Growth factor-driven mechanisms associated with resistance to estrogen deprivation in breast cancer: new opportunities for therapy

R I Nicholson, C Staka, F Boyns, I R Hutcheson and J M W Gee

Tenovus Centre for Cancer Research, Welsh School of Pharmacy, Cardiff University, Cardiff, UK

(Requests for offprints should be addressed to R I Nicholson; nicholsonri@cardiff.ac.uk)

Abstract

There is an increasing body of evidence demonstrating that elevated growth signaling in breast cancer cells can promote forms of endocrine resistance in either an estrogen receptor-dependent or -independent manner. The current article reviews what is known about such growth factor signaling networks and resistance to estrogen withdrawal and considers the many novel therapeutic opportunities that stem from this knowledge.

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Introduction

Within the last 5 years, clinical interest in estrogen deprivation strategies for the therapy of breast cancer has gained considerable momentum. Estrogen deprivation is achieved clinically through the use of ovarian ablation or suppression (e.g. luteinizing hormone-releasing hormone superagonists) in premenopausal women, and through aromatase inhibitors in postmenopausal women (Johnston & Dowsett 2003, Pritchard 2003). The 'third generation aromatase inhibitors' (AIs), exemplified by the non-steroidal competitive inhibitors of aromatase, anastrozole and letrozole, and the steroidal aromatase inactivator, exemestane, are potent and highly selective in their inhibition of the cytochrome P450 enzyme, aromatase. As a consequence, they efficiently block conversion of androgens (testosterone and androstenedione) to estrogens (estradiol and estrone respectively) in peripheral tissues and breast tumors (Santen 2003). Their induction of profound estrogen deprivation provides an effective therapeutic second line strategy in advanced postmenopausal estrogen receptor positive (ER α +) breast cancer (Johnston & Dowsett 2003). Moreover, emerging trial data indicate that AIs may be superior to the antiestrogen tamoxifen both as first line agents in advanced ER α +

patients and in the adjuvant setting (reviewed by Santen 2003). Thus, letrozole, anastrozole and exemestane have all achieved an increased tumor response rate in advanced disease, while in the ongoing ATAC (Anastrozole, Tamoxifen, Alone or in Combination) trial in early breast cancer, a significant improvement in 3-year disease-free survival with a reduced incidence of contralateral invasive breast cancers has been reported for anastrozole compared with tamoxifen (Baum *et al.* 2003, Wong & Ellis 2004). Clearly, longer follow-up for AI adjuvant trials such as ATAC remains essential if we are to judiciously assess whether these agents could be of routine value in the postmenopausal adjuvant setting (Winer *et al.* 2003). However, the clinical data are encouraging to date and there is hope that AIs may, in the future, replace tamoxifen as the 'gold standard' therapy for ER α +

disease and perhaps further improve breast cancer survival rates (Santen 2003). Our emerging knowledge of the molecular biology of ER α signaling sheds light on these promising observations. While the AF-2 activity of ER α is silenced, it is notable that weak AF-1-mediated agonistic effects are retained following tamoxifen binding to ER α . In contrast, under conditions of near-complete estrogen deprivation, it is predicted that ER α activity and hence ER α -mediated transcription would be fully abrogated (Johnston & Dowsett 2003).

Clearly, exciting advances in the endocrine therapy of breast cancer continue to be made. Importantly, however, as promising as the above prospects are, it is already

evident that a switch to estrogen deprivation as the preferred endocrine strategy will not provide the whole answer to effective treatment of breast cancer. Thus, to date, improvement in relapse-free survival in the adjuvant setting observed with AIs versus tamoxifen remains modest. Moreover, not all patients who are treated with AIs respond equally well, while relapse rates in advanced disease are still unfortunately often measured in months rather than years (Pritchard 2003). So where should we look for the next advance in the therapy of breast cancer? Clearly, accurate identification of the causative elements for primary (*de novo*) and acquired endocrine resistance is essential if we are to develop new therapeutic approaches to delay, prevent or potentially even reverse these undesirable conditions (Nicholson *et al.* 2003). The biology of endocrine resistance is highly complex and poorly understood. As such, diverse mechanisms have been implicated that are at various stages of experimentation and exploitation. Several groups have focussed on whether alterations occurring in the structure/function of ER α contribute to resistance. For example, studies by Fuqua *et al.* (2000) demonstrate that ER α mutation (Lys303Arg) enables increased co-activator recruitment to the receptor, conferring hypersensitivity in the presence of reduced estrogens. Moreover, in some model systems, aromatase is increased in response to estrogen deprivation in parallel with acquisition of estrogen hypersensitivity, while aromatase-transfected MCF-7 cells are also hypersensitive (Yue *et al.* 2001, 2003). However, the role of such events in invasive breast cancer and clinical endocrine resistance remains obscure (Karnick *et al.* 1994, de Jong *et al.* 2003). In contrast, there is considerable evidence that growth factor signaling pathways should be of high priority in our quest for new therapeutic targets in endocrine resistance (Nicholson *et al.* 2003). Thus, the current article reviews the growth factor signaling-driven mechanisms that have been implicated to date in resistance to estrogen withdrawal primarily from breast cancer model systems. Where possible, parallels will be drawn with resistance to antiestrogens. Based on these concepts, the article will suggest how we might in the future effectively and rationally target growth factor signaling pathways to enhance response to estrogen deprivation and improve breast cancer survival.

