) and is also found in radicicol (Roe et al., 1999). The 3,4-diarylpyrazole resorcinol

lead, CCT018159, was subsequently shown to inhibit human HSP90β with a similar potency but greater selectivity than 17-AAG (Sharp et al., 2007a). Consistent with our earlier studies using 17-AAG (Clarke et al., 2000; Hostein et al., 2001; Maloney et al., 2007), CCT018159 induced the molecular and cellular changes associated with HSP90 inhibition such as client protein depletion, induction of heat shock proteins such as HSP70, growth arrest and apoptosis, as well as reducing tumour cell invasion and angiogenesis (Sharp et al., 2007a). In addition, unlike 17-AAG, cellular sensitivity to CCT018159 was not affected by NQO1/DT-diaphorase expression nor was it a substrate for P-glycoprotein (Sharp et al., 2007a).

In a collaboration with Vernalis, structure-based design using X-ray crystallography resulted in the introduction of a 5-amide substitution which generated the more potent pyrazole amide CCT0129397/VER-49009 (Dymock et al., 2005) and the corresponding isoxazole CCT0130024/VER-50589 (Sharp et al., 2007b). The pyrazole to isoxazole switch did not affect the critical hydrogen bonding network, including essential water molecules, which we have previously shown to be vital to anchor the pyrazole resorcinol unit of these compounds to the N-terminal ATP binding site of HSP90 (Cheung et al., 2005; Dymock et al., 2005; Sharp et al., 2007a). As with 17-AAG and CCT018159, both compounds caused depletion of client proteins, induction of heat shock proteins, cell cycle arrest and apoptosis (Sharp et al., 2007b). Unlike 17-AAG but consistent with results for CCT018159, the cellular potency of both VER-49009 and VER-50589 was not affected by DT-diaphorase or P-glycoprotein expression (Sharp et al., 2007b).

In collaboration with Professor Laurence Pearl and Dr Chris Prodromou, we have used isothermal titration calorimetry (ITC) to show that the isoxazole had a greater binding affinity than the corresponding pyrazole with a dissociation constant (K_d) of 4.5 ± 2.2 nmol/L for VER-50589 compared to 78.0 ± 10.4 nmol/L for VER-49009 (Sharp et al., 2007b). In addition, the cellular uptake of the isoxazole was far greater than the pyrazole, resulting in more potent HSP90 inhibition and antiproliferative activity (Sharp et al., 2007b). Mean antiproliferative GI_{50} values for both the pyrazole and isoxazole were in the nanomolar range but the switch to the isoxazole resulted in an approximate nine-fold gain in potency (Sharp et al., 2007b). Based on our previous experience with cassette and individual compound dosing pharmacokinetic studies with CCT018159 and other early pyrazole compounds in mice (Smith et al., 2006), we investigated the pharmacokinetic properties of VER-49009 and VER-50589. Plasma clearance of both compounds was rapid; however, in vivo tumour cell uptake and HSP90 inhibition were confirmed by depletion of ERBB2 in an orthotopic human ovarian OVCAR3 carcinoma ascites model following treatment with either VER-49009 or VER-50589 (Sharp et al., 2007b). Extent and duration of pharmacodynamic changes using this in vivo model confirmed the superiority of VER-50589 over VER-49009 (Sharp et al., 2007b). Further studies using VER-50589 revealed that the good cellular uptake properties of the isoxazole resulted in tumour levels in HCT116 human colon tumour xenograft being above the in vitro GI_{50} for 24 h, resulting in approximately 30% growth inhibition (Sharp et al., 2007b).

Subsequent optimisation of the isoxazole series focused on maintaining or increasing potency while improving physiochemical, pharmacokinetic and pharma-

codynamic properties. This led to the identification of the resorcinylic isoxazole amide NVP-AUY922/VER-52296 (Brough et al., 2008; Fig. 2). X-ray co-crystal structures of NVP-AUY922 bound to the N-terminal domain of recombinant human HSP90α confirmed that this novel compound binds deep into the ATP pocket in a manner similar to CCT018159, VER-49009 and VER-50589 (Dymock et al., 2005; Sharp et al., 2007a; Sharp et al., 2007b). Replacement of the chlorine in the resorcinol ring present in VER-49009 or VER-50589 with an isopropyl group in NVP-AUY922 resulted in an additional hydrophobic interaction with Leu^{107} in the flexible lipophilic pocket of HSP90 (Eccles et al., 2008). In addition, replacement of the methoxy group of VER-49009 or VER-50589 with a morpholino side chain in NVP-AUY922 resulted in improved solubility whilst also providing additional hydrophobic interactions with Thr^{109} and Gly^{135} (Eccles et al., 2008).

NVP-AUY922 has excellent potency against HSP90 in a fluorescence polarisation binding assay with an IC_{50} of 21 nmol/L against the β isoform (Brough et al., 2008) and of 7.8 ± 1.8 nmol/L for the α isoform (Eccles et al., 2008). ITC demonstrated a very high binding affinity to HSP90β with a K_d of 1.7 ± 0.5 nmol/L which is three-fold lower than VER-50589 (Eccles et al., 2008). To our knowledge, NVP-AUY922 exhibits the tightest binding of any small molecule synthetic inhibitor yet reported. This can be explained in part by the improved bonding interactions described above along with superior entropy and enthalpy factors (Eccles et al., 2008). Studies with an analogue of NVP-AUY922 revealed a slow off-rate for binding to HSP90 (Brough et al., 2008). Profiling NVP-AUY922 against other ATPases, kinases, and a large panel of other enzymes and receptors showed a very high degree of selectivity towards HSP90 (Eccles et al., 2008).

Consistent with the other diaryl-pyrazoles and diary-isoxazoles described above, the cellular activity of NVP-AUY922 is independent of NQO1/DT-diaphorase and P-glycoprotein expression (Eccles et al., 2008). NVP-AUY922 inhibited in vitro proliferation of a panel of human cancer lines with nanomolar potency (Brough et al., 2008; Eccles et al., 2008). Inhibition of cell proliferation was accompanied by a G_1 or G_1 plus G_2-M phase cell cycle arrest in most cell lines, cell-line dependent apoptosis, HSP90 client protein depletion and heat shock protein induction, all of which are consistent with the molecular signature of HSP90 inhibition (Brough et al., 2008; Eccles et al., 2008). In addition, NVP-AUY922 potently inhibits tumour cell invasion, endothelial cell function associated with in vitro angiogenesis which include proliferation, motility, matrix invasion and tubular differentiation (Eccles et al., 2008). It also has good pharmacokinetic properties, with accumulation in tissues and especially tumour tissue (Eccles et al., 2008). Furthermore, NVP-AUY922 exhibits antitumour and antiangiogenic activity in a range of subcutaneous, orthotopic and metastatic human tumour xenograft models including colon (Brough et al., 2008), melanoma, glioblastoma, and breast, ovarian and prostate carcinomas (Eccles et al., 2008; Jensen et al., 2008). Figure 7.3 shows the activity of NVP-AUY922 against the BT474 human breast cancer xenograft that expresses both ERα and ERBB2 (Eccles et al., 2008). A prolonged growth inhibition and a significant number of regressions were observed in this model, consistent with depletion of these client proteins by the drug. Based on these promising preclinical studies, the optimised analogue NVP-AUY922 has now entered phase I clinical trial.

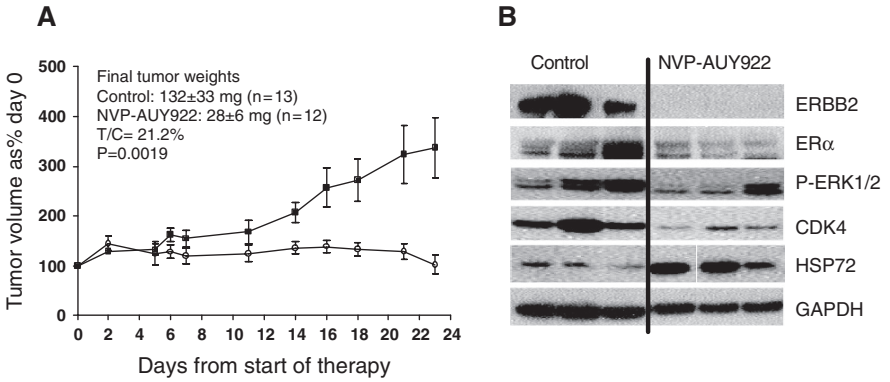

Fig. 7.3 Response of BT474 human breast cancer xenografts to NVP-AUY922. Tumour xenografts were established for ERBB2 + /ERα+ BT474 human breast carcinoma cells. Dosing with 50mg/kg of NVP-AUY922 or vehicle commenced 15 days after cell injection and continued daily for 23 days. (**A**) BT474 tumour xenograft growth curves, with (insert) final weights. *Solid squares*: vehicle controls; *open circles* NVP-AUY922 treated. (**B**) western blots showing biomarkers of HSP90 inhibition from representative control and treated BT474 xenografts. Reproduced with permission from Eccles et al., 2008

The design of NVP-AUY922 highlights the value of X-ray crystallography and structure-based design as a powerful approach to create novel HSP90 inhibitors. Also important in selecting NVP-AUY922 was the simultaneous optimisation of pharmacokinetic and pharmacodynamic properties, featuring a novel approach of determining tumour uptake in cassette dosing studies, together with pharmacodynamic biomarker determinations (Brough et al., 2008).

7.3.1 Agents That Inhibit HSP90 Function by Alternative Methods

Novobiocin is a member of the coumarin family of antibiotics which are known to bind to and inhibit the bacterial DNA gyrase B ATP binding site. However, it has also been shown to inhibit HSP90 function and induce client protein degradation (Marcu et al., 2000b). However, unlike the HSP90 inhibitors described so far, novobiocin is different in that it does not bind to the chaperone's N-terminal ATP-binding site (Marcu et al., 2000b). Instead novobiocin has been shown to interrupt HSP90 function by interacting with the C-terminal domain of HSP90 (Marcu et al., 2000b) and disrupting the interaction between HSP90 and its co-chaperones HSC70 and P23, both of which have been shown to be critical for the chaperone activity of HSP90 and both of which interact with the C-terminal domain of the chaperone (Marcu et al., 2000a). Novobiocin has also been proposed to inhibit HSP90 activity via an interaction with a proposed cryptic ATPase domain within the C-terminal domain of HSP90 (Marcu et al., 2000a). However, this domain has not yet been identified despite crystal structures for this region of the chaperone now being available (Dollins et al., 2005; Ali et al., 2006; Shiau et al., 2006).

Celastrol is a complex natural compound which been shown to inhibit the proteasome (Yang et al., 2006) and to restrict the growth of human prostate carcinoma (Yang et al., 2006), melanoma (Abbas et al., 2007) and glioma (Huang et al., 2008) xenograft models. Similar to HSP90 inhibitors, celastrol causes client protein depletion and induction of several heat shock proteins (Zhang et al., 2008; Hieronymus et al., 2006). Large scale gene expression studies revealed similarities between the molecular response to well documented HSP90 inhibitors and celastrol (Hieronymus et al., 2006). However, celastrol does not affect ATP or geldanamycin binding to HSP90, indicating that it does not associate with the N-terminal domain (Hieronymus et al., 2006). It has been suggested recently that celastrol inhibits HSP90 function by disrupting the association of CDC37 (Zhang et al., 2008), a co-chaperone which is required for loading of kinase clients onto HSP90 (Roe et al., 2004).

We have recently used a duplexed cell-based phenotypic assay (see below and Hardcastle et al., 2007) to screen our compound library and thereby to simultaneously identify compounds that inhibit HSP90 function and/or cellular acetylation in human colon carcinoma cells (Hardcastle et al., 2007). Using this approach we discovered CC002151 which induced the characteristic pattern of client protein depletion, heat shock induction and cell growth inhibition but did not inhibit HSP90 ATPase activity (Hardcastle et al., 2007). Further work is required to elucidate the mechanism of action of this compound.

7.4 Novel Approaches to Inhibit HSP90 Function

Inhibiting the ATPase domain of HSP90 has yielded significant information regarding the biological function of this chaperone, in addition to offering the most direct route to therapeutic manipulation. However, there are a number of alternative strategies to inhibit the function of this molecular chaperone which may broaden the therapeutic potential of chaperone modulation.

7.4.1 Targeting Individual HSP90 Isoforms

At present five isoforms of human HSP90 have been identified which differ in their cellular localisation (reviewed in Argon and Simen, 1999; Sreedhar et al., 2004a; Neckers et al., 2007). Evidence is also emerging of differences in specificity for particular client proteins and/or function, an aspect which could be potentially manipulated therapeutically to enhance the selectivity and reduce toxicity of HSP90 inhibitors. The two predominant cytoplasmic isoforms are HSP90α and HSP90β. HSP90β is constitutively expressed and considered to be important during cell differentiation and embryonic development (Sreedhar et al., 2004a). On the other hand, HSP90α basal expression is significantly lower than that of HSP90β, but its expression is significantly increased in response to stress and therefore it is considered

to more important than HSP90β for cytoprotection (Chen et al., 2005). Expression of either HSP90α or β as the sole isoform in yeast is sufficient to confer viability and to ensure stability of a number of, but not all, client proteins such as VSRC which more is reliant on HSP90α than HSP90β expression (Millson et al., 2007). In addition, activation of the heat shock factor-1 transcription factor (HSF1), which is known to be repressed by association with HSP90 (Shi et al., 1998), was more dependent on HSP90α expression than HSP90β (Millson et al., 2007). Interestingly, sensitivity to HSP90 inhibitors in yeast can be influenced by the expression levels of HSP90β. Expression of HSP90β as the sole isoform rendered yeast highly sensitive to radicicol, whereas sole expression of HSP90α did not (Millson et al., 2007). A further function which has been assigned solely to HSP90α is its unique ability to occupy a cell surface position and interact with the extracellular matrix protein, matrix metalloprotease-2, suggesting a potential role in cancer cell metastasis (Eustace et al., 2004).

Another cytoplasmic isoform is HSP90N which differs from HSP90α by the deletion of the N-terminal domain that is the site of the functional ATPase site (Grammatikakis et al., 2002). This isoform has been linked to cellular transformation via its association with CRAF (Grammatikakis et al., 2002). However, the details of its biological function remain to be defined.

The other major HSP90 isoforms are GRP94 (glucose regulate protein-1) in the endoplasmic reticulum (Argon and Simen, 1999) and TRAP1 (tumour necrosis factor receptor associated protein-1) in the mitochondrial matrix (Felts et al., 2000). There is limited literature regarding the specific functions of these isoforms. Although both GRP94 and TRAP1 share a similar overall structure to HSP90α and HSP90β, there is very little information regarding their interaction with or dependency on co-chaperones. GRP94 has been shown to play a role in the immune system by delivering peptides to MHC class I molecules for antigen presentation (Suto and Srivastava, 1995), ensuring immunoglobulin light chain formation and targeting unassembled subunits to the proteasome (Melnick et al., 1992; Melnick et al., 1994). GRP94 has also been associated with the maturation of receptor tyrosine kinases such as ERRB2 (Chavany et al., 1996) and the truncated EGFRvIII (Lavictoire et al., 2003) and also with the secretion of insulin-like growth factors (Wanderling et al., 2007). Finally, overexpression of GRP94 correlates with decreased sensitivity of cervical cancer cell lines to X-rays (Kubota et al., 2005) whereas reducing its expression increases the sensitivity of Jurkat cells to etoposide (Reddy et al., 1999). There is even less information on the biological function of the mitochondrial homologue TRAP1. Similar to HSP90α and HSP90β, TRAP1 has a functional ATPase domain; however, it does not associate with the co-chaperones P23 and HOP, potentially signifying a distinct mechanism of regulation (Felts et al., 2000). Despite its mitochondrial localisation, TRAP1 is implicated in the maturation of retinoblastoma protein (Felts et al., 2000). This interaction is unique to TRAP1 and is mediated by a LxCxE binding motif which is exclusive to this isoform (Felts et al., 2000). TRAP1 has also been linked to regulating mitochondrial function and protecting cells from mitochondrial-mediated apoptotic cell death induced by oxidative stress (Masuda et al., 2004; Pridgeon et al., 2007; Kang et al., 2007).

