

# Deciphering antihormone-induced compensatory mechanisms in breast cancer and their therapeutic implications

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## Abstract

Breast cancer inhibition by antihormones is rarely complete, and our studies using responsive models reveal the remarkable flexibility of breast cancer cells in recruiting alternative signalling to limit maximal anti-tumour effects of oestrogen receptor  $\alpha$  (ER) blockade. The recruited mechanism involves antihormone-induced expression of oestrogen-repressed signalling genes. For example, epidermal growth factor receptor gene (*EGFR*) is induced by antioestrogens and maintains residual kinase and ER phosphorylation, cell survival genes, and thereby allows incomplete antihormone response and emergence of resistance. Microarrays are revealing the breadth of antihormone-induced genes that may attenuate growth inhibition, including *NF $\kappa$ B*, *Bag1*, *14-3-3 $\zeta$*  and tyrosine kinases, such as HER2 and Lyn. Three concepts are emerging: first, some genes are induced exclusively by antioestrogens, while others extend to oestrogen deprivation; secondly, some are transiently induced, while others persist into resistance; finally, some confer additional adverse features when tumour cells are in an appropriate context. Among the latter is CD59 whose antioestrogen induction may permit evasion of immune surveillance *in vivo*. Also, induction of pro-invasive genes (including *NF $\kappa$ B*, *RhoE* and  $\delta$ -catenin) may underlie our findings that antioestrogens can markedly stimulate migratory behaviour when tumour intercellular contacts are compromised. Based on our promising studies selectively inhibiting EGFR (gefitinib), NF $\kappa$ B (parthenolide) or CD59 (neutralising antibody) together with antioestrogens, we propose that co-targeting strategies could markedly improve anti-tumour activity (notably enhancing cell kill) during the antihormone-responsive phase. Furthermore, subverting those induced signalling genes that are retained into resistance (e.g. *EGFR*, *NF $\kappa$ B*, *HER2*) may prove valuable in this state. Alongside future deciphering and targeting of genes underlying antioestrogen-promoted invasiveness, embracing of intelligent combination strategies could significantly extend patient survival.

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## Introduction

Antihormones that deplete oestrogen/oestrogen receptor  $\alpha$  (ER) signalling comprised the first targeted therapies for breast cancer and continue to be a mainstay in the management of this disease, promoting worthwhile tumour remissions and significant survival benefits in many ER+ patients. Some of these agents

act by competing with oestrogen for binding to its target receptor in breast cancer cells, exemplified by 'partial' antioestrogens (such as tamoxifen), and also 'pure' antioestrogens (such as faslodex) that additionally reduce ER level. Other agents, notably aromatase inhibitors, act to severely deplete the oestrogenic environment. Unfortunately, however, the efficacy of all antihormones present to date is compromised not only by a proportion of ER+ patients exhibiting *de novo* antihormone resistance, but also by the many more who acquire resistance following an initial therapeutic response (Gee *et al.* 2005, Nicholson &

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Johnston 2005, Nicholson *et al.* 2005). Sadly, in both the instances, resistance can be associated with increased metastatic capacity and poorer patient outlook. Based on studies primarily with tamoxifen (but now emerging with oestrogen deprivation), altered growth factor receptor signalling and its cross-talk with the ER pathway has been heavily implicated as a resistance mechanism (Nicholson *et al.* 2004, 2005). Clinical studies targeting aspects of this interplay are ongoing and there is much hope that this avenue of research will result in valuable new treatments for antihormonal resistant states.

However, an ultimate goal is to prevent emergence of resistant growth with its undesirable phenotype. Interestingly, the initial growth inhibitory effects of antihormones are rarely complete, with some cells evading growth inhibition during the drug-responsive phase, culminating in anti-tumour responses of a finite duration. Moreover, ER+ breast cancer model systems reveal that antihormones can exert anti-proliferative effects but generally promote only modest cell kill (Gee *et al.* 2003). Clearly, early 'protective' effects must be present in antihormone-treated cells, where these would be essential in maintaining the tumour cell cohort from which resistant growth ultimately emerges during treatment. Intrinsic genetic heterogeneity in the key signalling pathways is a likely contributory factor in limiting anti-tumour effect. However, our group has also accumulated evidence of a further important mechanism of tolerance, involving signal transduction genes normally repressed by oestrogen/ER signalling, that is induced during antihormone challenge (Gee *et al.* 2003, Nicholson *et al.* 2005). Since, in some instances, the identified genes have also been reported to be inducible by environmental stresses and other therapeutic approaches, they may comprise global cell survival mechanisms. Our studies with antihormones provide powerful evidence of the remarkable flexibility of the cancer cell in its ability to recruit 'compensatory' signalling to limit the impact of therapeutic blockade of any one pathway. Importantly, while the breadth of these antihormone-induced events remains largely unexplored, our emerging experience is that their intelligent targeting alongside antihormonal agents can confer previously unobtainable levels of suppression of proliferation and, critically, substantial induction of apoptosis (Gee *et al.* 2003, Nicholson *et al.* 2005). If this induced mechanism is recapitulated *in vivo*, it is our belief that such combination strategies have considerable potential to improve initial response and delay (or even prevent) resistance to antihormonal therapy.