Growth factor signaling associates with resistance to endocrine therapies, including estrogen deprivation

Developing breast cancer cells are bathed in a rich milieu of peptide growth factors that are produced locally or distally. Additionally, many neoplastic cells are able to produce growth factors by autocrine means and frequently express

several classes of growth factor receptors and their downstream signaling elements (Salomon *et al.* 1995, Nicholson *et al.* 2001b, Sachdev & Yee 2001, Gross & Yee 2003). Cancer cell responses to growth factors include enhancement of proliferation, cell survival, motility, invasiveness and angiogenesis (Wells 1999, Ciardiello & Tortora 2001, Sachdev & Yee 2001, Gross & Yee 2003). Thus, it is not surprising that inappropriate activation of growth factor signaling cascades, either through the enhanced supply of growth factor ligands or via up-regulation/increased activation of their target receptors or their recruited downstream signaling elements can associate with aggressive tumor biology and poor patient prognosis (Ciardiello & Tortora 2001, Nicholson *et al.* 2001a, 2004). Moreover, there is now considerable evidence that increased signaling through such pathways promotes *in vitro/in vivo* resistance to various cytotoxics (Dickstein *et al.* 1995, Gooch *et al.* 1999, Chen *et al.* 2000), radiotherapy (Dunn *et al.* 1997, Akimoto *et al.* 1999, Gee & Nicholson 2003), and notably endocrine strategies in breast cancer cells (Nicholson *et al.* 1994, 2004).

As an example, increased epidermal growth factor (EGF) receptor (EGFR) signaling is a relatively common feature in established cell models that have become refractory to antiestrogens (Vickers *et al.* 1988, Long *et al.* 1992, van Agthoven *et al.* 1994, van den Berg *et al.* 1996, McClelland *et al.* 2001, Knowlden *et al.* 2003b). Thus, our established MCF-7, and more recently T47D, human breast cancer sub-lines that have acquired resistance to tamoxifen or fulvestrant *in vitro* clearly demonstrate dominance of EGFR/HER2 signaling, with increased activation of the downstream growth factor signaling elements erk1/2 MAP kinase and Akt that are pivotal in driving antiestrogen resistant growth (McClelland *et al.* 2001, Knowlden *et al.* 2003b, Jordan *et al.* 2004). Extensive gene transfer studies further highlight the considerable potential for increased expression/activation of growth factor pathway elements to perturb endocrine response, notably to estrogen deprivation. These studies include overexpression of the growth factors heregulin- β 2 (Tang *et al.* 1996), fibroblast growth factor (FGF)-4 (McLeskey *et al.* 1993), insulin-like growth factor (IGF)-II (Daly *et al.* 1991, Abdul-Wahab *et al.* 1999) and transforming growth factor (TGF β)-1 (Arteaga *et al.* 1993), and also growth factor receptors such as EGFR and IGF-1R (Van Agthoven *et al.* 1992, Miller *et al.* 1994, Guvakova & Surmacz 1997, Abdul-Wahab *et al.* 1999). The receptor HER2 also appears to contribute to resistance either directly when over-expressed (Benz *et al.* 1993, Liu *et al.* 1995, Pietras *et al.* 1995) or indirectly as the preferred heterodimerization partner of liganded erbB receptor family members (Knowlden *et al.* 2003b). Furthermore, gene transfer of constitutively active growth factor signaling enzymes

including Ras family members (Yu & Feig 2002), Raf-1 (El-Ashry *et al.* 1997), MAP kinase (Donovan *et al.* 2001, Oh *et al.* 2001), Akt (Campbell *et al.* 2001, Kurokawa & Arteaga 2003), and protein kinase (PK) C (Tonetti *et al.* 2000) have all been associated with resistance, as has aberrant expression of nuclear transcription factors such as AP-1 (Smith *et al.* 1999).