The ATPase cycles of both GRP94 and TRAP1 have recently been determined and compared to the well documented ATPase cycle of the cytosolic HSP90 isoforms (Frey et al., 2007; Leskovar et al., 2008). Subtle differences have been observed in the ATPase cycles of all four isoforms which, along with differences in 3-dimensional structure and in the affinities of each isoform for nucleotide (Leskovar et al., 2008), may offer the potential for the development of isoform-specific inhibitors. The geldanamycin derivative 17-AAG exhibits moderate selectivity over GRP94 and is highly selective against TRAP1 (Eccles et al., 2008). A degree of isoform specificity has been observed with the diaryl-isoxazole resorcinol HSP90 inhibitor NVP-AUY922. The IC_{50} values for NVP-AUY922 against the HSP90 family members were 535 ± 51 nmol/L and 85 ± 8 nmol/L for GRP94 and TRAP1 respectively compared to 7.8 ± 1.8 nmol/L and 21 ± 16 nmol/L for HSP90α and HSP90β, respectively, indicating significantly reduced potency against the non-cytosolic isoforms (Eccles et al., 2008). These data reinforce the possibility of achieving inhibitors that are more specific for a particular isoform of the HSP90 family. Further work is required to elucidate the consequences of this for the treatment of cancer and for effects on normal tissues.

7.4.2 Modulating the Association of HSP90 Co-Chaperones

As shown in Fig. 7.1, HSP90 function is supported by a number of co-chaperones which are involved in substrate recruitment and/or regulation of ATPase activity (Pearl, 2005; Pearl et al., 2008). Targeting co-chaperone interactions may enable a particular subset of client proteins to be inhibited which would lead to a more selective and, as a consequence, potentially less toxic inhibitor, the nature of which could be tailored for individual tumour types. We have provided evidence to support this concept using a small-interfering RNA (siRNA) approach to selectively knockdown the expression of AHA1, a co-chaperone which studies at our institution have shown to stimulate the relatively weak intrinsic ATPase activity of human HSP90 (Panaretou et al., 2002). Reduction of AHA1 expression resulted in decreased CRAF activity and reduced phosphorylation of the downstream kinases MEK1/2 and ERK1/2 (Holmes et al., 2008). Interestingly, total levels of the HSP90 client protein CRAF were unaffected by AHA1 knockdown, importantly suggesting that reduced AHA1 association with HSP90 prevented CRAF activation rather than reducing its stability (Holmes et al., 2008). Results of overexpression of AHA1 have provided further evidence that AHA1 recruitment is required for client protein activation rather than stabilisation. Thus higher AHA1 levels and HSP90 binding resulted in increased AKT phosphorylation and immunoprecipitated AKT catalytic activity (Holmes et al., 2008). Also of interest was the observation that, as with CRAF, the expression of CDK4 or ERBB2 was unaffected following the knockdown of AHA1 (Holmes et al., 2008), highlighting the potential for molecular specificity using this approach which might be translated into differential effects on tumours with distinct molecular pathologies.

The observations described above have been attributed to altering the ATPase activity of HSP90 by reducing AHA1 association (Holmes et al., 2008). Another obvious strategy is to target the co-chaperones involved in recruiting the client proteins to the HSP90 complex. HSP70 has a well documented role during the early stages of substrate loading onto HSP90 (Wegele et al., 2004). It is also implicated in malignant transformation due to its antiapoptotic role (Mosser and Morimoto, 2004; Calderwood et al., 2006). We have used an siRNA approach to selectively and simultaneously reduce the expression of the major constitutive and inducible isoforms of the HSP70 family, HSC70 and HSP72, respectively. We have shown that simultaneous knockdown of both isoforms inhibits the activity of HSP90 to induce degradation of CRAF, CDK4 and ERBB2 in human colon and ovarian cell lines (Powers MV, Clarke PA and Workman P, unpublished observations). This was accompanied by inhibition of cell growth and induction of cell death, the extent of which was greater than that seen with 17-AAG. Importantly, the effect of the combinatorial knockdown was significantly less in a number of non-tumorigenic cell lines, providing the first evidence of tumour selectivity and potentially reduced toxicity using this approach (Powers MV, Clarke PA and Workman P, unpublished observations). Another way of inhibiting substrate recruitment by HSP70 is to target HOP, an adaptor protein which links the HSP70 and HSP90 chaperone cycles (Chen and Smith, 1998). An engineered HSP90-tetracopeptide repeat (TPR) binding module has been designed which disrupts the interaction between the HOP TPR domain and the C-terminal of HSP90 (Cortajarena et al., 2008). Preliminary evidence of activity has been demonstrated by depletion of the ERBB2 client protein and inhibition of cell proliferation in the BT474 breast cancer cell line (Cortajarena et al., 2008). In addition, inhibition of HSP90 function using this approach was not associated with the undesirable increase in HSP70 expression associated with conventional ATPase HSP90 inhibitors (Cortajarena et al., 2008).

CDC37 is also emerging as an interesting target for modulation based on its selective recruitment of protein kinase client proteins to the chaperone complex (Roe et al., 2004) and its possible role in malignant transformation (reviewed in Pearl, 2005). Agents which prevent CDC37 interaction with HSP90 may have a particular advantage in the treatment of tumours driven by overexpressed or mutated kinases. Compared to approaches that target all HSP90 functions, this approach could also have less toxicity to normal cells since inhibiting CDC37 function would not be expected to affect the activity of the large number of non-kinase HSP90 client proteins, which include the steroid hormone receptors. We have used an siRNA approach to selectively reduce the expression of CDC37 in human colon cancer cells. Knockdown of CDC37 resulted in reduced association of protein kinase clients with HSP90 and decreased expression of several of these including CDK4, CDK6, AKT, ERBB2 and CRAF (Smith JR, Clarke PA and Workman P unpublished observations). This resulted in decreased cell signalling through the kinase clients, as demonstrated by reduced phosphorylation of downstream substrates and a subsequent G1/S phase cell cycle arrest (Smith JR, Clarke PA and Workman P unpublished observations). Similar observations have been reported in prostate cancer cell lines which undergo irreversible growth arrest following the molecular silencing of CDC37 (Gray et al., 2007).

The evidence presented above reinforces the view that HSP90 co-chaperones represent potential targets in their own right. However, we and others have also investigated the consequences of combining co-chaperone interference with classical pharmacological HSP90 inhibitors such as 17-AAG. For AHA1, HSP70 and CDC37, we and others have demonstrated in each case that the response to 17-AAG, including depletion of client proteins, inhibition of cell growth and induction of cell death, can be dramatically enhanced by the combinatorial silencing of co-chaperone expression (Gabai et al., 2005; Guo et al., 2005; Gray et al., 2007; Holmes et al., 2008, Powers MV et al, unpublished observations, Smith et al, unpublished observations). These observations suggest a method to potentially increase the therapeutic benefit of existing HSP90 inhibitors. Since combining co-chaperone targeting with pharmacologic HSP90 inhibition increases apoptosis, this approach represents a promising form of synthetic lethality, with potential for greater effects in cancer versus normal cells. However, it is necessary, when considering the targeted disruption of co-chaperone interactions, to think about the potential complexities of this approach. It is technically more difficult to design compounds to disrupt protein-protein interactions than to inhibit ATP binding. However, crystal structures of HSP90 and its co-chaperones are now available which offer the opportunity for lead identification using techniques such as fragment-binding and virtual-screening.

7.4.3 Post-Translational Modification of HSP90

It has emerged in recent years that, alongside co-chaperone interaction, HSP90 function may be regulated by a series of post-translational modifications. Several studies have demonstrated client protein depletion and HSP70 induction following the inactivation of histone deacetylase (HDAC) enzymes (Kovacs et al., 2005; Bali et al., 2005). This is due to hyperacetylation of HSP90 which disrupts its molecular function, possibly via dissociation of the co-chaperone P23 (Bali et al., 2005; Kovacs et al., 2005). Acetylation of HSP90 is mediated by inhibition of HDAC6 (Kovacs et al., 2005). A critical residue involved in this modification is Lys^{294} which, when hyperacetylated, decreases the function of yeast Hsp90 by reducing the interaction with its client proteins and co-chaperones (Scroggins et al., 2007). It is currently not understood if acetylation is required for the normal regulatory function of HSP90, nor is it known which acetyltransferase is responsible for the modification.

As mentioned earlier, we have utilised a high-throughput screen to identify agents which inhibit cellular acetylation or HSP90 function (Hardcastle et al., 2007). We developed a high-throughput, duplexed, cell-based phenotypic screen which utilised a multiplexed time-resolved fluorescence cell immunosorbent assay (TRF-Cellisas) to simultaneously detect compounds which induce HSP70, as a mark of HSP90 inhibition, together with agents that modulate cellular acetylation (Hardcastle et al., 2007). Using this approach it may be possible to discover compounds which interrupt HSP90 function by modulating cellular acetylation (Hardcastle et al., 2007). Several hits were identified in both arms of the screen. The precise mechanism of action of these compounds remains to be further defined.

Alongside acetylation, HSP90 function can also be regulated by phosphorylation (Zhao et al., 2001). Serine and threonine phosphorylation have been reported to have a negative effect on the activity of HSP90 although the exact residues involved are unknown (Zhao et al., 2001). CDC37 also requires phosphorylation for its activity (Miyata and Nishida, 2005), indicating an alternative approach to inhibiting HSP90 function. A kinase-based strategy is reinforced by the observation of inhibition of HSP90 activity following blockade of phosphatase activity (Wandinger et al., 2006). Some initial success with this approach has already been achieved with the identification of compound IC101 which induces HSP90 dephosphorylation, client protein depletion and apoptosis (Fujiwara et al., 2004).

7.4.4 Substrate Mimetics

Inhibiting the association of a single client protein substrate with HSP90 would be an efficient approach to achieving a very high level of selectivity, particularly in the treatment of cancers driven by a single, dominant oncogenic protein, or perhaps a group of closely related clients. An example of an attempt to target the interaction of HSP90 with a particular client is the peptidometic shepherdin which was designed to interfere with the interaction between HSP90 and its client protein survivin (Plescia et al., 2005). Exposure of cancer cells to shepherdin caused depletion of client proteins, including survivin, induced apoptotic and non-apoptotic cell death, and brought about the eradication of acute myeloid leukaemia xenografts (Plescia et al., 2005; Gyurkocza et al., 2006). Subsequently, a non-peptidic small molecule inhibitor, AICAR, was developed which retained a similar profile of HSP90 inhibition (Meli et al., 2006). Although general client protein depletion was observed following treatment with shepherdin and AICAR, the molecular profile of HSP90 inhibition following their use was distinct from other HSP90 inhibitors in that it did not include the induction of HSP70. As described above, we and others have previously demonstrated that HSP70 induction dramatically reduces the cell death effects of 17-AAG (reviewed in Powers and Workman, 2007). Therefore, the lack of induction of HSP70 observed following shepherdin treatment may be of therapeutic benefit.

The design of inhibitors that very specifically block the interaction of individual client proteins with HSP90 will await the solving of what is probably the most outstanding and important problem in HSP90 biology: How does HSP90 recognise its client proteins (Pearl et al., 2008)?

7.5 Targeting HSP90 Function in Endocrine-Related Cancers

There is a growing body of evidence validating the potential of HSP90 inhibitors in the treatment of endocrine-related malignancies such as breast and prostate cancer. Expression of HSP90 has been shown recently to be elevated in breast cancer and

its increased expression is associated with decreased survival (Pick et al., 2007). In addition, many of the proteins implicated in breast cancer progression and resistance to therapy are chaperoned by HSP90 (reviewed in Beliakoff and Whitesell, 2004). These include the oestrogen receptor, members of the ERBB receptor tyrosine kinase family, AKT, HIF1α and mutant p53 (reviewed in Beliakoff and Whitesell, 2004). This collection of potential targets suggests that HSP90 inhibitors may have therapeutic potential for the treatment of both hormone-sensitive and hormone-insensitive breast cancer. This is reinforced by the recent observation of responses to 17-AAG plus trastuzumab in patients with trastuzumab-refractory breast cancers (Modi et al., 2007). We have also obtained promising results with the isoxazole resorcinol inhibitor NVP-AUY922 in a human breast tumour xenograft (Eccles et al., 2008), a result also confirmed by others (Jensen et al., 2008). Treatment of BT474 human breast tumours with pharmacologically relevant concentrations of NVP-AUY922 induced ERα and ERBB2 depletion which was accompanied by growth arrest and/or regression of tumours (Eccles et al., 2008).

HSP90 inhibitors may also be advantageous in the treatment of prostate cancer which can be reliant on androgen receptor expression or dependent on the PI3K pathway through loss of PTEN (reviewed in Majumder and Sellers, 2005). We have recently demonstrated, for the first time, that our HSP90 inhibitor, NVP-AUY922 can reduce the growth of established orthotopic PTEN-null, hormone-independent prostate carcinoma xenografts (Eccles et al., 2008). This is consistent with earlier reports using 17-AAG which demonstrated reduced growth of androgen-dependent and androgen-independent human tumour xenografts grown subcutaneously (Solit et al., 2002).

7.6 Conclusions, Future Prospects and Challenges

The potential to simultaneously deplete malignant cells of multiple client proteins and to modulate all six hallmark traits of cancer by inhibiting a single protein target has propelled HSP90 into the spotlight as an exciting anticancer drug target. Natural product HSP90 ATPase-inhibitors have been fundamental in elucidating the mechanism of action of the molecular chaperone. The geldanamycin analogue 17-AAG has completed phase I trials and provided proof-of-concept for HSP90 inhibition in the clinical setting. 17-AAG has shown early promise as the first-in-class HSP90 inhibitor with responses being observed in melanoma, breast and prostate carcinoma and phase II trials have now been initiated. A phase III study of 17-AAG plus trastuzumab in trastuzumab-refractory, ERBB2-positive tumours has been initiated. Phase III trials have also been instigated for the combination of 17-AAG and bortezomib for patients with multiple myeloma, where promising clinical activity has been seen in earlier studies.

Tumour samples from the clinical trials are now aiding the identification of factors which may influence patient response to HSP90 inhibitors. An example is our own recent identification of NRAS/BRAF mutation status as a potential factor in

the response of melanoma patients treated with 17-AAG (Banerji et al., 2008a). The promising activity of 17-AAG in trastuzumab-resistant ERBB2 positive breast cancer may relate to the depletion of ERBB2, but effects on other clients may also play a role. Combined effects on ERBB2 and the oestrogen receptor suggests potential in tumours with these characteristics but activity in ERBB2-/ER- breast cancers is also possible. In prostate cancer, effects on the androgen receptor and on the PI3K pathway are likely to be important. Activity in multiple myeloma, most notably in combination with bortezomib, appears to involve the unfolded protein response that is important in this disease (Davenport et al., 2007). Combined administration of 17-AAG and docetaxel has demonstrated promising clinical activity in patients with NSCLC (Solit et al., 2004), potentially related to effects on mutant epidermal growth factor receptor (EGFR) which is more sensitive to HSP90 inhibitors than the wild-type form (Sawai et al., 2008; Xu et al., 2007).

Despite the promising activity of 17-AAG it is not without its limitations, which include solubility and formulation issues and significant toxicity being observed in patients. It is not known how much of the toxicity is due to non-specific off-target effects related to the particular chemical scaffold of the inhibitor. For example, the liver toxicity seen with 17-AAG may be associated with the quinone moiety. As a result there is considerable interest in searching for novel, synthetic, small-molecule inhibitors of HSP90. Several have been described including purines and the potent isoxazole resorcinols that we discovered in collaboration with Vernalis. Of the latter, NVP-AUY922 is being developed by Novartis and has just entered phase I clinical trial.

It is possible that some of the side-effects seen with HSP90 inhibitors may be a consequence of inhibiting HSP90 activity. The combinatorial effect on multiple signal transduction pathways is one of the key strengths of HSP90 inhibitors but it may also obscure factors underlying toxicities associated with their on-target effects. For the same reason it is also difficult to deconvolute the precise mechanism of action of HSP90 inhibitors in a particular cancer. Because of the potential for more specific effects on certain cancers and for reducing normal tissue toxicity, alternative methods are being investigated to inhibit particular functions of HSP90. These include targeting individual isoforms of HSP90, altering the post-translational modifications of the chaperone, preventing the association of a defined subset of client proteins by the use of substrate mimetics, or modulating the association of a particular co-chaperone. Such co-chaperones include, amongst others, AHA1, CDC37 and HSP70. Silencing the expression of these proteins results in the inhibition of HSP90 function and sensitisation to pharmacologic HSP90 inhibitors. Developing the ability to selectively target a particular subset of client proteins may facilitate the tailored use of inhibitors which target different aspects of the HSP90 chaperone for the treatment of tumours with particular molecular drivers.