## Oestrogen-repressed genes: overview of molecular biology

Our signalling (and more recently microarray) studies are increasingly showing that there is a substantial cohort of genes whose expression is repressed by oestrogen/ER signalling, and in turn, induced during cellular response to antihormones in ER+ breast cancer cells. In agreement, microarray studies by Frasor *et al.* (2003) have revealed that transcriptional repression of genes actually comprises the bulk (70%) of the expression changes associated with oestrogen challenge in ER+ breast cancer models. In many instances, these repressive events can be antagonised by antioestrogens, particularly the pure antioestrogen faslodex (Frasor *et al.* 2004). However, despite oestrogen inhibitory effects on gene expression being surprisingly frequent, the underlying molecular biology of this phenomenon remains relatively poorly understood (Zubairy & Oesterreich 2005).

A mechanism that has been suggested is that the oestrogen/ER complex enters into protein/protein interactions with transcription factors, including nuclear factor  $\kappa$ B (NF $\kappa$ B), activator protein 1 (AP-1), CCAAT/enhancer-binding protein, specificity protein 1 (SP-1), GATA-binding factor-1 (GATA-1) or mothers against decapentaplegic (SMAD), which can lead to transcriptional repression at heterologous response elements. Most information is available for oestrogen repression of NF $\kappa$ B-driven transcriptional activity, where this event has been linked to the maintenance of bone density through subsequent blockade of the interleukin-6 promoter (Stein & Yang 1995, Kalaitzidis & Gilmore 2005). The areas of the ligand-binding domain, region D and the DNA-binding domain of ER are reported to be required for oestrogen/ER-mediated repression of NF $\kappa$ B-driven transcriptional activity (Valentine *et al.* 2000, Kalaitzidis & Gilmore 2005). The oestrogen/ER complex may interact with NF $\kappa$ B directly to block its DNA binding, or bind DNA-bound NF $\kappa$ B to interfere with its ability to activate transcription (Stein & Yang 1995, Kalaitzidis & Gilmore 2005); such gene-repressive effects are reported to be both promoter- and cell context-dependent (Cerillo *et al.* 1998).

Competition for co-activators, such as steroid receptor coactivator 1 (SRC1) or p300/CREB binding protein (CBP), may also be contributory to the mechanism of oestrogen/ER transcriptional repression (although this remains controversial with regards to transrepression of NF $\kappa$ B activity; Harnish *et al.* 2000, Valentine *et al.* 2000, Kalaitzidis & Gilmore 2005). For example, oestrogen repression of human epidermal growth factor receptor-2 (HER2) expression is reported

to involve the intron 1 region of the HER2 promoter and competition for SRC1 between the oestrogen/ER complex and the AP-2 transcription factor (Newman *et al.* 2000). The competition for p300/CBP family members has furthermore been described between ligand-bound steroid receptors and Fos/Jun proteins during transrepression of AP-1 sites (Kamei *et al.* 1996). Finally, and surprisingly, there is also some indication that the oestrogen/ER complex may actually be able to recruit repressors to some gene promoters. By example, oestrogen triggers recruitment of the DEAD box RNA helicase (DP97) co-repressor to the *HER2* gene to block its expression (Rajendran *et al.* 2003).

The repressive effects of the oestrogen/ER complex can be counteracted by antihormones, resulting in reinstatement of gene expression (Frasor *et al.* 2004). However, the consequences of these antihormone-induced events for the tumour cell have not been significantly explored. Frasor *et al.* (2004) have reported that many of the oestrogen-suppressed genes are anti-proliferative/pro-apoptotic and thus it seems likely that some of the antihormone-induced genes will play a role in their growth inhibitory mechanism. For example, our microarray studies (and those of Frasor *et al.* 2004) have demonstrated induction of transforming growth factor- $\beta$  family members and cell cycle inhibitors during antioestrogen response (Shaw *et al.* 2005). Paradoxically, however, our emerging experience from model systems indicates antihormones also induce expression of a number of oestrogen-repressed cell survival/proliferation-signalling genes, whose expression could serve to limit maximal anti-tumour activity of these agents in ER+ breast cancer cells (Gee *et al.* 2003, 2004, Shaw *et al.* 2005).

### **Antihormones induce expression of key signalling genes that may act to limit anti-tumour response in ER+ breast cancer cells**

#### **Proof of principle: antioestrogens induce epidermal growth factor receptor (EGFR) expression in parallel with their incomplete growth inhibitory response**

Oestrogen/ER signalling has been associated with the suppression of growth factor receptor tyrosine kinase EGFR at the transcriptional level in various ER+ breast cancer models *in vitro* (Yarden *et al.* 2001, Wilson & Chrysogelos 2002). The underlying mechanism of this profound oestrogen suppression of EGFR expression remains poorly defined, but seems to be associated with a negative regulatory element within the first intron of the *EGFR* gene in ER+ cells (Wilson & Chrysogelos 2002).