Several mechanisms appear able to contribute to the increases in growth factor signaling in breast cancer cells that can promote endocrine resistance. These include genetic alterations in their key components (gene amplification, mutation etc.), best illustrated by HER2, where gene amplification is a feature of 30% breast cancers *de novo* (Tsuda 2001). However, adaptive events can also contribute, known to be instigated by diverse therapeutic strategies. Since growth factor pathways have been demonstrated to influence, and be influenced by, ER α signaling (Nicholson & Gee 2000, Nicholson *et al.* 2001b, 2003, 2004, Hutcheson *et al.* 2003, Johnston *et al.* 2003), it is perhaps not surprising that adaptive events in growth factor pathways also occur when breast cancer cells are subjected to therapies that suppress ER α signaling. Thus, *in vitro* data have demonstrated that upregulation of EGFR (Chrysogelos *et al.* 1994, de Fazio *et al.* 1997, Yarden *et al.* 2001, Wilson & Chrysogelos 2002, Gee *et al.* 2003) and HER2 (Russell & Hung 1992, Bates & Hurst 1997) occurs in several ER α + endocrine responsive breast cancer cells when they are subjected to estrogen deprivation, while in clinical disease increases in HER2 have been detected in serum during ovarian ablation (Luftner *et al.* 2003). A particularly common adaptive feature during response to long-term estrogen deprivation of *in vitro* and *in vivo* models appears to be increased MAP kinase activation (Coutts & Murphy 1998, Jeng *et al.* 2000, Martin *et al.* 2003, Santen *et al.* 2004). Parallel adaptive data have been described for antiestrogens, again exemplified by our MCF-7 cell studies demonstrating that the initial response to tamoxifen (or fulvestrant) is associated with de-repression of the inhibitory effect of estrogen exposure on EGFR and HER2, with resultant increases in MAP kinase and also Akt signaling occurring during treatment (McClelland *et al.* 2001, Gee *et al.* 2003). Our extensive observations of these cells have clearly demonstrated that these adaptive events facilitate cell survival in the presence of anti-hormones (explaining incomplete initial anti-tumor activity) and ultimately promote proliferation and hence development of resistance (Gee *et al.* 2003, Nicholson *et al.* 2004). Similarly, emerging clinical studies are confirming up-regulation of various growth factor signaling elements, including HER2, that are apparent at the time of tamoxifen relapse (Gee *et al.* 1999, 2002, Johnston *et al.* 1999, Dowsett *et al.* 2003).

Growth factor signaling clearly has considerable potential to promote endocrine resistance. In the following sections, data will be presented demonstrating that altered growth factor signaling (either apparent *de novo*, or adapting during estrogen depletion) can contribute to development and growth of phenotypes resistant to estrogen deprivation. This appears to occur by a number of mechanisms that are also likely to be relevant when considering resistance to antiestrogens. The first of these resistance mechanisms is ER α -dependent, where increased growth factor signaling elements act to hypersensitize breast cancer cells to residual estrogens (and in the case of challenge with the anti-estrogen tamoxifen, enhance its agonistic capability). The second mechanism, proposed by our own studies, indicates again that growth factor signaling elements can promote resistance to estrogen deprivation in an ER α -dependent manner, but not by obviously increasing sensitivity to residual estrogens. Subsequent responses to anti-hormonal measures that further deplete ER α signaling (e.g. fulvestrant) are observed in both these forms of resistance, since interplay between ER α and growth factor signaling elements is a unifying feature. Thirdly, however, an ER α -independent mechanism can be employed where growth factor signaling promotes proliferation in the presence of endocrine agents under conditions of markedly reduced ER α expression and/or signaling. Indeed, at its extremes, there is tantalizing evidence to suggest that growth factor signaling may even allow progression to an ER α negative (ER α -) phenotype.