Over the last few years HSP90 has progressed from a fringe target that many if not most considered too risky to one that has taken centre stage in many companies and academic drug discovery groups. Proof-of-concept for target modulation in patients and clear signs of clinical activity have reduced the perceived risk and stimulated considerable interest. A range of N-terminal ATP site inhibitors are now

emerging from screening and structure-based design programmes and are entering the clinic. This in turn has encouraged alternative technical approaches to attack HSP90 and stimulated the biomedical research community to think more widely about additional targets in chaperone biology and protein quality control. The next few years will see considerable activity in the evaluation of HSP90-targeted agents in various cancers, both endocrine and non-endocrine, as well as potentially in the treatment of other diseases. We can no doubt continue to expect to be surprised by what we do not yet know about the basic biology, as well as the pharmacology and therapeutics, of the HSP90 molecular chaperone.

7.7 Conflict of Interest

PW has been involved in a funded research collaboration with Vernalis to develop HSP90 inhibitors that were licensed to Novartis. PW has been a consultant to Novartis.

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Abbreviations

17-AAG:	17-allylamino-17-demethoxygeldanamycin
17-DMAG:	17-dimethylaminoethylamino-17-demethoxygeldanamycin
GRP:	glucose regulated protein
HSP:	Heat shock protein
siRNA:	short interfering RNA
AKT:	protein kinase B
ERBB2:	epidermal growth factor receptor 2
HSP70:	heat shock protein 70
IPI-504:	17-allylamino-17-demethoxygeldanamycin hydroquinone hydrochloride (retaspimycin)

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Chapter 8
Adverse Features of Acquired Antihormone Resistance and Their Targeting

Stephen Hiscox, Nicola Jordan, Liam Morgan, Chris Smith, Lindy Goddard, Julia M.W. Gee and Robert I. Nicholson

Contents

Abstract Endocrine therapy is the treatment of choice in hormone receptor-positive breast cancer. However, the effectiveness of these agents is limited by the development of drug resistance, ultimately leading to disease progression and patient mortality. Cell models of endocrine resistance have demonstrated a role for altered growth factor signalling in the development of an endocrine insensitive phenotype. Significantly, recent studies have revealed that the acquisition of endocrine resistance in breast cancer is also accompanied by the development of an adverse cellular phenotype, with resistant cells exhibiting altered adhesive interactions, enhanced migratory and invasive behaviour, and a capacity to induce angiogenic responses

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in endothelium. Since invasion and metastasis of cancer cells is a major cause of mortality in cancer patients, elucidation of molecular mechanisms underlying the adverse cellular features that accompany acquired endocrine resistance and their subsequent targeting may provide a means of limiting the progression of such tumours in vivo.

Keywords Invasion · Migration · Metastasis · Cell adhesion · Cadherin · Src · Fak

8.1 Introduction

Endocrine therapy is the treatment of choice for early stage and metastatic breast cancers that are hormone receptor positive. In such cases, these agents are effective at reducing breast cancer recurrence rates and improving patient survival. Unfortunately however, whilst a substantial proportion of breast tumours will display an intrinsic resistance to hormone therapies despite being hormone receptor positive (de novo resistance), more than a third of patients with endocrine-responsive, early stage breast cancer and almost all of those with metastatic disease will develop hormone resistance during the course of their disease (acquired resistance) (Conte et al., 2007; Howell et al., 2008; Nicholson and Johnston, 2005).

Much research has been undertaken in order to understand the mechanisms that underlie the phenomenon of endocrine resistance and to reveal markers that predict for response to, or early relapse on, treatment and identify potential therapeutic targets through which endocrine resistance may be delayed or prevented. Through these studies it is increasingly apparent that the tumour cells' ability to harness a variety of growth factor signalling pathways to drive growth in the presence of endocrine agents plays a major role in promoting a resistant phenotype. Moreover, it is becoming clear that anti-hormones themselves can promote the expression of a number of growth factors and their receptors in the drug-responsive phase, which subsequently play key roles in the regulation of tumour growth during the drug-resistant phase (Arpino et al., 2008; Nicholson et al., 2004 and see Chapter 4). Indeed, the role of growth factor signalling in endocrine resistance has gained significant attention over the past decade and there is now compelling evidence which suggests that the inappropriate activation of growth factor signalling cascades can readily promote anti-hormone failure in breast cancer cells. In such cases, the enhanced expression of growth factor signalling pathways and networks are likely to contribute to endocrine resistance through cross-talk with the ER resulting in a ligand-independent activation of the ER and sustained cellular growth (Britton et al., 2006; Nicholson et al., 2004; Wilson and Slamon, 2005). Overexpression of members of the erbB family of receptor tyrosine kinases including the epidermal growth factor receptor (EGFR), HER2, HER3 and the insulin-like growth factor-1 receptor (IGF1R) together with several of their ligands have all been suggested to play a central role in mediating an endocrine resistant state in some situations (Knowlden et al., 2003; Kurokawa and Arteaga, 2001; Nicholson et al., 2004; Parisot et al., 1999; Tovey et al., 2005).

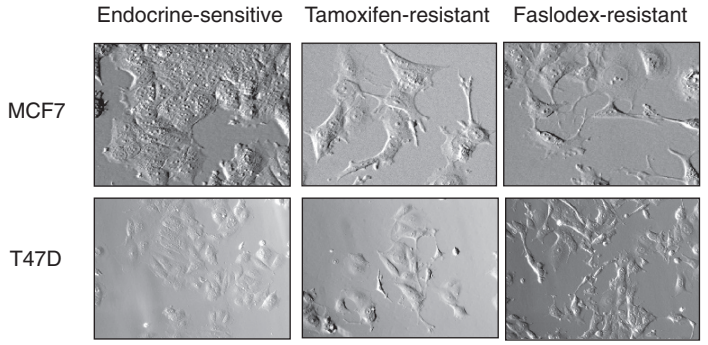

Fig. 8.1 Acquisition of endocrine resistance in ER-positive MCF7 and T47D breast cancer cells is accompanied by a change in cellular morphology. Resistant cells demonstrate high levels of membrane activity (lamella and filopodia, and membrane ruffling) in addition to a loss of cell-cell adhesion

Growth factor signalling pathways that promote the proliferation of breast cancer cells in an endocrine resistant context are also known also to play prominent roles as promotors of cellular migration and invasion in other cell systems (Arora et al., 2008; Ueno et al., 2008; Wells et al., 2002) and it therefore follows that resistance to endocrine agents in breast cancer may result in the development of an adverse cellular phenotype. Indeed, our recent observations have demonstrated this to be the case, with multiple breast cancer cell models of acquired drug resistance demonstrating a highly invasive phenotype in vitro (Fig. 8.1) (Hiscox et al., 2006b, 2004a; Jones et al., 2004). However, since inhibition of the dominant growth regulatory pathways in these models results only in modest suppression of their invasive phenotype (Hiscox et al., 2004a) other, as yet unidentified, mechanisms must be present that control an adverse cellular behaviour. A number of key elements with pro-invasive roles are now known to be induced by a range of endocrine treatments and, if recapitulated in vivo, suggest that anti-hormones themselves may further augment the cells' metastatic capacity and promote tumour progression. Several key molecular elements are described in this chapter that regulate these processes and which may present future targets through which endocrine resistance and an associated adverse cell phenotype may be prevented.

8.2 Acquired Resistance to Endocrine Therapies is Accompanied by Altered Cell Morphology and a Change in Their Adhesive Characteristics

In in vitro models of breast cancer cells which have developed resistance to the endocrine agents tamoxifen and fulvestrant commonly show a more angular, dedifferentiated morphology with numerous lamellipodia and membrane ruffling in

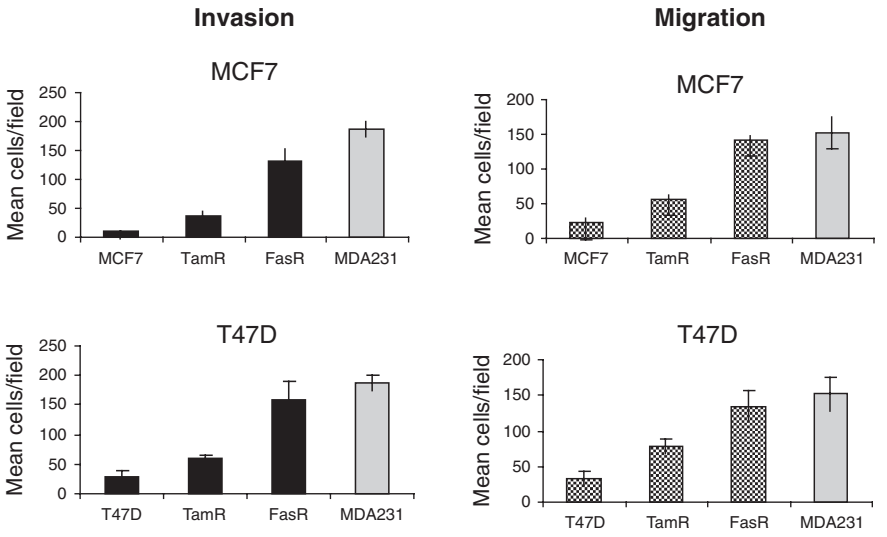

Fig. 8.2 Acquired tamoxifen- and fulvestrant- resistant MCF7 and T47D cells possess a highly migratory and invasive phenotype in vitro. In the case of fulvestrant-resistant cells, their aggressive behaviour approaches that observed in the highly-metastatic MDA-MB-231 cell line

addition to growing as loose, disorganised colonies in which cells appear to have partially-dissociated cell-cell contacts (Fig. 8.2; (Hiscox et al., 2006a, 2004a). Such cellular morphology is reportedly characteristic of a migratory phenotype, where it may be indicative of a dynamic regulation of focal adhesions and actin within the cell underlying a migratory phenotype (Carragher and Frame, 2004). This is interesting in light of the fact that our microarray analysis of endocrine-resistant breast cancer cells has revealed changes in the expression of Rho (Shaw et al., 2005), a GTPase which regulates actin dynamics, stimulating membrane protrusions (Aspenstrom et al., 2004) and is involved in the endosomal trafficking of receptor and non-receptor tyrosine kinases involved in the regulation of cellular motility (Sandilands and Frame, 2008).

The apparent change in epithelial cell morphology and colony integrity observed within endocrine-resistant cell cultures may further be reflective of a loss in intercellular adhesion, suggesting that these cells might be undergoing epithelial-to-meshenchymal transition (EMT), a process well associated with a more aggressive cell phenotype (Hugo et al., 2007). Although loss of E-cadherin is a hallmark of EMT and well associated with a more aggressive cell phenotype, its expression does not appear to be altered in our cell models of acquired endocrine resistance (Hiscox et al., 2006a). However, tamoxifen- and fulvestrant-resistant cells display changes in β-catenin, a key interacting partner of E-cadherin and a modulator of EMT (Hiscox et al., 2006a).

Microarray and signalling studies have revealed that β-catenin expression is increased at both the mRNA and protein level, whilst its phosphorylation status is significantly modified (elevated tyrosine phosphorylation, decreased serine/threonine

phosphorylation) in resistant cells compared to their endocrine sensitive counterparts. In tamoxifen-resistant cells, this deregulation is associated with PI3K/AKT-induced inactivation of GSK3β resulting in reduced association of β-catenin with E-cadherin, disruption of cell-cell contacts and increased migration and invasion (Hiscox et al., 2006a). Recently, we have identified a key role for Src kinase in these phenomena. Furthermore, failure of GSK3β/ubiquitin-mediated degradation of β-catenin in these cells results in elevated intracellular levels of β-catenin, promoting its nuclear translocation and interaction with the TCF/LEF-1 transcription factor. This triggers increased transcription of β-catenin/TCF/LEF-1 target genes such as CD44 (Hiscox et al., 2006a) that may further modify invasive cellular responses (See Section 8.3.2 below). Interestingly, NF-κB expression is induced by both tamoxifen and fulvestrant (Chapter 4 and (Shaw et al., 2005)) and recent reports have linked increased NF-κB levels with the induction of EMT in breast cancer (Radisky and Bissell, 2007). The development of EMT-like changes in these models may have bearing on the cells' response to targeted therapies since in other cancer types an induction of EMT has been associated with a decreased response to small molecule inhibitors (lung cancer cells) (Rho et al., 2008) and a determinant of sensitivity to EGFR inhibitors (hepatocellular carcinoma cells) (Fuchs et al., 2008).

In addition to modulations in cell-cell adhesive interactions, we have identified that acquired endocrine resistance in breast cancer cells is accompanied by a change in integrin expression (Hiscox et al., 2004b). Consequently, the intrinsic ability of these cells to adhere to, and migrate over, components such as collagen, laminin and fibronectin are enhanced (Hiscox et al., 2007) whilst this attachment and migration is suppressed in the presence of neutralising antibodies to integrins αv, β1 and β6. Clearly, this has potential significance in an in vivo context, where adhesive interactions between tumour cells and extracellular matrix proteins are paramount to successful tumour dissemination. These observations are further interesting given that integrin signalling is implicated in hormone-dependent cell proliferation. For example, high levels of $\alpha_5\beta_1$ (fibronectin integrin) expression are detectable during periods of steroid-induced proliferation but decreased during late pregnancy and lactation and following ovariectomy (Haslam and Woodward, 2003). Thus alterations in integrin expression profile may modify the cells' response to oestrogenic signals and endocrine agents in the appropriate environment. Indeed, such effects have recently been reported in breast cancer cells where enhanced integring expression contributes to tamoxifen resistance through a mechanism involving HER3 and Akt (Folgiero et al., 2008).

8.3 Endocrine Resistant Breast Cancer Cells Overexpress Cell Surface Receptors that May Sensitize them to the Tumour Microenvironment

As mentioned above, certain signalling pathways are known to be overexpressed in endocrine resistance (e.g. EGFR/HER2 pathway) that, in addition to playing a

prominent role as a driver of resistant cell growth, may also promote invasive response. It follows that targeting of these may be an effective means of suppressing the invasive phenotype in addition to cellular proliferation. However, pharmacological targeting of erbB signalling in these cells exerts only a modest inhibitory effect on the cells' invasive capacity ((Hiscox et al., 2004a); L. Morgan, unpublished observations) suggesting that erbB signalling contributes to, but is not essential for, their invasive in vitro phenotype. Moreover, prolonged exposure to such inhibitors ultimately results in the development of a further drug-resistant state, with a further gain in cellular invasiveness (Jones et al., 2004). We have now identified a number of key molecules that appear to play a central role as mediators of an intrinsic invasive phenotype in endocrine resistance in vitro. However, a further consequence of their overexpression in resistant cells is to sensitize these cells to factors commonly found within the tumour microenvironment.

In vivo, the tumourogenic potential of cancers is profoundly influenced by their microenvironment. The release of soluble factors from stromal fibroblasts allows paracrine regulation of epithelial cell behaviour including growth, differentiation, migration and invasion; this reciprocal communication between cells is often deregulated in cancer progression (reviewed in (Bhowmick and Moses, 2005; Bhowmick et al., 2004)). The majority of observations on endocrine resistant breast cancer cells models derive from 2D, in vitro cultures of individual cell lines. Although these represent relatively 'pure' experimental systems and do not accurately reflect the complexity of the tumour microenvironment in vivo, data from co-culture systems is beginning to reveal that endocrine resistant breast cancer cells appear to be sensitized to factors commonly found, and frequently overexpressed, within the tumour microenvironment raising the possibility that that the adverse phenotype of resistant cells may be further enhanced in an in vivo context.

8.3.1 C-Met Receptor

One such case is exemplified by the c-met receptor which we have identified as being overexpressed in fulvestrant-resistant MCF7 and T47D cells. the c-met receptor tyrosine kinase is the cell surface receptor for hepatocyte growth factor (HGF, also known as scatter factor (SF)) and its activation results in disruption of intercellular adhesion, cell migration and invasion and promotion of angiogenesis (Comoglio et al., 2008). subsequently, we have shown that co-culture of fulvestrant-resistant cells with stromal fibroblasts, known producers of HGF/SF (Jiang et al., 2003), or in fibroblast-conditioned medium, results in the activation of akt and the production of MMP2 and MMP9 and a further enhancement of these cells' invasive behaviour (Hiscox et al., 2006a; Y. Khirwadkar personal communication); although fibroblasts secrete a range of growth factors and cytokines that may modulate epithelial cell behaviour, our sirna data demonstrated that these effects are specific to c-met activation (Hiscox et al., 2006b).