Immunostaining studies performed by our group in MCF-7 breast cancer cells confirm that expression of EGFR is largely absent after 17 $\beta$ -oestradiol treatment ( $10^{-9}$  M; Gee *et al.* 2003). In turn, however, blockade of oestrogen/ER signalling upregulates the expression of EGFR in MCF-7 cells during their antihormone response. While induction was relatively small with oestrogen deprivation (using phenol red-free medium containing charcoal-stripped serum that depletes exogenous oestrogen to  $\sim 10^{-13}$  M), more obvious increases in EGFR expression occurred from as early as by 1 week's treatment with the antioestrogen 4-OH tamoxifen ( $10^{-7}$  M). Prominent membrane immunostaining for the receptor was achieved in MCF-7 cells by 3 weeks. Parallel data were obtained with  $10^{-7}$  M faslodex, although significant inductive events were seen even earlier with this pure antioestrogen in keeping with its more profound blockade of ER (McClelland *et al.* 2001, Gee *et al.* 2003). While there was some depletion of MAP kinase (MAPK) and protein kinase B (AKT) phosphorylation (probably a consequence of the inhibitory effects of antihormones on the insulin-like growth factor receptor pathway), the tamoxifen-induced EGFR was able to maintain residual downstream activity through these kinases in MCF-7 cells. We showed that these kinases in turn cross-talked with ER, maintaining residual phosphorylation of serine 118/serine 167 of the ER and low levels of the ER-regulated gene *bcl-2* in the presence of tamoxifen (Gee *et al.* 2003). This persistent kinase activity, ER activity and residual levels of a key anti-apoptotic gene would be predicted to maintain some cell survival and proliferation during the tamoxifen-responsive phase. In accordance with this concept, the drug's anti-tumour effects were incomplete, with a 35% fall in proliferation and only a 15% induction of apoptosis, culminating in approximately 50% inhibition of growth (Gee *et al.* 2003). By week 12, 60% of tamoxifen-treated cells demonstrated increased expression and activity of EGFR and a substantial gain in downstream kinase-signalling activity. These events were paralleled by acquisition of tamoxifen-resistant growth at this time, coincident with substantial recovery in ER activity and expression of ER-regulated EGFR ligands (e.g. amphiregulin), completing an EGFR autocrine-signalling loop that provides the dominant growth mechanism for the emerging resistant cells (Knowlden *et al.* 2003, Britton *et al.* 2006). Studies from Yarden *et al.* (2001) are in agreement with our work, where there was again a significant increase in EGFR expression during response to antihormones and also subsequent enhanced growth sensitivity to EGFR ligands.

In total, these signalling studies provide proof of principle that induction of oestrogen-repressed gene

products can comprise an important mechanism for promoting residual growth and cell survival-signalling activity during antihormone challenge. This acts to limit anti-tumour response and subsequently permits the emergence of resistance. The mechanism of antioestrogen-induced EGFR expression in ER+ breast cancer cells seems unlikely to be driven by selection of a population of pre-existing EGFR+ cells since it arises rapidly within the first week of treatment, and moreover is partially reversible on tamoxifen withdrawal (Gee *et al.* 2003). Yarden *et al.* (2001) have furthermore reported that the EGFR gain can be reversed by prolonged re-exposure to oestrogens in their ER+ models. Equivalent deciphering of EGFR/kinase signalling during antihormone treatment and at the time of acquisition of resistance is now clearly important in clinical breast cancer, but remains notoriously problematic because of the difficulty in obtaining sequential treated breast cancer samples (Gee & Hutcheson 2005, Gee *et al.* 2005). Nevertheless, while the data remain controversial, emerging clinical studies indicate that there may indeed be some upregulation of various elements in the EGFR pathway by antioestrogens. Thus, modest increases in various components of EGFR/HER2/kinase signalling are apparent by the time of tamoxifen relapse (Gee *et al.* 1999, 2005, Gee & Hutcheson 2005, Gutierrez *et al.* 2005), suggesting clinical relevance of such induced mechanisms to a proportion of patients.

#### **Microarrays are revealing the breadth of antihormone-induced signalling genes that may limit growth inhibitory response**

It has only been possible for us to begin to appreciate the extent of signalling mechanisms induced by antihormone treatment in ER+ breast cancer cells through our recent embracing of microarray technologies. The arrays are proving to be of increasing value to associate gene signature with key cancer cell endpoints in model systems, as well as in discriminating previously unrecognised tumour subsets and markers of therapeutic response/failure/prognosis in clinical disease. Several studies have employed microarrays to decipher the transcriptional impact of oestrogen during its promotion of breast cancer cell growth, and in turn have evaluated how effective different classes of antihormones are in reversing these profiles (Inoue *et al.* 2002, Levenson *et al.* 2002, Cunliffe *et al.* 2003, Frasor *et al.* 2003, Hodges *et al.* 2003). However, these studies have to date primarily focussed on profiling the

inductive events of antioestrogens that underlie their growth inhibitory effects, rather than exploring the concept of 'compensatory' increases in oestrogen-repressed signalling genes that may serve to attenuate antihormone response. In this regard, our array studies specifically aiming to reveal induced genes that may prove valuable targets alongside antihormones are, to our knowledge, unique (Gee *et al.* 2004, Shaw *et al.* 2005).