In vivo, the c-Met receptor is primarily expressed by epithelial cells and its overexpression in node-positive breast cancer identifies patients with poor clinical outcome (Lengyel et al., 2005). This is not surprising given the ability of c-Met to be activated in a paracrine fashion by HGF/SF-secreting stromal fibroblasts. Indeed, this mechanism has been implicated as a major contributory factor for tumour progression with studies demonstrating the ability of HGF/SF to regulate EMT and metastasis (Thiery, 2002). Furthermore, the therapeutic value of c-Met in breast cancer has been demonstrated through studies that have used retroviral ribozyme transgenes to target HGF/SF expression in fibroblasts or the Met receptor in mammary cancer cells to inhibit paracrine stromal-tumour cell interactions (Jiang et al., 2003). Since tumour invasion and spread may thus be critically influenced by paracrine influences arising from the surrounding stroma, these observations suggest that, in vivo, overexpression of c-Met in anti-hormone-resistant epithelial breast cancer cells may significantly affect tumour progression.

Interestingly, as well as being overexpressed in the endocrine resistant state, c-Met gene and protein expression is induced by fulvestrant in the drug-responsive phase. Such an event may act to limit the response of these cells to fulvestrant by providing a mechanism to drive cellular growth in the absence of functional ER (induced by fulvestrant) as evidenced by out preliminary studies using fulvestrant-treated MCF7 cells (S. Hiscox, unpublished observations). An intriguing question is to how fulvestrant might modulate c-Met expression in breast cancer cells. A role for the ER is unlikely, since c-Met expression does not correlate with ER status in breast cancer tissues (Ghoussoub et al., 1998; Lengyel et al., 2005). However, transcription of the c-Met gene in known to be regulated by members of the widely expressed Sp family of transcription factors (Zhang et al., 2005, 2003) with Sp1 activity itself influenced by ER signalling (Kim et al., 2005; Sumi and Ignarro, 2005) and thus fulvestrant treatment. Indeed, fulvestrant-induced p21Waf1 expression has been recently demonstrated in MCF7 cells through an Sp1-mediated mechanism (Varshochi et al., 2005). Interestingly, we have observed alterations in Sp1 and Sp3 expression in MCF7 cells on exposure to fulvestrant (S. Hiscox and N. Jordan, unpublished observations) which may thus represent one mechanism by which c-Met overexpression can be achieved.

8.3.2 CD44

In contrast to the overexpression of the c-met receptor which appears to be an effect specific to one particular endocrine agent (fulvestrant), a common feature of acquired resistance to multiple endocrine agents (tamoxifen and fulvestrant) and to oestrogen deprivation is the overexpression of cell surface receptors of the CD44 family (Harper et al., 2005), a group of transmembrane glycoproteins implicated in the progression and spread of breast cancer. alternative splicing and variation in glycosylation results in structural and functional diversity amongst this group of proteins (Screaton et al., 1992) with several CD44 variants being associated with invasive breast cancer. for example, expression of the CD44 variant 3 (cd44v3) cor-

relates with lymphatic spread in breast cancers (Rys et al., 2003), soluble cd44v6 is associated with lymph node metastases (Mayer et al., 2008) whilst cd44v7 is associated with a reduction in disease-free survival (Watanabe et al., 2005). however, whilst a wealth of evidence implicates CD44 variants in tumour progression, the case for the standard form of CD44 (cd44s) is controversial. whereas some studies report that increased expression of the cd44s correlates with patient survival (Gong et al., 2005), recent studies have demonstrated that expression of cd44s in non-metastatic MCF7 breast cancer cells promotes their migration and invasion in vivo (Ouhtit et al., 2007).

In tamoxifen and fulvestrant-resistant cell models, CD44s, together with the v3, v6 and v10 isoforms, are overexpressed at the gene and protein level. The relevance of overexpression of CD44 in these model systems has been demonstrated by siRNA knockdown experiments which reveal that loss of CD44 has an inhibitory effect on the cells' intrinsic migratory capacity in vitro (Hiscox et al., 2008a; Jordan et al., 2008). CD44 is also reported to associate, and form stable complexes with, a number of growth factor receptors including those of the erbB family providing a system through which cellular migration and invasion can be augmented (Bourguignon et al., 1997; Tsatas et al., 2002). This is interesting in light of our knowledge that such receptors are also overexpressed in endocrine resistance (Hiscox et al., 2006a). Indeed, we have seen that CD44v3, and to a lesser extent CD44s, associate with the EGFR and HER2 in tamoxifen-resistant cells and the c-Met receptor in fulvestrant-resistant cells. The effect of this is to significantly augment the cellular invasive response to exogenous erbB ligands (in tamoxifen resistance) or HGF (in fulvestrant resistance) (Hiscox et al., 2008a; Jordan et al., 2008). A caveat to these data is that CD44 siRNA is not specific for any particular CD44 isoform but rather results in the knockdown of all forms of CD44 expressed. It is thus not possible to determine the relative contribution to the cell's aggressive phenotype from individual CD44 family members. However, it is interesting to note that examination of CD44v3 protein expression in a small series ($n = 77$) of clinical tissue revealed an association with HER2 expression, poor survival and shortened response to endocrine therapy in ER+ patients (Hiscox et al., 2008a).

In addition to growth factors and cytokines, tumour cells are in contact with a number of extracellular matrix components in an in vivo situation. A number of these can act as ligands for cell surface receptors providing additional means through which the epithelial cell phenotype can be modulated. Our recent observations have revealed that activation of CD44 by hyaluronic acid (HA), an important structural component of extracellular matrices known to be concentrated in regions of high cell division and invasion (Toole and Slomiany, 2008), promotes erbB invasive signalling in tamoxifen-resistant cells (B. Baruha, unpublished observations) which may again promote an adverse cellular phenotype. Together these observations suggest that acquired resistant cells are sensitized to many factors commonly found within the tumour microenvironment such as erbB ligands, HGF/SF and the matrix components themselves. The fact that many of these factors are increased in breast cancer tissue and serum may have significant bearing on the progression of tumours following relapse on therapy.

8.3.3 Antihormones Induce Pro-Invasive Responses During the Drug-Responsive Phase which, in the Appropriate Cell Context, May Contribute to an Adverse Cell Phenotype

An intriguing observation is that increased cellular invasiveness is observed in response to short term anti-hormone treatment in ER+, endocrine-sensitive breast cancer cells (Borley et al., 2007). Although modest, these antihormone-induced, pro-invasive effects become significant under conditions of E-cadherin deficiency, where the gain in cellular invasion is greatly augmented (Borley et al., 2007). Such data highlight a previously unreported effect of tamoxifen (and potentially further antioestrogens), in that these agents appear able to induce breast cancer cell invasion in a specific context (absence of good cell-cell contacts); this may have major clinical implications for those patients with tumours where there is inherently poor intercellular adhesion.

These observations suggest that pro-invasive gene/pathway changes can be induced by endocrine agents at an early stage, the effects of which may be further augmented by changes in cell context: for example, the presence of exogenous growth factors within the tumour microenvironment that may suppress cell-cell adhesive interactions. The interaction between tumour cells and the microenvironment can have a substantial effect on tumour cell behaviour by influencing cell-cell as well as cell-matrix contacts although the underlying molecular mechanisms are not well characterised at present. Several factors within the tumour microenvironment, including growth factors such as TGFβ and erbB ligands in addition to protein components of the surrounding extracellular matrix itself, have been identified as being able to cause the disruption of the E-cadherin adhesion complex and reduced E-cadherin expression (Giehl and Menke, 2008). These factors activate a number of pathways within the tumour cells (integrins, Src kinase, focal adhesionkinase and PI3K) that may regulate EMT like behaviour. Sensitization of breast cancer cells to these microenvironmental factors, brought about through acquired endocrine resistance, may further facilitate the complex interactions between tumour cells and the surrounding stroma may create conditions permissive for further pro-invasive actions of antihormones and ultimately promote disease progression and spread.

8.4 Src Kinase as a Therapeutic Target in Breast Cancer

Although the precise molecular mechanisms involved in the acquisition of endocrine resistance in breast cancer cell still remain to be elucidated, data is emerging which implicates Src kinase as a key mediator of resistance in breast cancer, through its role as a key intermediary in both growth factor and ER signalling pathways, and as a regulator of cross-talk between the ER and growth factor receptors and other downstream signalling elements resulting in ligand-independent activation of the ER and tumour cell growth.

8.4.1 Interaction with EGFR Family Receptors

Physical interactions between Src and growth factor receptors are reported in breast cancer tissues and cells, particularly with receptor tyrosine kinases of the erbB family, known to be overexpressed in endocrine-resistant cell lines and tissues. Both EGFR and HER2 are frequently over-expressed in breast cancers (up to 60%, (Nicholson et al., 2001)), often with concomitant Src overexpression (Biscardi et al., 2000; Ishizawar and Parsons, 2004; Summy and Gallick, 2003). Indeed, Synergism between Src and the EGFR enhances neoplastic growth of mammary epithelial cells (Biscardi et al., 2000; Maa et al., 1995) and elevated expression of both EGFR and Src occur in a subset of late-stage breast cancers where interaction between these two molecules promotes an aggressive disease phenotype (Biscardi et al., 1998). Activation of Src following HER2 stimulation promotes cellular invasion through a mechanism involving FAK and the production of proteases (Mitra et al., 2006; Vadlamudi et al., 2003) and has been implicated in tumour spread (Tan et al., 2005). Such data may have clinical bearing given that high levels of activated c-Src correlate with HER2 positivity in breast cancer tissue (Wilson et al., 2006; Vadlamudi et al., 2003).

Although a wealth of data supports the interaction between Src and EGFR/HER2 in breast cancer, the role of Src in HER3- and HER4-mediated signalling is less clear. There is evidence that HER3/c-Src signalling protects breast cancer cells against radiation-induced apoptosis (Contessa et al., 2006) and Src has been shown to enhance HER2/HER3 signalling and subsequent biological effects by promoting HER2/HER3 heterodimerisation (Ishizawar et al., 2007). Together, these findings reveal Src as a modifier of the oncogenic function of several EGFR family members which may have relevance in breast cancer subtypes over-expressing them.

8.4.2 Interaction with Steroid Hormone Receptors

Src is able to potentiate the AF-1 dependent gene transcription function of the ER both by indirect phosphorylation of nuclear ER via ERK 1/2 (Feng et al., 2001) and Akt (Campbell et al., 2001; Shah and Rowan, 2005) and through regulation of FAK-P130cas-JNK pathway activity and the subsequent activation of co-activator molecules including CBP and GRIP1 (Feng et al., 2001). Interestingly, these effects of Src can occur in the absence or presence of liganded ER; in the latter case, this ligand can be oestrogen or tamoxifen.

In addition to its genomic action, however, it is becoming clear that the ER can also exert rapid, non-genomic effects which are initiated in the cytosolic/membrane compartment of the cell (Acconcia and Kumar, 2006). Activation of distinct cytoplasmic protein kinase cascades in this manner results in the regulation of numerous cellular processes such as proliferation, differentiation, apoptosis and vasorelaxation. In human breast cancer cells, ligand binding to the ER results in the rapid activation of the ERK and Akt pathways in a Src dependent manner (Migliaccio et al.,

1996; Castoria et al., 2001; Wessler et al., 2006). Furthermore, in both ER-positive breast cancer cells and in cells which transiently express ER, oestradiol has been shown to induce rapid (within minutes) activation of Src-dependent signalling pathways (Castoria et al., 2001; Migliaccio et al., 1996) which regulate cellular proliferation and survival (Castoria et al., 1999; Migliaccio et al., 2000). The physical interactions that occur between Src and the ER can enhance oestrogen-mediated gene transcription and may be facilitated by intermediate adapter-molecules such as the recently-described MNAR protein (Wong et al., 2002). Significantly, specific targeting of ER-associated Src using phosphopeptides disrupts these interactions and prevents the induction of DNA synthesis following treatment with oestradiol (Varricchio et al., 2007).

8.4.3 Involvement in Steroid Receptor-Growth Factor Receptor Crosstalk

Significant levels of cross talk exist between ER and growth factor receptor signalling pathways in breast cancer. For example, EGF treatment can activate genes regulated by estrogen-responsive elements (Ignar-Trowbridge et al., 1993; Migliaccio et al., 2005) whilst the induction of DNA synthesis following EGFR activation may be inhibited by endocrine agents such as fulvestrant (Migliaccio et al., 2006). Additionally, EGFR/HER2 activation can potentiate ER signalling in the absence of oestradiol promoting the phosphorylation of the AF-1 domain of ER, recruitment of co-activators and subsequent gene transcription (Bunone et al., 1996; Lannigan, 2003; Deblois and Giguere, 2003). Moreover, growth factor binding to receptor tyrosine kinases such as the EGFR results in Src activation and subsequent oestrogen-independent regulation of ER activity and cell proliferation (Shupnik, 2004; Levin, 2003; Osborne and Schiff, 2003).

There is strong evidence linking crosstalk between the ER and growth factor receptor signalling pathways in the development of endocrine resistance (Osborne and Schiff, 2003) by providing a mechanism of ligand-independent activation of the ER and promotion of tumour growth (Britton et al., 2006). Indeed, our own studies demonstrate that ERα can maintain EGFR signalling and modulate the growth of tamoxifen-resistant cells through the regulation of the production of EGFR ligands (Knowlden et al., 2003).

Both oestrogen- and growth factor receptor-mediated signalling require common downstream signalling intermediates and thus several ‘nodes’ of cross talk exist between them. Given its intimate involvement with both the ER and growth factor pathways, Src kinase is emerging as a key facilitator of crosstalk between them, with recent studies are beginning to show the potential therapeutic effectiveness of Src-ER complex disruption (Varricchio et al., 2007) and Src kinase activity inhibition as a means to prevent oestradiol- or growth-factor-induced breast cancer cell growth.

Given that we have recently identified that Src kinase activity is induced in response to short-term (10 days) treatment with a range of endocrine agents and that

elevated Src activity appears to represent a generic feature of an acquired resistant state, where it plays a key role in maintaining an aggressive, invasive phenotype in multiple forms of resistance (Hiscox et al., 2006c, d), the ability to inhibit Src function may have therapeutic benefits for both endocrine-responsive and endocrine-resistant breast cancer.

8.4.4 Targeting Src in Endocrine-Sensitive Breast Cancer

That Src activity may be induced by endocrine agents in the drug-responsive phase (Acconcia et al., 2006) suggests that the ability to inhibit Src activity, alongside targeting of the ER with anti-oestrogens, may enhance the anti-tumour activity seen with each agent alone. Indeed, two recent studies provide compelling evidence to support such a hypothesis, demonstrating the short-term effectiveness of such combination treatments in preventing the growth of MCF7 cells (Herynk et al., 2006; Planas-Silva and Hamilton, 2006). Importantly, studies from our own group demonstrate that targeting of Src kinase in ER-positive MCF7 and T47D cells using the novel Src/Abl inhibitor, AZD0530 (Hennequin et al., 2006) alongside the ER (using tamoxifen) significantly delays the emergence of tamoxifen resistance (Hiscox et al., 2008b). A further benefit of combining Src inhibition with tamoxifen was the ability to suppress the development of the highly migratory and invasive characteristics seen to accompany the development of resistance; this would have has significant implications in vivo, where the development of aggressive characteristics, even in the absence of cellular proliferation, may favour cell dissemination and tumour spread. These studies are now being extended to other ER-positive cell lines and with additional hormonal agents to further determine the effectiveness of co-targeting Src and the ER as a treatment strategy to circumvent the phenomena of acquired endocrine resistance.

8.4.5 Targeting of Src in Endocrine-Resistant Breast Cancer

Of particular importance is the emerging role of Src, and therefore the potential benefits of its inhibition, in endocrine-resistant breast cancer. A role for Src kinase in acquired endocrine resistance has been suggested by several groups, where it may contribute to cellular growth via regulation of Cas-mediated EGFR signalling (Riggins et al., 2006) or through interplay with focal adhesion kinase (FAK) (Planas-Silva et al., 2006). Additionally, our own studies have revealed elevated Src activity to be a unifying feature of acquired resistance to hormonal therapies (Hiscox et al., 2004b). Furthermore, we have observed that the expression of constitutively-active Src is sufficient to confer resistance to tamoxifen in MCF7 breast cancer cells and, subsequently, inhibition of Src activity re-sensitises both these and tamoxifen-resistant MCF7 cells to tamoxifen (L. Morgan, unpublished data). These studies are confirmed by others who have demonstrated that co-targeting of Src and the

ER results in sensitization of ER+ breast cancer cells to tamoxifen and a greater inhibition of cellular proliferation (Herynk et al., 2006; Planas-Silva and Hamilton, 2006; Fan et al., 2007).

In these models of endocrine resistance, Src plays a key role in mediating cellular migration and invasion through its interplay with focal adhesion kinase (FAK) (Hiscox et al., 2007; Planas-Silva et al., 2006). Subsequently, pharmacological and siRNA-mediated inhibition of Src significantly suppresses the invasive phenotype of endocrine-resistant cells. Interestingly, inhibition of Src activity in endocrine resistant cells appears to restore their morphology to that of their parental, endocrine-sensitive cells, a process which likely involves suppression of β-catenin tyrosine phosphorylation (A. Wadhawan, unpublished observations).

Further to these observations, we have recently observed that endocrine resistant breast cancer cells also show increased expression of a number of angiogenic factors (e.g. VEGF, IL-8) in addition to a reduction in the expression of angiostatic factors. Our preliminary studies have shown that human umbilical vein endothelial cell (HUVEC) cultures stimulated by conditioned medium from resistant cells show enhanced proliferation compared with conditioned medium from endocrine-sensitive counterparts and this is accompanied by an elevation in HUVEC ERK1/2 activity. Significantly, conditioned medium from endocrine-sensitive MCF7 cells engineered to express constitutively active Src also stimulate HUVEC proliferation whereas conditioned medium from antioestrogen-resistant cells treated with a Src kinase inhibitor fails to elicit angiogenic activity in HUVEC cultures. This is interesting in the light of recent reports that Src signalling via FAK has been identified as a mechanism for the production of VEGF and subsequent blood vessel growth in vivo (Mitra and Schlaepfer, 2006). Notably, pharmacological inhibition of Src can reduce FAK tyrosine phosphorylation in a number of tumour cell types, including our acquired resistant cells (Summy and Gallick, 2006), suppress VEGF and IL-8 expression (Han et al., 2006; Trevino et al., 2006) and prevent VEGF-induced proliferation of endothelial cells (Ali et al., 2006).

Of particular interest are data which show that acquired resistance to tamoxifen may involve gradual loss of the ER protein, which occurs in a Src dependent manner (Chu et al., 2007a). Consequently, the inhibition of Src kinase can impair proteolytic degradation of ER and restore expression. These observations may provide an explanation as to why ER protein levels are apparently restored in weakly ER-positive, long-term tamoxifen-treated MCF7 cells following inhibition of Src activity (A. Bensmail and I. Hutcheson, personal communication). Furthermore, whereas anti-oestrogens can suppress cell proliferation (MacGregor and Jordan, 1998), Src promotes cell cycle progression via the activation of FAK (Oktay et al., 1999), PI3K (Castoria et al., 2001) and/or ERK 1/2 (Riley et al., 2001) in addition to inducing proteolysis of p27, a major inducer of cell senescence. Consequently, inhibition of Src activity can increase cellular levels of p27 restoring the growth inhibitory effects of tamoxifen (Chu et al., 2007b). These observations may highlight mechanisms though which Src kinase mediates the development and maintenance of an endocrine-resistant phenotype.

8.5 Conclusions

Evidence is increasing which reveals that prolonged exposure to endocrine agents results in a number of changes within breast cancer cells that favour an adverse, pro-invasive phenotype in vitro. Such changes include the overexpression of a number of cell surface receptors which may sensitize these cells to factors found within the tumour microenvironment.Indeed, the concept that the development of endocrine resistance in breast cancer cells sensitizes these cells to stromal-produced factors is further supported by experimental data showing the ability of conditioned medium from primary fibroblast cells to promote the migration of endocrine-resistant breast cancer cells compared to their endocrine-sensitive counterparts (see chapter 5) although it is not currently clear which fibroblast-secreted factors and/or epithelial cell receptors are involved in this process. However, these observations have clear implications for the development and spread of tumours in an in vivo context.

Several potential targets for intervention have been identified through which these adverse cellular features may be suppressed; although there are few inhibitors available for c-Met and CD44 is not yet developed as a target, the targeting potential of these individual molecules has been demonstrated through siRNA studies. However, the ability to reveal generic targets in acquired resistance, if present, will probably yield the best therapeutic targets through which acquired resistance may be significantly delayed or prevented. Src kinase, a critical regulator of diverse signalling pathways key to tumour progression, is emerging as one such potential target. Of particular importance is the potential use of pharmacological Src inhibitors in breast cancer where they may be combined with standard chemotherapies to achieve greater response. However, although Src inhibitors have shown encouraging effects in preclinical studies in breast cancer, further investigation of the clinical effectiveness of Src inhibitors is needed in selected patient groups in order to assess whether their use would provide benefit alongside existing endocrine therapies.

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List of Abbreviations

EGFR:	Epidermal growth factor receptor
HER2:	Human epidermal growth factor receptor 2
HER3:	Human epidermal growth factor receptor 3
FAK:	Focal adhesion kinase
ER:	oestrogen receptor
IGFR:	Insulin-link growth factor receptor
GSK3β:	glycogen synthase kinase 3β
EMT:	epithelial to mesenchymal transition

NFκB:	nuclear factor of kappa light polypeptide gene enhancer in B-cells
TCF/LEF:	T-cell factor/Lymphoid enhancer-binding factor 1
HUVEC:	humkan umbillical vein endothelial cells
ERK1/2:	extracellular regulated kinase 1/2
HGF/SF:	hepatocyte growth factor/scatter factor
c-Met:	hepatocyte growth factor/scatter factor receptor
VEGF:	vascular endothelial growth factor receptor
IL8:	Interleukin 8
PI3K:	phosphoinositide 3-kinase
AKT:	protein kinase B

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Chapter 9
Identifying Modifiers of Tamoxifen Sensitivity Using High-Throughput Genetic and Chemical Screens

Elizabeth Iorns, Christopher J. Lord and Alan Ashworth

Contents

Abstract Endocrine therapies, which inhibit estrogen receptor α (ERα) signalling, are the most common and effective treatments for ERα positive breast cancer. However, the utility of these agents is limited by the frequent development of resistance. The precise mechanisms underlying endocrine therapy resistance remain incompletely understood. In our laboratory, an RNA interference (RNAi) screen was used to identify modifiers of sensitivity to the most commonly used endocrine therapy, tamoxifen. The cyclin-dependent kinase 10 (CDK10) gene was identified as an important determinant of resistance and the mechanism whereby this gene modulates sensitivity to tamoxifen was investigated further. Silencing of CDK10 gene expression was shown to activate the MAPK signalling pathway, circumventing the reliance of breast cancer cells upon estrogen signalling. Patients with ERα positive breast tumours that express low levels of CDK10 were shown to relapse early on tamoxifen and methylation of the CDK10 gene promoter was observed

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in a significant proportion of patients, suggesting a mechanism for loss of CDK10 expression in tamoxifen resistant tumours. By suppressing gene expression RNAi, to a certain extent, models the pharmacological inhibition of a target protein. We performed parallel small molecule screens alongside the RNAi screen to identify compounds that sensitise to tamoxifen. Both the RNAi and small molecule screens identified the PDK1 pathway as a potential target for sensitisation to inhibit the development of endocrine therapy resistance.

Keywords Cdk10 · Endocrine therapy resistance · pdk1 · rna interference screen · Small molecule screen

9.1 Endocrine Therapy

Approximately 70% of breast tumours express estrogen receptor α (ERα) and, of these, most are dependent on estrogen signalling for their growth (EBCTG 1998). Therefore, patients with ERα positive tumours can be treated with endocrine therapies that target this dependence. The most commonly used endocrine therapy is tamoxifen, which has shown significant patient benefit in the treatment of ERα positive breast cancer (EBCTG 1998). In patients with ERα positive disease, tamoxifen treatment results in a 51% reduction in recurrence and a 28% reduction in death (EBCTG 1998). However, despite its widespread use, the effectiveness of tamoxifen is limited by the development of resistance; all patients with metastatic disease and 40% of early stage breast cancer patients treated with adjuvant tamoxifen eventually relapse with tamoxifen resistant disease (Jordan 1995; Ring and Dowsett 2004).

9.1.1 Endocrine Therapy Resistance

Despite intense study, the molecular alterations that underlie endocrine therapy resistance are not fully understood and this has limited the development of effective approaches for preventing and overcoming resistance. Two major mechanisms have been proposed by which resistance may arise. First, continued ERα signalling in the presence of an ERα antagonist or the absence of estrogen may occur, an effect termed ligand independent ERα activation (Shou et al. 2004). Second, the reliance of tumours upon ERα signalling may be circumvented by the activation of non ERα growth promoting pathways (Tang et al. 1996; El-Ashry et al. 1997; Oh et al. 2001). Many molecular alterations have been implicated in these fundamental mechanisms, however, none entirely explain resistance in the majority of cases and alternative approaches are therefore required to identify the key molecular alterations driving resistance.

9.2 Experimental Approaches

Several approaches have been utilised experimentally to determine the causes of endocrine therapy resistance. The vast majority of studies have used a candidate-based approach, where the role of specifically chosen genes is studied. These approaches have been successful in identifying a series of proteins that most likely modulate resistance, including several components of growth factor signalling cascades (Benz et al. 1992; Liu et al. 1995; Guvakova and Surmacz 1997; Kurokawa et al. 2000; McClelland et al. 2001; Stephen et al. 2001; Knowlden et al. 2003; Nicholson et al. 2004). While the study of individual proteins has been informative, a complementary approach is to perform large scale, relatively unbiased, studies to rapidly examine the role of many genes in parallel. The comparatively recent development of RNA interference (RNAi) reagent libraries now provides an effective tool for this type of analysis (Iorns et al. 2007).

9.2.1 RNA Interference (RNAi)

Rnai is an endogenous physiological mechanism that regulates gene expression at the post-transcriptional level. The phenomenon of RNAi was originally described in plants in the early 1990s (Napoli et al. 1990) and is thought to have evolved to protect the host against viruses and rogue genetic elements such as transposons that utilise double stranded RNA (dsRNA) for self-propagation (Cerutti and Casas-Mollano 2006; Stram and Kuzntzova 2006). Experimentally, long dsRNA can be used to silence target gene expression in various organisms including the nematode *Caenorhabditis elegans*, the fruit fly *Drosophila melanogaster* and several plant species. However, in mammalian cells, the introduction of long dsRNAs into cells induces an interferon response, which activates protein kinase R (PKR). PKR, in turn, phosphorylates and inactivates eukaryotic initiation factor 2, inhibiting mRNA translation and resulting in the global shutdown of Protein Synthesis (Manche et al. 1992; Provost et al. 2002). To circumvent this problem, experimental RNAi can be effected in mammalian cells by the use of small interfering RNA (siRNA) duplexes that silence gene expression without inducing the inhibitory interferon response (Brummelkamp et al. 2002; Paddison et al. 2002; Meister et al. 2004). siRNAs can either be directly introduced into cells by transfection or can be generated within the cell by transfecting plasmids that express short-hairpin RNA (shRNA) precursors of siRNAs (Fig. 9.1). shRNAs are processed by the DICER enzyme into siRNAs, which are incorporated into the RNA-induced silencing complex (RISC), a multi-protein endoribonuclease. A helicase within RISC unwinds duplex siRNA allowing its antisense strand to bind mRNA with a high degree of sequence complementarity. An RNase within RISC degrades the target mRNA by cleavage, which results in the specific silencing of gene expression (Meister et al. 2004; Meister and Tuschl 2004). This characteristic of RNAi makes it a valuable laboratory research tool to selectively silence specific proteins in mammalian cells.

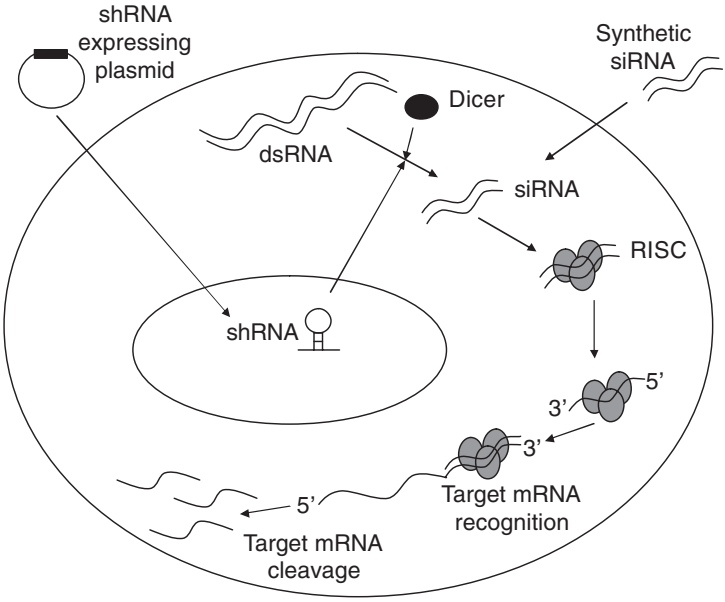

Fig. 9.1 The mechanism of experimental RNA interference (RNAi). Double stranded RNA (dsRNA) or short hairpin RNAs (shRNAs) encoded by plasmids are processed by the RNase III-like enzyme, DICER, into short interfering RNA (siRNA) duplexes 21–28 nucleotides in length with dinucleotide 3′ overhangs (Tuschl et al. 1999). Alternatively synthetic siRNAs can be synthesized chemically and introduced directly into the cell using transfection or electroporation. siRNAs are incorporated into the RNA induced silencing complex (RISC), a multiprotein endoribonuclease. A helicase within RISC unwinds duplex siRNA allowing its antisense strand to bind messenger RNA (mRNA) with a high degree of sequence complementarity. An RNase within RISC degrades the target mRNA by cleavage resulting in silenced gene expression and reduced protein production (Meister and Tuschl 2004). Modified from Iorns et al. 2007

Experiments using individual siRNAs and shRNAs have been widely used to identify the functional role of specific proteins in numerous phenotypes, demonstrating the power of RNAi as a research tool (Pan et al. 2005; Zhang et al. 2006). The development of RNAi libraries, composed of reagents that allow the targeting of a wide range of transcripts, has made it possible to conduct high throughput screens that systematically interrogate phenotypes associated with the silencing of gene expression on a large scale (Iorns et al. 2007).

9.3 RNAi Screen

We performed high throughput RNAi and compound screens to identify modifiers of tamoxifen sensitivity (Iorns et al. 2008). For the RNAi screen we used a library of siRNA oligonucleotides to identify kinases, that when silenced, modulate sensitivity to tamoxifen. Focusing on a subset of the genome, in this case protein kinases, is obviously more time and cost effective than screening the entire genome. Kinases

were analysed primarily because of the number of signalling cascades involving kinases previously implicated in the development of tamoxifen resistance (Gee et al. 2001; Pérez-Tenorio et al. 2002). Furthermore, protein kinases represent druggable targets as they contain structural features that favour interactions with drug-like chemical compounds (Hopkins and Groom 2002). As such, an RNAi screen of kinases could potentially identify pharmacologically tractable targets for tamoxifen sensitisation. siRNA screens have proven highly effective in the unbiased identification of novel genes involved in biological processes (Aza-Blanc et al. 2003; Mukherji et al. 2006) and have recently been used to identify key determinants of resistance to chemotherapeutic drugs including paclitaxel (Swanton et al. 2007; Whitehurst et al. 2007). Therefore, this approach provided a method to characterise potentially novel mechanisms of tamoxifen resistance and to identify potential targets for tamoxifen sensitisation. We identified several kinases that modulate tamoxifen sensitivity, including CDK10, a novel determinant of tamoxifen resistance, and PDK1, a new potential target for tamoxifen sensitisation.

9.4 CDK10

Cyclin-dependent kinase 10 (CDK10) was identified in the RNAi screen as a potential determinant of tamoxifen resistance (Iorns et al. 2008). Further investigation demonstrated that silencing of CDK10 causes resistance to multiple endocrine therapies including tamoxifen, fulvestrant and estrogen deprivation. In addition, we also identified a mechanism by which CDK10 may modulate sensitivity to these agents. CDK10 normally binds and represses the ETS2 transcription factor (Kasten and Giordano 2001). We identified a novel ETS2 binding site in the *c-RAF* promoter and used chromatin IP (ChIP) to demonstrate that both CDK10 and ETS2 bind to this site. In the absence of CDK10, *c-RAF* transcription was significantly upregulated, most likely due to the relief of ETS2 repression. This increase in c-RAF expression leads to activation of downstream components of the MAPK pathway, including MEK1,2 and ERK MAPK. Activation of these latter kinases increases the expression of cyclin D1 (Lavoie et al. 1996). This, in turn allows cells to progress through the cell cycle, circumventing arrest caused by therapies that target ERα, resulting in drug resistance (Fig. 9.2). Therefore, the fundamental mechanism underlying resistance in CDK10 silenced cells is most likely circumvention of the reliance of tumour cells upon ERα signalling by the activation of non ERα growth promoting pathways, in this case the MAPK pathway (Tang et al. 1996; El-Ashry et al. 1997; Oh et al. 2001).