Our quest for antihormone-induced genes initially focused on antioestrogens, employing triplicate cell preparations, BD Clontech Atlas Human Cancer 1.2K gene cDNA arrays (nylon platform) and web-based analysis software (GeneSifter). This approach revealed over 150 genes induced by both 4-OH-tamoxifen and faslodex ( $10^{-7}$  M) in ER+ MCF-7 breast cancer cells versus oestrogen challenge (Shaw *et al.* 2005). Of these, several had an adverse signalling ontology, some apparently having the capacity to contribute in a positive manner to cell survival and/or proliferation (Gee *et al.* 2004, Shaw *et al.* 2005). Very recently, we have extended these studies to larger Affymetrix microarrays (U133A GeneChip; ~25K genes) applied to triplicate cell preparations, in order to be able to ultimately reveal the full breadth of antihormone-induced signalling genes that may contribute towards cell survival in the presence of such treatments. Alongside challenge with antioestrogens, these latter studies have encompassed gene profiling of MCF-7 cells subject to oestrogen deprivation versus oestrogen challenge. In the first instance, simple clustering and profiling of the Affymetrix data has been employed specifically to study those genes (~90) known to comprise the tyrosine kinase (TK) category of the 'kinome' (as defined by the landmark paper by Manning *et al.* 2002 and the online 'KinWeb' resource). Receptor (membrane) and non-receptor TKs comprise important signalling mediators implicated in neoplastic development and progression, and as such these oncogenes are being intensively studied as targets for anti-cancer drug development, exemplified by EGFR, HER2, vascular endothelial growth factor (VEGF) receptors, Src and Abl (Vieth *et al.* 2005). Our Affymetrix array studies have revealed that the expression of several TKs (including EGFR) is induced during antihormone response, where ontological examination indicates that 15 have potential to promote compensatory signalling. Cumulatively, our various array approaches are revealing a number of broad features of antihormone-induced events that may have important implications for future treatment of breast cancer.

*Some signalling genes on the arrays are induced by antioestrogens only, while others are also induced by oestrogen deprivation*

Our use of the nylon array platform, while representing only limited gene coverage, has been able to reveal several previously unrecognised signalling genes induced by the ER blockade associated with both partial and pure antioestrogens (Gee *et al.* 2004, Shaw *et al.* 2005). An obvious oestrogen-suppressed, antioestrogen-induced profile for these genes has subsequently been verified at the mRNA and protein levels. Ontological search suggests a positive role in proliferation/cell survival for some of these genes, where previous studies have provided further links with cell survival in the presence of the environmental stresses of hypoxia, heat shock and in limiting radio/chemoresponse. As such, their induction may indeed be important in the early evasion of maximal antioestrogen-associated growth inhibition. Among these antioestrogen-induced genes are the co-chaperone bcl-2-associated athanogene 1 (*Bag1*) and an adapter molecule, 14-3-3 $\zeta$  (Shaw *et al.* 2005). In both instances, induction was common to tamoxifen and faslodex, although subsequent verification studies revealed that more substantial inductive events were apparent when treating with the pure antioestrogen, in agreement with the reports of more substantial de-repressive effects of this agent (Frasor *et al.* 2004). *Bag1* is reported to interact with heat shock proteins to enhance protein refolding, with bcl-2, ER, growth factor receptors and Raf-1, and to promote proteasomal degradation of denatured proteins (Cutress *et al.* 2002, Townsend *et al.* 2005). Interestingly, *Bag1* has also been shown to be present in clinical breast cancers (Cutress *et al.* 2002), although the impact of antioestrogens on this gene and its definitive prognostic associations in clinical disease are not yet known. As an adapter molecule, 14-3-3 $\zeta$  is reported to bind phospho-serine/threonine-containing motifs, interplaying with AKT and sequestering apoptotic proteins (Subramanian *et al.* 2001). As a consequence of these diverse interactions, both *Bag1* and 14-3-3 $\zeta$  can promote cell survival in the presence of various stresses and limit chemo/radioresponse in cancer cells (Qi & Martinez 2003, Townsend *et al.* 2005), features extending to ER+ breast cancer cells for the former gene.

As we previously noted for EGFR, induction of *Bag1* and 14-3-3 $\zeta$  in MCF-7 cells was substantial during antioestrogen treatment but not with oestrogen deprivation (Shaw *et al.* 2005). This may be reflective of their repression by the traces of oestrogens ( $\sim 10^{-13}$  M)

remaining in the charcoal-stripped serum employed for our oestrogen deprivation studies. Interestingly, however, this was not the case for all the induced genes revealed on the nylon arrays, since verification of NF $\kappa$ B1(p105) revealed that both antioestrogens and oestrogen deprivation were capable of increasing expression of this gene. Our subsequent studies have revealed antihormone increases in active NF $\kappa$ B1(p50) DNA binding and NF $\kappa$ B transcriptional activity (Gee *et al.* 2004, Shaw *et al.* 2005). NF $\kappa$ B1 lies within the Rel transcription factor subunit family whose members dimerise to promote transcription of NF $\kappa$ B-dependent genes and where such signalling is known to be oestrogen-repressed at multiple levels (Kalaitzidis & Gilmore 2005). The molecule bcl-3 can co-activate NF $\kappa$ B1 homodimers and is again oestrogen-repressed (Pratt *et al.* 2003); interestingly, this gene was also elevated by antihormones alongside NF $\kappa$ B1 in our MCF-7 cells. NF $\kappa$ B signalling has again been implicated substantially in promoting cell survival/proliferation under conditions of environmental stress, in chemo/radioresistance (Wu & Kral 2005) and furthermore in ER+/ER- resistance to antioestrogens or oestrogen deprivation in breast cancer (see below). As such, it seems likely that NF $\kappa$ B would allow cells to evade maximal anti-tumour effect during the antihormone-responsive phase.

In total, these nylon array studies show that, in addition to EGFR, further oestrogen-repressed signalling genes with potential roles in cell survival are induced in response to antihormone challenge. Our observations suggest that signalling genes (such as *Bag1* and 14-3-3 $\zeta$ ) that are selectively induced exist and their protective role may be exerted according to the conditions of ER blockade, while the induction of others (such as NF $\kappa$ B) may play a dominant signalling role in limiting response to diverse antihormonal strategies. This concept is borne out by our emerging experience with the Affymetrix platform that we have used to discriminate antihormone-induced TK genes. Out of the 15 oestrogen-repressed TKs identified with interesting ontology, a proportion was increased only by antioestrogens. These included various ephrin receptors, where some members of this large TK family have previously been implicated in tumorigenesis and are of increasing interest in breast cancer (Fox & Kandpal 2004). In addition, however, further TKs were induced by all antihormonal strategies, exemplified by HER2. This erbB receptor has been established as an oestrogen-repressed, antihormone-induced gene in several ER+-responsive breast cancer models (Bates & Hurst 1997, Newman *et al.* 2000), including by microarrays (Frasor *et al.* 2003, 2004), and is a key

player in pathways driving cell survival/proliferation and endocrine failure in breast cancer. A further TK induced by all antihormones in our array studies was the Src family member Lyn. This is reported to be a glucose stress-activated TK and interestingly has been implicated in the growth of leukaemia, hormone-refractory prostate tumours (Goldenberg-Furmanov *et al.* 2004) and chemoresistance in colon cancer (Bates *et al.* 2001).

*Some signalling genes on the arrays are transiently induced during antihormone response, while others persist on acquisition of resistance*

We have extended profiling of the various oestrogen-repressed, antihormone-induced genes identified on the arrays into acquired resistance using our sublines resistant to antioestrogens (FASR and TAMR cells; McClelland *et al.* 2001, Knowlden *et al.* 2003) or severe oestrogen deprivation (MCF-7X cells; Staka *et al.* 2005). This approach has revealed two distinct cohorts of antihormone-induced signalling genes. First, we observed genes whose potential cell survival/proliferative effects may be contributory only during the antihormone-responsive phase, since their induction is completely lost following acquisition of resistance. Among the TKs, this cohort of transiently induced genes on the arrays includes *Brk* (breast tumour kinase/PTK6; Harvey & Crompton 2004), a gene previously implicated in cell survival/proliferation and subversion of chemoresponse. However, most of the antihormone-induced genes revealed by our array studies persisted at elevated levels into the acquired resistant variants. This second gene cohort was exemplified by EGFR whose expression (and activity) is substantial in both FASR and TAMR cells (McClelland *et al.* 2001, Knowlden *et al.* 2003), and also by HER2 that is retained at high levels within the latter model. EGFR and HER2 increases have been associated with both ER<sup>-</sup> and ER<sup>+</sup> antihormone resistance in additional breast cancer models (Benz *et al.* 1993) and clinical disease (Gee *et al.* 2005). Further induced TKs maintained at high levels into acquired antioestrogen resistance on the arrays included Lyn. Several of the induced TKs were also elevated within the oestrogen-deprived resistant MCF-7X cell line and analysis of the nylon array genes showed that Bag1 and NFκB1 were retained at high expression/activity in both the MCF-7X and the FASR cells. Interestingly, constitutive NFκB activity has been reported in further ER<sup>+</sup> models of acquired oestrogen independence (LCC1; Pratt *et al.* 2003) and resistance to faslodex (LCC9, Riggins *et al.* 2005), as

well as in ER<sup>-</sup> cells (Biswas *et al.* 2000). Furthermore, in clinical breast cancer, there is constitutive activity of NFκB in ER<sup>+</sup> disease destined for relapse despite adjuvant tamoxifen treatment, and again in ER-tumours (Zhou *et al.* 2005).