9.4.1 Clinical Significance of CDK10

Having identified *CDK10* as a modifier of tamoxifen sensitivity, we examined the expression of *CDK10* in breast tumours of patients subsequently treated with tamoxifen. Patients with the lowest levels of tumour-associated *CDK10* transcript tended

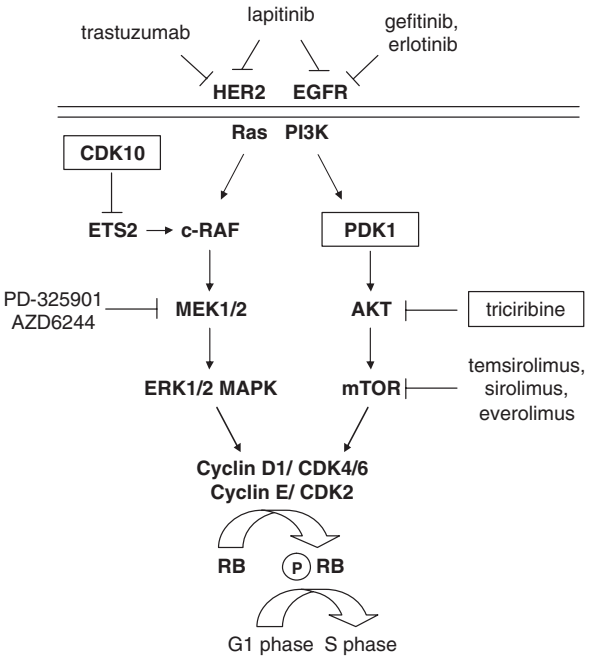

Fig. 9.2 Endocrine resistance mediated by signal transduction cascades. Tamoxifen induces G1 cell cycle arrest. Activation of signal transduction molecules overcomes tamoxifen induced G1 arrest by increasing expression of cyclin D1. Cyclin D1 drives G1 to S phase cell cycle progression, resulting in resistance to endocrine therapy. CDK10 loss drives ERK MAPK pathway activity through transcriptional upregulation by ETS2 of c-RAF. PDK1 drives AKT pathway activity. Signal transduction inhibitors targeting these pathways May enhance endocrine response. Molecules identified from RNAi and compound screens are boxed. Modified from Swanton and Downward, 2008

to relapse with tamoxifen-resistant disease much earlier than those with the higher levels of *CDK10*, an observation that was consistent with the functional effects of silencing *CDK10* expression in in vitro models (Iorns et al. 2008). In addition, methylation of the *CDK10* gene promoter in breast tumours correlated with low *CDK10* expression. Methylation of promoter sequences is a well-known mechanism of controlling gene expression and therefore this suggests a means by which *CDK10* expression might be suppressed in tumours, leading to tamoxifen resistance (Iorns et al. 2008). Therefore, *CDK10* represents a potential biomarker for sensitivity to endocrine therapies and further clinical validation of these observations is now underway.

A significant issue in translating these observations into clinical practice will be the routine quantification of CDK10 expression in tumours. The expression of biomarkers is generally assessed by immunohistochemistry (IHC) or enzyme-linked immunosorbent assay (ELISA), both of which require a specific antibody. How-

ever, no antibody is currently available that can detect CDK10 in this way and new reagents will need to be developed to address this issue.

As activation of the MAPK signalling pathway most likely explains the mechanism of resistance in CDK10 silenced cells in vitro, it is possible that targeting the MAPK signalling pathway with inhibitors may reinstate endocrine therapy sensitivity in patients (Fig. 9.2). Furthermore, since drugs are now available that can efficiently reverse epigenetic silencing, for example the DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine (decitabine), these may also prove valuable in regenerating CDK10 expression and endocrine agent response. Future work should determine whether these approaches are effective in in vitro and in vivo breast cancer models, paving the way for similar strategies in patients.

9.5 Tamoxifen Sensitisers

A parallel small molecule screen was performed alongside the RNAi screen to identify compounds that modulate tamoxifen sensitivity. In addition to identifying mechanisms of resistance, potential targets for sensitisation to tamoxifen were identified from both the RNAi and small molecule screens. The identification of targets for sensitisation to tamoxifen is key, as studies in experimental models of hormone sensitive breast cancer have shown tamoxifen sensitisers can delay the emergence of resistance when used in combination with tamoxifen (Fig. 9.2; Gee et al. 2003; Boulay et al. 2005; Treeck et al. 2006; Martin et al. 2007). For example, the EGFR inhibitor gefitinib, when combined with 4OH tamoxifen or fulvestrant enhanced tumour growth inhibition and prevented the development of resistance in an in vitro model of breast cancer (Gee et al. 2003). The proposed mechanism of enhanced endocrine therapy efficacy in this model was via the inhibition of increased EGFR activation. EGFR activation is known to occur during treatment with endocrine therapies, and is thought to promote resistance. Similarly, mTOR pathway activation occurs during the development of endocrine therapy resistance and a combination of the mTOR inhibitor RAD001 with endocrine therapy resulted in significantly greater inhibition of tumour growth in breast cancer models (Boulay et al. 2005; Treeck et al. 2006). Importantly, the results of a recent phase I clinical trial combining RAD001 with letrozole suggested enhanced anti-tumour activity with no adverse pharmacokinetic interactions, indicating this approach may have clinical utility (Awada et al. 2008). Therefore, the identification of novel targets that sensitise to tamoxifen, may, in the future, be exploited in the form of combination therapies that limit the development of resistance.

On this basis, we screened a library of existing drugs to identify agents that sensitise to tamoxifen. The advantage of using existing drugs for such a screen is that the pharmacokinetic and safety profiles of many of the agents in the screen are known and many of the drugs are also approved for clinical use (Chong and Sullivan 2007). This means that potentially, sensitisation effects identified in a screen could be rapidly evaluated in phase II clinical trials, eliminating much of the toxicological

and pharmacokinetic assessment that is required for novel compounds (DiMasi et al. 2003). This not only has the potential to improve the speed of clinical application of the identified drug but could also significantly reduce the cost of bringing a drug combination into clinical use (Chong and Sullivan 2007).

9.5.1 Triciribine

Triciribine, a relatively non-toxic inhibitor, was identified from the compound screen as a potent sensitiser to tamoxifen. Triciribine is a tricyclic nucleoside that was first synthesised in 1971 (Schram and Townsend 1971) and identified as a potential anticancer drug using in vitro models (Schweinsberg et al. 1981). Early clinical trials showed that low concentrations of triciribine resulted in neither response nor toxicity in advanced breast, colon, and lung cancer patients (Feun et al. 1993; Mittelman et al. 1983; Hoffman et al. 1996). Later work identified AKT activation as a triciribine target and triciribine was shown to be selective for human tumour cell lines with constitutive AKT activiation (Yang et al. 2004). The early reports of lack of efficacy in clinical trials may be explained by a failure to stratify patients according to tumour-associated AKT activation. A phase I clinical trial to assess the efficacy of tricirbine for tumours with activated AKT has now begun, using low, non-toxic concentrations of triciribine (VioQuest Pharmaceuticals NCT00363454; Cheng et al. 2005).

The most likely mechanism of tamoxifen sensitisation by triciribine is inhibition of AKT activation. Consistent with this hypothesis, previous studies have shown that inhibition of the AKT activator, phosphoinositide-3 kinase (PI3K), can sensitise to endocrine therapies in in vitro and in vivo models and that activation of AKT causes resistance to tamoxifen in breast cancer models and tumours (Campbell et al. 2001; Clark et al. 2002; Pérez-Tenorio et al. 2002; Sabnis et al. 2007).

Interestingly, both the compound and RNAi screens identified activators of AKT as important targets for tamoxifen sensitisation. In addition to triciribine, the specific inhibitor of AKT activation identified from the compound screen as a potent sensitizer to tamoxifen and other endocrine therapies, analysis of the sensitising hits from the RNAi screen identified 3-phosphoinositide-dependent protein kinase 1 (PDK1), the kinase that phosphorylates and activates AKT, as a potential target for tamoxifen sensitisation. Our data confirms previous studies showing the importance of the PI3K signalling pathway in modulation of tamoxifen sensitivity, but importantly identifies PDK1 as a specific component that may be a novel putative target for tamoxifen sensitisation, as well as identifying triciribine, a drug with clinical potential.

9.5.2 PDK1

The most potent tamoxifen sensitising component of the PI3K pathway identified from the RNAi screen was PDK1 (aka PDPK1). PDK1 is activated by PI3K and reg-

ulates the AGC (cAMP-dependent or cGMP-dependent protein kinases and protein kinase C) family of protein kinases (Mora et al. 2004), which includes AKT. Interestingly, PDK1 is highly expressed in many human cancer cell lines (Fry 2001) and breast tumours (Lin et al. 2005), suggesting a role in breast cancer tumorigenesis. However, few studies have evaluated PDK1 as a potential target for cancer therapy. We discovered that silencing of PDK1 increases sensitivity to multiple to endocrine therapies including tamoxifen, fulvestrant and estrogen deprivation and determined a potential mechanism by which PDK1 modulates sensitivity to these agents. Inhibition of ERα signalling induces arrest at the G1 checkpoint by decreasing the expression of proteins that promote cell cycle progression (Wilcken et al. 1997) and increasing the expression of proteins that inhibit it, such as cyclin dependent kinase inhibitor proteins. One of the cyclin dependent kinase inhibitors upregulated by tamoxifen treatment is $p21^{CIP1}$ (Pestell et al. 1999; Cariou et al. 2000). AKT, which is activated by PDK1 phosphorylation (Alessi et al. 1997), phosphorylates $p21^{CIP1}$ and targets it for cytoplasmic localisation and degradation (Zhou et al. 2001). In the absence of PDK1, AKT is not active, and subsequently $p21^{CIP1}$ levels are not degraded, enhancing tamoxifen-induced G1 arrest and resulting in sensitisation.

The observation that inhibition of PDK1 sensitises to a range of estrogen signalling inhibitors, suggests that the combination of PDK1 inhibitors with tamoxifen, fulvestrant or aromatase inhibitors merits investigation in the clinic. Clinical trials combining signal transduction inhibitors with tamoxifen and aromatase inhibitors are currently underway for many targets including EGFR, mTOR and HER2 (Fig. 9.2; Johnston 2005). Although not currently in clinical use, PDK1 specific inhibitors have recently been developed and may be suitable for this purpose in the future (Zhu et al. 2004; Feldman et al. 2005).

9.6 Utility of Parallel RNAi and Compound Screens

Both the RNAi screen and compound screens identified AKT activators as important targets for tamoxifen sensitisation. This provides an example of how parallel RNAi and chemical screens performed in mammalian cells may complement each other, identifying compounds and gene-specific siRNA reagents that inhibit the same targets to cause similar cellular phenotypes (Iorns et al. 2007). Given the complexity of biochemical pathways and the number of protein-protein interactions now described for each of the proteins within a cell, deconvoluting the mechanism by which one RNAi screen hit controls a phenotype can be difficult. Using the parallel chemical screen, it was possible to simultaneously validate data from the RNAi screen and suggest potential mechanisms by which RNAi hits determine a given cellular phenotype. Conversely, these results suggest RNAi screens could be used to partially deconvolute hits from parallel small molecule screens (Iorns et al. 2007). While in vitro small molecule screens are generally used to identify compounds that inhibit a validated protein target, the inhibitors identified may perform poorly in living cells. One solution is to perform compound screens in cells, but this approach is also

limited as it requires the cellular targets of inhibitors to be identified, which can be challenging. However, small molecule screens in cells could become more informative if combined with RNAi screens. By performing parallel RNAi and compound screens, small molecules and gene-specific siRNA reagents can be identified that cause similar cellular phenotypes, simplifying target identification. A previous study illustrated this principle using a small molecule screen to identify inhibitors of cytokinesis in *Drosophila melanogaster* (Eggert et al. 2004). This screen demonstrated that small molecules could affect this phenotype but the actual target(s) of the hit compounds remained unknown. In parallel, an RNAi screen for the same phenotype suggested that inhibitors of Aurora B could affect cytokinesis. By cross-comparing these results, one compound from the small molecule screen was identified as an Aurora B inhibitor (Eggert et al. 2004). Similarly, in our laboratory, parallel RNAi and small molecule screens identified inhibitors of AKT activation as potential tamoxifen sensitisers. This proof-of-principle suggests that a combination RNAi/chemical approach in human cell lines could streamline the development of small molecules into drugs by improving compound target deconvolution (Iorns et al. 2007).

9.7 Conclusion

The utilisation of high throughput RNAi and compound screens has allowed us to identify novel determinants of tamoxifen resistance, including CDK10, a clinically significant mediator of resistance to multiple endocrine therapies, and PDK1, a new potential target for tamoxifen sensitisation. Further studies will establish whether CDK10 is a useful biomarker for sensitivity to endocrine therapies and determine whether PDK1 can be targeted therapeutically to inhibit the development of tamoxifen resistance. Finally, our work demonstrates the strength of RNAi screens as a tool for identifying key mechanisms underlying the behaviour of cancer cells.

Abbreviations

RNAi:	RNA interference
CDK10:	cyclin-dependent kinase 10
MAPK:	Mitogen activated protein kinase
ERα:	estrogen receptor α
dsRNA:	double-stranded RNA
PKR:	protein kinase R
shRNA:	short-hairpin RNA
RISC:	RNA-induced silencing complex
ChIP:	chromatin IP
ELISA:	enzyme-linked immunosorbent assay
mTOR:	mammalian target of rapamycin
AKT:	Protein kinase B

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Chapter 10
Endocrine Resistance in Breast Cancer – Where Are We Now With Intelligent Combination Therapies?

Stephen R.D. Johnston

Contents

Abstract Despite the improvements in breast cancer brought about by endocrine therapy, their success clinically is limited by a significant number of patients which continue to acquire resistance and die of the disease. An increased understanding of the various biological mechanisms responsible for the development of endocrine resistance has identified new therapeutic targets, providing the rationale for combining signal transduction inhibitors with endocrine therapies to delay the emergence of acquired resistance and enhance the efficacy of current endocrine treatments. Although therapeutic targeting of mTOR, Ras activation and erbB family members alongside the ER have shown promise in pre-clinical models, clinical results have been disappointing, partly due to poor patient selection. The application of rigorous trial design and tumour selection criteria to future clinical trials may allow more accurate evaluation of intelligent combination therapies in breast cancer patients.

Keywords Combination therapy · mTOR inhibitors · Endocrine therapy · Signal transduction inhibitors

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10.1 Introduction

Currently available endocrine strategies include targeting the ER itself with the selective oestrogen receptor modulator tamoxifen or the ER downregulator fulvestrant, or suppressing the amount of available ligand (estrogen) for the receptor either with gonadal suppression in pre-menopausal women (ovariectomy or luteinizing hormone-releasing hormone agonists), or with aromatase inhibitors in post-menopausal women. Given their proven efficacy and generally favourable side effect profile, endocrine therapies are widely used in the treatment of both early-stage and recurrent/metastatic breast cancer (Early Breast Cancer Trialists' Collaborative Group 1998). Unfortunately, despite documented levels of ER in recurrent disease, up to 50% of patients with metastatic disease do not respond to first line endocrine treatment (de novo resistance), while the remainder will eventually relapse despite an initial response (*acquired* resistance) (Ring and Dowsett 2004). In the last two decades there have been major efforts to understand the various biological mechanisms responsible for the development of endocrine resistance, with the ultimate aim of identifying new therapeutic strategies to enhance the efficacy of current treatment strategies for hormone receptor positive breast cancer (Ali and Coombes 2002; Osborne and Schiff 2005).

Various theories, each supported by pre-clinical and in some instances clinical data, have been suggested to explain endocrine resistance. These include mechanisms that have a sustained dependence on ER-mediated signalling, while others implicate growth factor mediated mitogenic signalling which may or may not cross-talk with existing ER-signalling pathways. The strong likelihood is that even in ER+ve disease there will be no single unifying mechanism for endocrine resistance. Therefore, identifying which resistance mechanism is operational in an individual patient could become clinically relevant in tailoring the most appropriate subsequent therapy, e.g. targeted treatment against various signalling pathways, further endocrine manipulation, or a combination of both. However, developing intelligent combinations for the clinic has proven somewhat challenging, and this article reviews the progress that has been made to date. First, it is important to briefly summarise the signalling pathways that are functional in endocrine resistant breast cancer.

10.2 ER Signalling in Hormone Resistance

Most in vitro and clinical observations suggest that even following the development of endocrine resistance, ER signalling continues to play an important role in the proliferation of breast cancer (Encarnacion et al. 1993; Johnston et al. 1995). In the clinic, biopsies of tumours from breast cancer patients who have relapsed on an antiestrogen show a functional ER that is still able to bind to DNA, indicating that ER-mediated signalling remains functional (Johnston et al. 1997). While ER expression is an obligate requirement for sensitivity to endocrine therapy, loss of ER either

due to the clonal selection of ER negative cells or transcriptional suppression of ER gene expression could account for acquired endocrine resistance associated with progressive disease (Kuukasjarvi et al. 1996). Laboratory studies have suggested that transcriptional repression of the PgR gene by signaling through the insulin-like growth factor (IGFR) and epidermal growth factor receptor families (EGFR/HER2) may be the cause of PgR down-regulation in some tumors (Petz et al. 2004).

ER silencing as a result of promoter hypermethylation has been documented in a proportion of breast cancers, and demethylating agents or histone deacetylase (HDAC) inhibitors can reactivate expression of a functional ER in cell lines with ER silencing due to promoter methylation (Ferguson et al. 1995). These observations are quite provocative and have obvious clinical implications for a proportion of patients with ER-ve tumours who might potentially benefit from endocrine therapy if ER expression could be reactivated. There is also evidence that enhanced peptide growth factor signalling due to over-expression of HER2 and subsequent MAPK activation can directly suppress ER expression (Creighton et al. 2006), which in turn may eventually lead to complete loss of ER. ER positive cell lines stably transfected with full length HER2 demonstrate downregulation in ER, while quantitative measurements of ER levels in tumour samples show consistently lower levels of the receptor among patients with HER2 amplified breast cancer (Konecny et al. 2003). Furthermore, interruption of hyperactive mitogen activated protein kinase (MAPK) signalling or epidermal growth factor receptor (EGFR) has been shown to re-induce ER expression both in cell lines and xenograft models. In fact in a small study of 10 ER − ve/HER2 + ve patients treated with trastuzumab, three patients acquired ER expression in sequential biopsies during treatment (Munzone et al. 2005). Additionally, studies with the dual EGFR/HER2 tyrosine kinase inhibitor lapatinib have shown that long-term treatment was associated with an adaptive increase in ER signalling (Xia et al. 2006). This dynamic interaction between ER and growth factor signalling certainly supports using ER and growth factor targeted therapies in combination, or in fact in sequence, as one may sensitize to the other and thus enhance/retain endocrine responsiveness longer than would otherwise occur.

The genomic activity of ER may also be altered in association with endocrine resistance. ER transcription is tightly regulated by the balance of co-activators (NCOAs) and co-repressors (NCORs) within individual cells. The co-activator NCOA3, also known as AIB1 (Amplified in Breast Cancer-1) is overexpressed in 50% of breast carcinomas and amplified in 5% of tumours (Bautista et al. 1998). In HER2 amplified breast cancer, AIB1 has been associated with a poorer outcome with tamoxifen – this might be explained by the fact that HER2 activates AIB1 and enhances the agonist effects of tamoxifen (Osborne et al. 2003). Similarly, decreased levels of NCORs have been shown to enhance tamoxifen agonism by shifting the balance towards ER transcriptional activity (Lavinsky et al. 1998). These data suggest that mitogenic signalling via other pathways (ie. HER2) can alter the ratio of NCOAs/NCORs, and result in an altered response of ER to endogenous E2 or to exogenous tamoxifen, in particular enhancing an agonist response.

In addition to directly binding with DNA and increasing “classical” genomic transcription of ER dependent genes, ligand-bound ER may also complex with

other transcriptional factors, such as fos/jun via AP-1 "non-classical" genomic activity. Stress and/or cytokine signaling pathways can contribute to AP-1 signalling, and thus have been associated with resistance to tamoxifen (Kushner et al. 2000). Laboratory and clinical studies suggest that elevated levels of phosphorylated jun N-terminal kinase (JNK) are associated with tamoxifen resistance, and preliminary data have also implicated activated p38 MAPK (Gutierrez et al. 2005). Although the mechanisms by which signaling through these pathways might contribute to tamoxifen resistance in clinical breast cancer are not well defined, preliminary evidence in human tumours and MCF7 xenografts has suggested an association of p38 MAPK with hormonal resistance. In tissue microarrays (TMAs) from 39 patients with paired biopsies before and after acquired resistance to tamoxifen, all ER+ tumours that over-expressed HER2 originally or at resistance expressed high levels of phosphorylated p38 MAPK. In 3 patients with ER + ve tumours that were HER2−ve initially, at the time of relapse on tamoxifen they had converted to HER2 + ve, including conversion to FISH + ve in two cases. In the tamoxifen resistant xenograft tumors high ER expression was preserved, and, like the clinical samples, there was a striking increase in phosphorylated p38 MAPK. These data support the concept that adaptive changes in ER genomic signaling occur during development of hormonal resistance to tamoxifen, and as discussed further below implicate various cross-talk between mitogenic signaling and ER pathways in the underlying process. This has obvious implications for which intelligent combination therapies to choose to try and prevent development of endocrine resistance.

While loss of ER may occur in some tumours during prolonged endocrine therapy, it is clear that in many instances signalling through ER is retained. In particular the biological mechanisms contributing to resistance following long term estrogen deprivation (LTED) using aromatase inhibitors or gonadal suppression have been associated with retention and enhanced ER signalling. Adaptation to LTED may lead to upregulation in ER, and in vitro models have shown that part of the adaptive process involves an increase in ER expression and E2 hypersensitivity to very low levels of residual estrogen (Masamura et al. 1995). Data from several groups support this hypersensitivity concept as a means of escape from estrogen deprivation. While wild-type MCF7 cells respond maximally to doses of estradiol of c.10^{-11} to 10^{-10} M, cells exposed to LTED adapt and instead respond maximally at c.10^{-13} M (Martin et al. 2003; Santen et al. 2001). In part this is caused by an adaptive increase in ER expression and function, but there is additional evidence for increased "cross-talk" between various growth factor receptor signaling pathways and ER at the time of relapse, with ER becoming activated and super-sensitised by a number of different intracellular kinases, including mitogen-activated protein kinases (MAPKs) and the insulin-like growth factor (IGF)/AKT pathway. Increased expression of HER2/HER3, MAPK, and IGFR signalling in cells that become resistance to LTED may activate residual and enhanced levels of ER in a manner similar to that observed in acquired tamoxifen resistant cells. Thus, it would appear that the ER remains an integral part of signalling, even following failure of aromatase inhibitors.

10.3 Growth Factor Signalling and Hormonal Resistance

Membrane peptide growth factor receptors such as the epidermal growth factor receptor (EGFR), the human epidermal receptors-2 (HER2) or the insulin growth factor 1 receptor (IGF1R) have been implicated in endocrine resistance. Over-expression of HER2 due to gene amplification occurs in approximately 15 to 20% of all human breast cancers (Slamon et al. 1987), and has been associated with poor prognosis and de novo resistance to tamoxifen in the neoadjuvant setting. Similarly, EGFR is over-expressed in a number of breast cancers and has also been associated with poor response to tamoxifen. As discussed above, in-vitro models of acquired resistance to both tamoxifen and oestrogen deprivation (ED) have shown that the development of resistance over time is associated with an adaptative up-regulation in growth factor signalling pathways, whereby cells enhance their dependence on EGFR or HER2 signalling pathways.

Activation of these membrane receptors stimulates two major intracellular kinase signalling cascades—the ras/mitogenic-activated protein kinase (MAPK) pathway and the phosphatidylinositol 3-kinase (PI3K)/Akt pathway (Fig. 10.1). These pathways activate downstream effectors leading to a cascade of signals involved in malignant growth and survival, and can be involved in endocrine resistance by a number of mechanisms including down-regulation and loss of ER expression (described above), a total switch to ER independent growth using these pathways, or bi-directional cross-talk between ER and mitogenic signalling. Growth factor mediated activation of MAPK or Akt can potentiate E2 mediated ER classical transcriptional activity by directly phosphorylating AF-1. Importantly both MAPK and Akt have been shown to phosphorylate ER within AF-1, at serine 118 and serine 167 respectively, in the absence of E2, thereby contributing to ligand-independent ER transactivation (Chen et al. 2002; Kato et al. 1995; Campbell et al. 2001).

Conversely, in addition to its effects on transcription, oestrogen bound ER has been also been shown to result in non-genomic effects via membrane interaction with growth factor receptors (Fan et al. 2007; Kahlert et al. 2000). This may result in rapid activation of EGFR, IGF1R, HER2 or the cleavage of membrane bound growth factor receptor ligands such as EGF or TGFalpha. This bi-directional interaction between ER and growth factor pathways creates a self-reinforcing synergistic loop that potentiates pro-survival signals and may allow breast cancer to escape normal endocrine responsiveness (Fig. 10.1).

Thus, it would appear that both ER and various peptide growth factors are an integral part of signaling even following failure of estrogen deprivation therapies, and that a possible successful approach to overcoming hormonal resistance could involve the use of the ER downregulator fulvestrant or various signal transduction inhibitors (STIs) to remove ER and/or activation of ‘cross-talk’ ER signalling, respectively. Furthermore as discussed below, evidence is now emerging that such drugs may be more effective when given in combination with existing endocrine therapies in an attempt to delay or prevent resistance occurring.

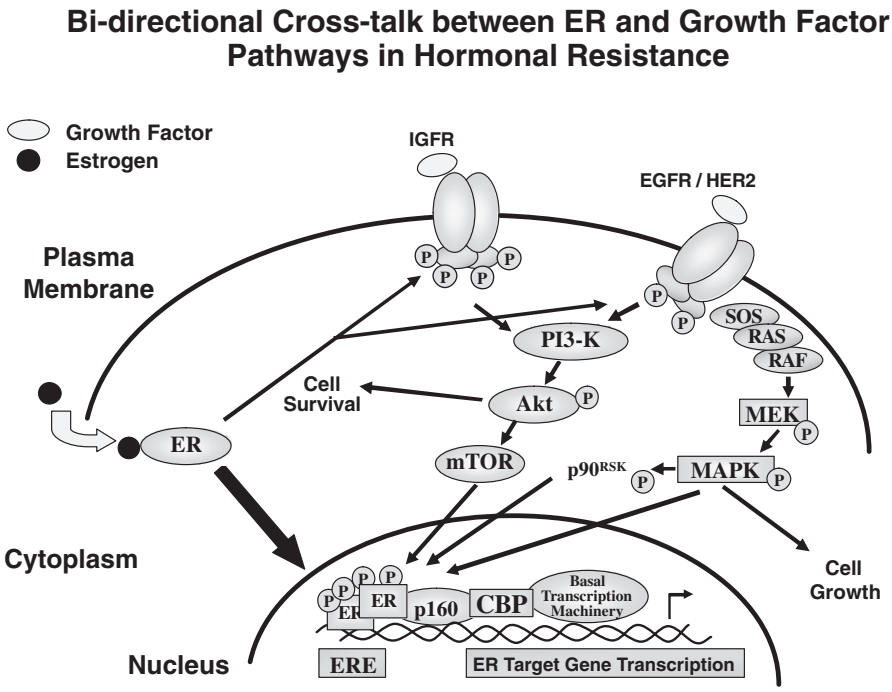

Fig. 10.1 Bi-directional cross-talk between ER and growth factor pathways in hormonal resistance

10.4 Intelligent Combinations to Overcome Hormonal Resistance – Progress to Date

The clinical implications of a retained, albeit an altered or hyperactive ER signalling pathway with or without 'cross-talk' activation of peptide growth factor pathways, are that further endocrine therapies can be used after development of hormonal resistance in combination with novel signalling agents. This treatment strategy has been tested in the clinic with several approaches including utilising of an ER down-regulator (fulvestrant), anti-growth factor receptor antibodies (trastuzumab) and small molecule tyrosine kinase inhibitors (gefitinib, erlotinib, lapatinib), farnesyltransferase inhibitors (tipifarnib), and mTOR antagonists (everolimus, temsirolimus). Progress to date with these approaches are summarised below:

10.4.1 Fulvestrant – Targeting Activated ER in Hormonal Resistance

Fulvestrant is a novel type of estrogen receptor (ER) antagonist that unlike tamoxifen, has no known agonist effects (Wakeling et al. 1991). Fulvestrant binds to the ER, but due to its steroidal structure and long side-chain, induces a different conformational shape with the receptor to that achieved by the non-steroidal

anti-estrogen tamoxifen. Because of this, fulvestrant prevents ER dimerisation and leads to the rapid degradation of the fulvestrant–ER complex, producing the loss of cellular ER (Dauvois et al.). Thus fulvestrant, unlike tamoxifen, inhibits ER binding with DNA and produces abrogation of estrogen-sensitive gene transcription. It has been shown that due to its unique mechanism of action, fulvestrant delays the emergence of acquired resistance compared with tamoxifen in an MCF-7 hormone-sensitive xenograft model (Osborne et al. 1995). The lack of agonist effects means that fulvestrant did not support the growth of tumors that became resistant to, and subsequently stimulated by, tamoxifen.

Clinical data from three phase II studies in a total of 293 postmenopausal women with advanced breast cancer suggest some modest efficacy for fulvestrant in a second/third-line setting (Perey et al. 2007; Steger et al. 2005; Ingle et al. 2006). Many of these patients had progressed on prior treatment with several endocrine agents, and these results imply that disease progression after non-steroidal aromatase inhibitors may not preclude subsequent treatment with fulvestrant. This was confirmed in the large randomised Phase III 'Evaluation of Faslodex vs Exemestane Clinical Trial' (EFECT) study that demonstrated similar efficacy for fulvestrant vs exemestane in patients who have progressed on treatment with non-steroidal AIs (Gradishar et al. 2006).

The efficacy of fulvestrant, especially in the setting of endocrine resistance where activated ER signaling may be dominant, may critically depend on the background estrogen environment in which the cells exist. Recent experiments with tamoxifen-stimulated breast cancer xenografts demonstrated paradoxical effects on tumour growth dependent on whether fulvestrant was administered in the presence or absence of estrogen (Osipo et al. 2003). While wild-type MCF-7 xenografts were growth stimulated by estrogen and inhibited both by tamoxifen and fulvestrant, in contrast long-term tamoxifen-treated (MCF-7TAMLT) tumours which became resistant and growth stimulated by tamoxifen were inhibited by estradiol. The addition of fulvestrant to estradiol-treated tumours reversed these effects and actually stimulated growth of MCF-7TAMLT tumours. However, when fulvestrant was given to these tumours on its own in a low estradiol environment, tumours did not grow. Similar results have been reported in LTED-R cells in-vitro where maximal growth inhibition of cells was observed with a dose of 10^{-8}M fulvestrant, yet the titration back of increasing amounts of estradiol resulted in re-growth of cells which fulvestrant was no-longer able to effectively antagonize (Martin et al. 2005). On the basis of these findings, an ongoing phase III trial (SoFEA) will compare progression-free survival in patients who have progressed on a non-steroidal AI, and who are subsequently treated with either fulvestrant plus continued anastrozole, or with fulvestrant alone.

10.4.2 Endocrine Therapy in Combination with Anti-Growth Factor Receptor Therapies

Growth factor signalling has been extensively implicated in endocrine resistance, and in some cases the interaction between ER and mitogenic pathways can be

described as a dynamic inverse relationship, where inhibition of one results in compensatory increase in the other. Thus growth factor inhibition may increase ER expression or function and re-sensitize breast cancer cells to endocrine therapy and would support combination, or in fact sequential treatment. Alternatively, growth factor signalling can interact synergistically with ER and augment both genomic and non-genomic functions of the estrogen receptor. This would provide a strong rationale for simultaneous blockade of both ER and mitogenic pathways using various signal transduction inhibitors (STIs).

Most of the experimental data in support of this concept has come from HER-2 positive tamoxifen-resistant models rather than LTED-resistant scenarios, but similar principles may apply. It has been shown that signal transduction blockade using a HER2 tyrosine kinase inhibitor (AG1478) or a MAPK inhibitor (UO126) may abrogate antiestrogen resistance, while combined treatment with tamoxifen and either STI was significantly more effective than either therapy alone, not only at inhibiting estrogen-mediated gene transcription and tumor colony survival in vitro, but also at delaying tumor xenograft growth in vivo (Kurokawa et al. 2000). Others have shown that hormone-resistant MCF-7 cells with up-regulated HER2 signaling are sensitive to the TKI gefitinib, and that combined therapy of gefitinib and tamoxifen provided maximal growth inhibition and significantly delayed the time to progression of the disease (Shou et al. 2004). Using an in-vivo model of MCF-7/HER2 over-expressing xenografts, similar effects were seen with gefitinib combined with estrogen deprivation, which provided greater inhibition of growth and substantially delayed acquired resistance compared with estrogen deprivation alone (Massarweh et al. 2006).