Cumulatively, these data suggest that alongside those antihormone-induced genes likely to provide an initial cell survival/proliferation benefit, there are others whose growth contribution may remain critical following acquisition of resistance to various anti-hormonal strategies and also in inherently refractory states. This retention of elevated expression into resistance provides further smoking-gun evidence that a proportion of antihormone-induced signalling genes do exert positive, rather than tumour-suppressive, activity in breast cancer cells.

*Some genes induced on the arrays during antihormone response may confer additional adverse features on breast cancer cells when in an appropriate context*

*Evasion of immune surveillance.* CD59 is a membrane regulatory protein of the complement cascade, where such complement regulatory proteins (CRPs) normally protect against inappropriate immune-mediated cell death (Rushmere *et al.* 2004). CRPs are commonly elevated in malignancy and during progression allowing tumour cells to escape immune attack, where our recent studies have shown that CRPs are readily detectable in ER<sup>+</sup> clinical breast cancer and cell models such as MCF-7 (Rushmere *et al.* 2004). Interestingly, our nylon array studies have revealed that, while there was no obvious induction with oestrogen deprivation, there was a substantial increase in CD59 expression during response of MCF-7 cells to the antioestrogens tamoxifen or faslodex (Gee *et al.* 2004, Shaw *et al.* 2005). This induction has been re-confirmed at the mRNA and the protein levels. Subsequent studies revealed that the CD59 induction was transient during the acute phase of antioestrogen inhibition, with gene expression level subsequently declining once therapeutic resistance was acquired. The CD59 induction by antioestrogens could be functionally important in the *in vivo* context, where tumour cells would be vulnerable to the complement cascade. This protein may defend tumour cells from immune surveillance during early treatment and thereby limit cell kill in the presence of antioestrogens. Interestingly, induction of this gene also occurred during growth blockade of breast cancer cells by further anti-tumour agents, implying it may provide a fundamental cell survival mechanism. In support of

such CD59-promoted cell survival, we have recently shown that CD59 induction during exposure of MCF-7 cells to the antioestrogens tamoxifen or faslodex does indeed promote increased resistance to complement-mediated lysis (Gee *et al.* 2004, Shaw *et al.* 2005). There is now a need to perform clinical studies to confirm that such CRPs are induced during anti-hormone treatment *in vivo* and to explore further their biological importance in this context.

**Invasive behaviour.** It has been reported that oestrogen and ER confer a protective effect on invasiveness and motility in breast cancer cells, and in general ER+ disease is associated with a more favourable prognosis (Platet *et al.* 2004). Matrigel assays confirm an inherently low migratory behaviour of ER+ models, such as MCF-7, in comparison with their ER- counterparts with a small further protective effect of oestrogen challenge on ER+ cell invasiveness (Platet *et al.* 2004, Hiscox *et al.* 2007). The mechanism(s) whereby ER suppresses breast cancer cell invasiveness remains largely unknown. The protective effect of oestrogen challenge is reported to require integrity of the hormone binding, DNA binding and AF-2 regions of ER (Platet *et al.* 2000). As such, oestrogens may in part promote their protective effect through priming expression of anti-invasive, oestrogen response element-containing genes (e.g.  $\alpha$ 1-anti-chymotrypsin; Platet *et al.* 2004). However, it is also feasible that ER protein-protein interactions and transrepression events at heterologous response elements may be contributory if they limit expression of key pro-invasive elements in ER+ breast cancer cells (Platet *et al.* 2000, 2004).

Interestingly, we (and others; Platet *et al.* 2000, 2004) have recognised that ER blockade using the antioestrogens tamoxifen or faslodex is associated with a small, early induction of MCF-7 cell invasiveness *in vitro* (Hiscox *et al.* 2007). Our array studies focussing on antihormone-induced, oestrogen-repressed genes have also revealed increased expression during treatment of several elements whose ontology implicates them in epithelial-mesenchymal transition, motility and invasiveness (Shaw *et al.* 2005). Of obvious interest in this regard is NF $\kappa$ B (Wu & Kral 2005); in addition, we also observed antioestrogen induction of RhoE and  $\delta$ -catenin, with changes subsequently verified at the protein level. RhoE is an anti-proliferative member of the Rnd subfamily of small Rho-related GTP-binding proteins that can also promote actin cytoskeleton changes, cell rounding and augment cell migratory speed (Guasch *et al.* 1998). Ectopic expression of  $\delta$ -catenin, an adhesive junction protein, in turn can