Based on the evidence outlined above, a number of trials were initiated with either the HER2 monoclonal antibody trastuzumab or the EGFR/HER2 tyrosine kinase inhibitors (TKIs) gefitinib, erlotinib or lapatinib in combination with endocrine therapy (Table 10.1). While some of these trials are in patients with established hormonal resistance where activated growth factor pathways may be operative, many of the trials are in the first-line ER + ve hormone-sensitive setting in combination with an aromatase inhibitor, where clinical and experimental data have shown that TKIs alone may have limited activity. Therefore, the primary endpoint for these trials is to investigate whether time to disease progression (TTP) can be significantly prolonged by the addition of an STI to endocrine therapy, thus delaying the emergence of resistance as demonstrated in various preclinical models described above.

Gefitinib and erlotinib are both small molecule tyrosine kinase inhibitors of the ATP binding site of the EGFR and have been shown to delay the development of tamoxifen resistance in vitro (Shou et al. 2004). Two studies have explored the potential benefit for combining either gefitinib or erlotinib with an aromatase inhibitor (Mita et al. 2005; Mayer et al. 2006). Neither study showed significant clinical efficacy. A randomized neo-adjuvant trial of anastrozole alone or in combination with gefitinib given for 3 months prior to surgery in 206 postmenopausal patients with ER+ primary breast cancer was also negative (Smith et al. 2007), although this study also failed to select patients for EGFR over-expression. In contrast, a pre-operative trial of gefitinib versus gefitinib combined with anastrozole for 4–6 weeks prior to

Table 10.1 Reported results from clinical trials of combinations of endocrine therapies with targeted biological agents in ER+ postmenopausal breast cancer

Clinical Setting	Trial Phase & Number Patients	Intervention	Clinical Endpoints	Biological Correlates	Author and Reference
Combination with gefitinib (GEF) or erlotinib (ERL)					
MBC Hormone-refractory	**II** N = 15	ANA + GEF	PR = 0 SD = 0	None available (NA)	Mita et al. (2005)
MBC Hormone-sensitive	**II** N = 150	LET + ERL	CBR = 11/20 so far	Will record EGFR/HER2 and ER phosphorylation	Mayer et al. (2006)
MBC Hormone-sensitive	**II RCT** N = 206 Stratum 1	TAM vs TAM + GEF	PFS 10.9 mo (TAM + GEF) vs 8.8 mo (TAM), p = 0.31 CBR 50.5% (TAM + GEF) vs 45.5% (TAM), p = 0.74	To be conducted	Osborne et al. (2007)
Neo-adjuvant EBC	**II RCT** N = 206	ANA vs ANA + GEF × 16 weeks	ORR = 61% (ANA) vs 48% (ANA + GEF), p = .067	Reduction in Ki67 = 83.6% (ANA) vs 77.4% (ANA + GEF), p = 0.164	Smith et al. (2007)
Pre-operative EBC EGFR+ only	**II RT** N = 56	GEF vs GEF + ANA × 4–6 weeks	ORR = 50% (GEF) vs 54% (ANA + GEF)	Reduction in Ki67 = 92.4% (GEF) vs 98% (ANA + GEF), p = .005	Polychronis et al. (2005)

Table 10.1 (continued)

Clinical Setting	Trial Phase & Number Patients	Intervention	Clinical Endpoints	Biological Correlates	Author and Reference
Combination with trastuzumab (TRAS)					
HER2 + MBC (note: all pts were TRAS and AI naïve)	**II** N = 33	TRAS + LET	PR = 26% SD = 26%	NA	Marcom et al. (2007)
HER2 + MBC (all pts were IHC 3+ or FISH+)	**III RCT** N = 207	ANA vs ANA + TRAS	PFS = 2.4mo (ANA) vs 4.8mo (ANA + TRAS), p = .0016 ORR = 6.8% (ANA) vs 20.3% (ANA + TRAS), p = .018	NA	Mackey et al. (2006)
Combination with farnesyltransferase inhibitors: tipifarnib (TIP)					
MBC Tamoxifen resistant	**I** N = 20	TAM + TIP	PR = 1/20 SD > 6mo = 4/20	NA	Dalenc et al. (2005)
MBC Tamoxifen resistant	**II RCT** N = 120	LET vs LET + TIP	PR = 38% (LET) vs 30% (LET + TIP) CBR = 62% (LET) vs 49% (LET + LAP)	NA	Johnston et al. (2007)
Combination with mTOR inhibitors: everolimus (EVE) or temsirolimus (TEM)					
MBC	**II** N = 92	LET vs LET + TEM 10mg daily vs LET + TEM 30mg intermittent	ORR = 45% (LET) vs 33% (LET + TEM10) vs 40% (LET + TEM30) PFS = 11.6mo (LET) vs 11.5mo (LET + TEM10) vs 13.2mo (LET + TEM30)	NA	Baselga et al. (2005)

Table 10.1 (continued)

Clinical Setting	Trial Phase & Number Patients	Intervention	Clinical Endpoints	Biological Correlates	Author and Reference
Pre-operative EBC	**II RCT** N = 270	LET vs LET + EVE 10 mg/d × 4 months	Clin ORR 68% (LET + EVE) vs 59% (LET)	If High S6Kinase	Baselga et al. (2007)
			Clin USS 58% (LET + EVE) vs 47% (LET)	ORR 82% (LET = EVE) vs 68% (LET)	Gardner et al. (2007)
MBC	**III RCT** N = 992	LET vs LET + TEM 30 mg intermittent	ORR = 24% (LET) vs 24% (LET + TEM) SD = 19% (LET) vs 16% (LET + TEM) PFS = 9.2m (LET) vs 9.2mo (LET + TEM)	NA	Chow et al. (2006)

MBC: metastatic breast cancer; EBC: early (primary) breast cancer;
RCT: randomized controlled trial; RT: randomized trial;
PR: partial response; SD: stable disease >6months;
ORR: objective response rate (PR + CR); CBR: clinical benefit rate (ORR + stable disease > 6months);
USS – ultrasound PFS: progression free survival; OS: overall survival;
TAM: tamoxifen, EVE everolimus; TEM temsirolimus; TIP tipifarnib; TRAS: trastuzumab
ANA: anastrozole; LET: letrozole; GEF: gefitinib; ERL: erlotinib

surgery was conducted in women with known ER+ and EGFR+ primary breast cancer (Polychronis et al. 2005). This study showed that both treatments effectively reduced the size of breast tumors and levels of ER phosphorylation, and that combined treatment induced the greatest reduction in tumor proliferation. These studies of EGFR therapies illustrate the importance of selecting tumors with the known target for combined STI endocrine therapy, although the reported rates for EGFR expression in primary breast cancer do vary quite dramatically among studies (range 15–90%) (Atkins et al. 2004).

The results of a randomised, double-blind placebo-controlled phase II trial of tamoxifen with/without gefitinib in 290 patients with ER + ve metastatic breast cancer were recently presented (Osborne et al. 2007). This study set out to prove the pre-clinical concept that combination therapy could delay the onset of acquired resistance to endocrine therapy, as demonstrated both in xenograft models in-vivo (Shou et al. 2004; Massarweh et al. 2006). Patient's disease was either endocrine naïve or had developed greater than a year after completion of adjuvant tamoxifen (Stratum 1, n = 206), or had developed during or after AI therapy (Stratum 2 n = 84). In the endocrine naïve patients (stratum 1) there was a numerical increase in progression-free survival from 10.9 to 8.8 months (hazard ratio 0.84, 95% CI 0.59–1.18, p = 0.31) which met the pre-defined criterion of a 5% improvement in PFS. Clinical benefit rate was also numerically superior (50.5% vs 45.5%). Patients that had been pre-exposed to AIs did not gain any benefit from the combination, suggesting that difference in patient populations are crucial in selecting an appropriate populations to test in these studies. Further randomised trials in metastatic disease of gefitinib and anastrozole versus anastrazole alone are in progress to see if a delay in acquired resistance to estrogen deprivation can be delivered by combined therapy.

Clinical evidence exists that trastuzumab may restore both ER expression and endocrine responsiveness in advanced breast cancer (Munzone et al. 2005). A phase II clinical trial of letrozole and the monoclonal antibody trastuzumab in patients with ER + /HER2+ metastatic breast cancer revealed that the combination was well tolerated and had a clinical benefit rate (PR + SD) of 50% (Marcom et al. 2007) (Table 10.1). A randomized phase II trial in 207 patients with known ER + /HER2+ metastatic breast cancer recently reported a doubling of progression-free survival with the addition of trastuzumab over anastrozole alone (4.8mo vs 2.4 mo, P = .0016) (Mackey et al. 2006).

Lapatinib is a potent oral tyrosine kinase inhibitor of both EGFR and HER2. As a dual inhibitor it may have the potential for greater anti-tumor effect than strategies targeting a single receptor, and in-vitro data have demonstrated that estrogen deprivation significantly enhances the antiproliferative effects of lapatinib in HER2 amplified breast cancer cell lines (Xia et al. 2006; Leary et al. 2006). Preclinical evidence suggests that lapatinib can significantly enhance sensitivity to tamoxifen in cell lines with acquired tamoxifen resistance (Chu et al. 2005). A Phase III trial has completed recruitment of 1200 patients with metastatic ER+ breast cancer who were randomized to receive either letrozole alone or letrozole combined with lapatinib. Importantly, patients were selected regardless of their known EGFR/HER2 status in the primary tumor, but were stratified according to the time interval since

adjuvant tamoxifen (> or < 6 months). This large study may offer an important insight into the subgroups of patients most likely to benefit from a lapatinib-endocrine combination, such as, known HER2 + /ER+ breast cancer with potential de novo endocrine resistance (at least 200 such patients should be included in the study), or tumors that might develop acquired resistance to letrozole during treatment due to adaptive HER2 up-regulation. To identify the latter, all patients had serum taken at baseline entry for assessment of circulating extracellular domain (ECD) HER2 which has been reported to be a predictor of poorer outcome with endocrine therapy, with sero-conversion occurring during endocrine therapy in up to 25% of patients with ER+ metastatic disease treated with either letrozole or tamoxifen (Lipton et al. 2003). Thus, correlative biomarker studies will be crucial to the interpretation of which ER+ tumors derive benefit from combined STI-endocrine therapy.

10.4.3 Endocrine Therapy Combined with Farnesyltransferase Inhibitors

Interfering with the downstream effectors of growth factor receptors has emerged as another effective anti-tumor strategy. Ras proteins are membrane bound GTP-binding proteins that are frequently aberrantly expressed in breast cancer, and act as mitogenic switches between growth factors receptors and downstream intracellular signaling via Raf/MAPK (Clark and Der 1995). This reaction is catalyzed by the farnesyltransferase enzyme. FTIs such as tipifarnib and lonafarnib were developed in an effort to interrupt this pathway by inhibiting farnesylation, the first step in Ras activation. Based on encouraging results in cell line and tumor xenograft models (Martin et al. 2007) trials have been conducted in combination with tamoxifen or aromatase inhibitors (Table 10.1). A small phase I/II study that included patients with endocrine resistance suggested evidence of efficacy (Dalenc et al. 2005). Unfortunately a larger randomized phase II study of letrozole alone or in combination with tipifarnib failed to show added benefit for the combination (Johnston et al. 2007). Mistakes in this trial included underpowering with inappropriate clinical endpoints of response rate rather than disease stabilization. However, the true target for FTIs remains poorly understood, with up to 30 proteins that require farnesylation having a role in cellular growth and survival.

10.4.4 Endocrine Therapy Combined with mTOR Antagonists

The PI3K/Akt/mTOR pathway is activated by a number of growth factors, including insulin, insulin-like growth factor I (IGF-1), basic fibroblast growth factor (bFGF), EGF and vascular epidermal growth factor (VEGF). Inhibiting this key effector of multiple pro-survival signals has therefore emerged as a viable therapeutic strategy in cancer. Mutations in the catalytic domain of PI3K have been identified in 20–25% of breast cancers (Wu et al. 2005; Bachman et al. 2004). A further 15–35% of breast cancer patients demonstrate reduced expression of PTEN (phosphatase and tensin

homolog deleted on chromosome Ten), a known inhibitor of the PI3K/AKT pathway which may be associated with poor prognosis in patients with ER+ breast cancer treated with tamoxifen (Saal et al. 2005; Shoman et al. 2005). As such these cancers may be resistant to strategies targeting upstream growth factor receptors, but particularly sensitive to PI3K or mTOR inhibition. Furthermore, preclinical studies have demonstrated that the combination of letrozole with an mTOR inhibitor results in synergistic growth inhibition and apoptosis in ER+ breast cancer cell models (Boulay et al. 2005).

While PI3K inhibitors are still in the early stages of development, mTOR inhibitors have been tested in breast cancer in combination with endocrine therapies (Table 10.1). A randomized phase II study of letrozole alone or in combination with another inhibitor, temsirolimus, has also been reported (Baselga et al. 2005). Preliminary results suggested a modest benefit to the combination in terms of median progression free survival (13.2 mo vs 11.6 mo). Unfortunately, the resulting large phase III randomized trial of letrozole alone or in combination with temsirolimus in 992 postmenopausal women was terminated early after an interim analysis demonstrated a lack of benefit for the combination (Chow et al. 2006). As with gefitinib, the inability to identify patients in whom the tumors demonstrate dependence on PI3K-mTOR activation severely limited the likelihood of success for this large phase III trial. Likewise, concern has been expressed that mTOR inhibition may induce a feedback loop via S6kinase and IGFR which enhances further Akt activation, thus overcoming the effects of mTOR inhibition.

Further studies in the neoadjuvant setting have evaluated the benefit of adding the mTOR inhibitor everolimus (RAD-001) to letrozole. In a randomized phase II study in 270 postmenopausal women with ER+ve primary operable breast cancer (> 2 cm in size), the combination of letrozole 2.5 mg/day and everolimus 10 mg/day for 4 months pre-surgery resulted in a significantly greater tumour shrinkage as judged by ultrasound (58% vs 47%, $p = 0.03$) and a greater reduction in cell proliferation as measured by changes in Ki-67 after 15 days therapy (Baselga et al. 2007). In associated biomarker studies to determine those tumours most likely to respond to combined mTOR antagonists and AI, elevated levels of one of the downstream biomarkers of mTOR activation (pS6240 kinase) was associated with a greater chance of response to the combination (odds ratio 2.1) (Gardner et al. 2007). These types of clinical studies in primary breast cancer are more likely to yield informative biomarker data than correlative studies in advanced disease, and as such may help select appropriate patients for combination strategies which attempt to overcome endocrine resistance pathways.

10.5 Conclusion

A number of theories have been proposed as contributing to endocrine resistance, and it is unlikely that there is any single dominant mechanism in the clinic. There is an increasing body of evidence to suggest that ER signalling survives, and that growth factor receptor and downstream kinases often operate in conjunction with

ER to account for both de-novo and acquired endocrine resistance. The nature of the interaction between ER and mitogenic signalling likely varies over time and from one patient to another. In some activated growth factor mediated signalling suppresses ER expression and function, raising the possibility that growth factor targeted therapy may directly restore endocrine responsiveness. In other cases, ER and growth factor signalling may interact synergistically providing the basis for combination strategies. Despite the strong pre-clinical data and rationale, translation of these hormone resistance hypotheses into clinical studies of combined STI and endocrine therapies have yielded disappointing results to date, which may be in part attributable to a poor selection of patients. It is unlikely that patients will respond to combination with specific inhibitors unless the intended target is a significant driver of endocrine resistant growth. A number of trials are currently exploring the benefit of various targeted agents in combination or in sequence with endocrine therapy and include biological analyses that may shed further light on the clinically relevant mechanisms of endocrine resistance, and ultimately show us the intelligent way to combat the various hormonal resistance pathways that cancer cells utilise to survive.

Abbreviations

ER:	estrogen receptor
EGFR:	epidermal growth factor receptor
HER2:	epidermal growth factor receptor 2
IGFR:	insulin-like growth factor receptor
HDAC:	histone deacetylase
MAPK:	mitogen activated protein kinase
FISH:	Fluorescent in situ hybridization
LTED:	long term estrogen deprivation
MCF-7TAMLT:	long-term tamoxifen-treated MCF7 cells
STI:	signal transduction inhibitor
TTP:	time to disease progression
IGF-1:	insulin-like growth factor I
bFGF:	basic fibroblast growth factor
EGF:	epidermal growth factor
VEGF:	vascular epidermal growth factor
PTEN:	phosphatase and tensin homolog deleted on chromosome Ten
AI:	aromatase inhibitor

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