enhance growth factor-promoted cell scattering, increase cell spreading and formation of lamellipodia and filopodia (Lu *et al.* 1999). Similarly, many of the antihormone-induced TKs revealed by our Affymetrix studies have been implicated previously in cell migratory behaviour (including ephrin receptors (Fox & Kandpal 2004), Lyn (Suzuki *et al.* 1998) and HER2). It is thus feasible that these various genes may contribute towards the small inductive effect of invasiveness exhibited in the presence of antioestrogens. Moreover, they may play a role when acquired antioestrogen resistance subsequently emerges with its inherently increased aggressiveness (Hiscox *et al.* 2007) since, as stated above, many of these induced TKs, NF $\kappa$ B1, RhoE and  $\delta$ -catenin are retained at increased levels in our TAMR and/or FASR cell lines.

It is clear that an apparently substantial induction of such pro-migratory genes in MCF-7 cells does not translate out into substantial increases in invasiveness during antioestrogen response. Intriguingly, however, our emerging data indicate the full impact of these antioestrogen-induced genes may be manifested under conditions of poor cell-cell contact. MCF-7 cells have inherently good cell-cell contacts maintained by functional E-cadherin, in keeping with their low invasiveness (Hiscox *et al.* 2007). However, neutralisation of E-cadherin-mediated intercellular adhesion (for example, by exposure to E-cadherin antibody; Hiscox *et al.* 2007), which in itself affords only a small increase in invasiveness, dramatically enhances (up to 40-fold) the ability of antioestrogens to induce invasive behaviour of MCF-7 cells. This effect has now been reproduced across several replicate experiments in our laboratory. Our data indicate that this early antioestrogen-promoted aggressive behaviour is independent of EGFR signalling, suggesting the need to evaluate the role of alternative signalling pathways and, in particular, the antihormone-induced genes linked with an aggressive ontology that have arisen from our array studies (Shaw *et al.* 2005, Hiscox *et al.* 2007). Clearly, while antioestrogens confer only small increases in invasiveness under conditions of good cell-cell contact, this may become substantial in an appropriate context/environment where cell-cell contact is compromised. These observations may have major implications when using antioestrogens in ER+ tumours with inherently poor cell-cell contacts and aberrant E-cadherin. For some of these patients, despite potentially substantial growth inhibitory responses as a consequence of ER blockade, antioestrogen-induced aggressive behaviour of any surviving cells could nevertheless translate out into poorer prognosis. Interestingly, although patient

cohorts are small and reports few, survival benefits of tamoxifen have been reported by some groups as inconclusive in lobular cancers, which are ER+ but exhibit E-cadherin loss and disrupted cell attachments (Jirstrom *et al.* 2005).

#### Discussion of implications for future therapy

Our *in vitro* signalling/microarray studies are revealing an increasing number of oestrogen-repressed, anti-hormone-induced genes (including several tyrosine kinases) whose ontology implies a positive contribution to cell survival/proliferation and as such may act to limit maximal anti-tumour response to antihormones and maintain the cellular cohort from which resistance subsequently emerges. Of course, full profile verification and pharmacological/molecular manipulation of these genes in our models remains essential to definitively prove they promote undesirable events with antihormones. It will also be important to address to what degree the various induced events are interlinked, in particular whether they lie within the EGFR pathway or are independent of this induced mechanism and thus likely to provide independent therapeutic targets. Nevertheless, based on our proof of principal studies with EGFR, NFκB and also CD59, we believe that future targeting of such genes together with ER blockade could prove extremely fruitful in improving the anti-tumour (notably cell kill) properties of antihormones.

Since EGFR increased during antioestrogen challenge, we targeted this pathway in the presence of tamoxifen in MCF-7 cells using gefitinib. Co-treatment blocked antihormone-induced EGFR signalling (Gee *et al.* 2003, Nicholson *et al.* 2005), abrogating activity not only of EGFR but also of its dominant heterodimer partner HER2, fully depleting downstream residual MAPK, AKT and ER activity, and eliminating *bcl-2* survival gene expression. In parallel, this strategy was superior in promoting cell death (30% induction) and inhibiting proliferation (75% fall) versus tamoxifen alone, culminating in a markedly improved anti-tumour effect and very substantial delay, and in some instances, prevention of emergence of resistance *in vitro*. These data not only confirm the key cell survival role for EGFR signalling during tamoxifen challenge, but also clearly demonstrate the considerable therapeutic potential of such a combination strategy to improve quality and duration of response in ER+ disease. Our studies have shown that this concept is highly reproducible. It also extends to other ER+ breast cancer models, to combination treatment with faslodex plus gefitinib (McClelland *et al.* 2001,

Gee *et al.* 2003), indicating the EGFR survival/resistance mechanism and hence co-targeting potential is equally applicable to non-steroidal and steroidal antioestrogens, and to combined treatment with antihormone plus a MAP kinase kinase 1 (MEK1) inhibitor to block the EGFR-driven residual kinase signalling. Supportive *in vivo* model data have also been described for various anti-EGFR plus antihormone strategies (Shou *et al.* 2004). In total, these data indicate that targeting EGFR-signalling elements together with antihormones should now be examined as a matter of priority in antihormone-responsive breast cancer patients, and excitingly several such combination strategies are presently under clinical evaluation (Johnston 2006). Our studies suggest co-treatment should be of considerable value in the context of antioestrogens. However, it is possible that combination treatment of anti-EGFR strategies with oestrogen deprivation may not prove so valuable since there was only a small induction of EGFR with this strategy, and our growth studies have not to date shown substantial anti-tumour effect of this particular co-treatment in MCF-7 cells. To our knowledge, preclinical studies showing improved activity of oestrogen deprivation when combined with EGFR blockade have only been described in ER+ tumour cells overexpressing erbB receptors *de novo* (e.g. BT474 or MCF-7/HER2; Shou *et al.* 2004), observations recently paralleled by inhibitory effects of aromatase inhibitor plus gefitinib in the ER+/EGFR+ clinical setting (Polychronis *et al.* 2005).

There is likely to be value in the targeting of further induced genes revealed by our array studies, in combination with antihormones. An amenable avenue may be to target the activity of the various induced TKs. In this regard, there is clinical interest in evaluating combined treatment with inhibitory agents of HER2 (or of its dimerisation) with diverse antihormones based on preclinical data (Johnston 2006). Inhibitors able to target multiple members of the Src family (e.g. dasatinib), including Lyn (Nam *et al.* 2005), are also being explored in various cancer types. Moreover, among the various antihormone-induced genes revealed by our nylon array studies, we have recently obtained encouraging combination therapy data for CD59 and NFκB. Excitingly, we have been able to show that complement-mediated lysis in MCF-7 cells in the presence of antioestrogens can be restored by co-treating with a CD59-neutralising antibody (Gee *et al.* 2004, Shaw *et al.* 2005). These data suggest such combination therapy to enhance anti-tumour response to antioestrogens should be explored *in vivo* if feasible. With regards to NFκB, we have

noted that the sesquiterpene lactone parthenolide, a relatively specific I $\kappa$ B kinase inhibitor, is able to substantially deplete activity of this transcription factor that is induced by faslodex in MCF-7 cells (Gee *et al.* 2004, Shaw *et al.* 2005). In parallel, parthenolide plus faslodex combination treatment substantially improved growth inhibitory effects of the antihormone in triplicate experiments (Gee *et al.* 2004, Shaw *et al.* 2005). Faslodex-induced NF $\kappa$ B transcriptional activity was also partially inhibited by co-treatment with gefitinib, indicating that this transcription factor contributes to the antihormone-induced EGFR-signalling pathway, where a link between NF $\kappa$ B and EGFR has also been proposed in ER cells (Biswas *et al.* 2000). Importantly, however, there was an additional EGFR-independent NF $\kappa$ B contribution, and as such NF $\kappa$ B signalling targeting alongside EGFR may provide a powerful approach to improve antihormone response. Since NF $\kappa$ B is induced by both antioestrogens and oestrogen deprivation, such targeting may prove valuable in combination with diverse approaches for ER blockade.

Inhibiting these new gene targets, where their increased expression is retained, may also prove valuable in established resistance. As proof of principle, treatment with gefitinib or trastuzumab confirms the key role for further increased EGFR/HER2 signalling on acquisition of antioestrogen resistance in our TAMR and FASR cell lines (McClelland *et al.* 2001, Knowlden *et al.* 2003), as well as in additional resistant models such as MCF-7/HER2 (Shou *et al.* 2004). The value of anti-erbB therapies in clinical breast cancer is presently being investigated in various antihormone-resistant states (Johnston 2006). A further example is NF $\kappa$ B, where our FASR cells, which exhibit elevated activity of this transcription factor, can be markedly inhibited by parthenolide, observations supported by studies in the LCC9 ER + FasR line (Riggins *et al.* 2005). NF $\kappa$ B blockade is also growth inhibitory and restores tamoxifen response in ER + MCF-7/HER2, BT474 (Zhou *et al.* 2005) and cells constitutively overexpressing AKT (deGraffenried *et al.* 2004), as well as ER – models (Biswas *et al.* 2000). Since there is already clinical interest in targeting of NF $\kappa$ B signalling in cancer (e.g. using proteasomal inhibitors such as bortezomib (Zhou *et al.* 2005) to subvert NF $\kappa$ B nuclear translocation), we propose such studies should consider both co-treatment with antihormones and monotherapy in diverse antihormone resistant states.

Finally, since under certain cell contexts antioestrogens can encourage aggressive behaviour in residual cells, it is critical that combination therapies should

aim to bring about maximal cell kill to subvert progression. Alongside future deciphering and targeting of the genes underlying antioestrogen-promoted invasive behaviour, embracing of intelligent combination strategies may significantly extend breast cancer patient survival.

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