Increased growth factor signaling elements contribute to adaptive hypersensitivity in breast cancer cells by interplay with ER α

Adaptive estrogen hypersensitivity during estrogen deprivation

Estrogen deprivation of various endocrine responsive breast cancer cell models (including MCF-7 and T47D) *in vitro* and *in vivo* is associated with an initial inhibitory response. However, studies from several laboratories have demonstrated that estrogen deprivation in the long-term is associated with development of an estrogen hypersensitive state, whereby the exquisitely low (non-physiological) amounts of estrogens remaining after estrogen deprivation appear sufficient to allow development and maintenance of tumor cell growth. Indeed, in one instance (Santen *et al.* 2004), dose-response data show that a 4-log lower concentration of estradiol (10^{-14} M) is able to stimulate the growth of the resistant cells in comparison with the parental MCF-7 cell line. Furthermore, since the estrogen hypersensitivity dose-response curve is bell-shaped,

physiological levels of estradiol (10^{-10} M) are growth inhibitory (an event mediated through estradiol-induced Fas/FasL and hence apoptosis; Fernandez *et al.* 1998, Song *et al.* 2001, Martin *et al.* 2003, Song & Santen 2003, Santen *et al.* 2004). Clinical observations imply that an adaptive shift in estrogen sensitivity also occurs in breast cancer patients when estrogen/ER α signaling is compromised and that this is important in the emergence of resistance. Thus, endocrine therapies that systematically reduce estrogen signaling can be sequentially effective in breast cancer patients after resistance to initial anti-hormonal challenge develops (i.e. 'manipulating the estrostat'; Lonning 2001).

Based on various model system data, overlapping explanations involving the predominant interplay between growth factor elements and non-genomic or genomic ER α signaling pathways have been proposed for the phenomenon of adaptive estrogen hypersensitivity that is associated with acquisition of resistance to estrogen deprivation. Unifying features of these hypersensitivity mechanisms are increased ER α and enhanced activation of MAP kinase and phosphatidylinositol-3-OH kinase (PI3K) signaling, phenotypic changes that appear to very commonly arise during prolonged estrogen deprivation. Indeed, such elements appear critical since, as will be described below, all these models show second-line responses to agents depleting such signaling (i.e. the selective ER α down-regulator, fulvestrant, and signal transduction inhibitors respectively).

Dominant 'non-genomic' ER α /growth factor signaling mechanism

An MCF-7 model of long-term estrogen deprivation (LTED) has been derived by Richard Santen's group by use of phenol red-free medium containing charcoal-stripped serum, where the estradiol concentration is reduced to 10^{-13} M. Following a growth inhibitory phase of 3–6 months, LTED cells acquired resistance and resumed substantial proliferation (Santen *et al.* 2004). Such cells are markedly hypersensitive when challenged with estradiol, as described above, and *in vivo* xenograft model system data from the group parallel these *in vitro* findings (Masamura *et al.* 1995). In these cells that have adapted to grow in the presence of residual estrogens, Santen has placed emphasis on increased utilization of 'nongenomic' cell membrane ER α . His group has shown that ER α translocated to the plasma membrane is markedly elevated in LTED cells in comparison with parental MCF-7 cells. On binding of estrogen, this membrane ER α is able rapidly to recruit classical growth factor signal transduction (Santen *et al.* 2004). The estrogen-primed membrane ER α is thus able efficiently to associate with, and phosphorylate, the adaptor protein

Shc in LTED (Santen *et al.* 2004). It is notable that Shc is a key modulator of several tyrosine kinase receptors including EGFR and IGF-1R (Pelicci *et al.* 1995), and such growth factor receptors may contribute to the increased translocation of ER α to the membrane and participate in its coupling to Shc on estrogen binding. Of note in the LTED cells is IGF-1R, where Santen and colleagues have shown that both Shc and ER α co-immunoprecipitate using an anti-IGF-1R antibody, and that binding of ER α to the IGF-1R occurs within minutes of the addition of estradiol (Santen *et al.* 2004, Song *et al.* 2004). Shc subsequently binds to the adaptor proteins Grb2 and Sos and rapidly activates the Ras/Raf/Mek/MAP kinase signaling cascade (Santen *et al.* 2001, 2003 2004, Song *et al.* 2002a,b). Estradiol was thus shown to stimulate Shc and marked increase MAP kinase phosphorylation in a time- and dose-dependent fashion in LTED. These events were blocked by the ER α down-regulator, fulvestrant, indicating that ER α is a key upstream component of such signaling rather than MAP kinase activation being derived from constitutive, ER α -independent growth factor receptor activity/growth factor secretion in LTED cells (Santen *et al.* 2001, 2003, 2004, Song *et al.* 2002a). Certainly there is no evidence of constitutive TGF α over-expression in LTED cells from several studies (Herman & Katzenellenbogen 1994, Jeng *et al.* 1998, 2000, Santen *et al.* 2001, 2004).

Similarly, the blockade of MAP kinase activation in LTED by PP-2, a specific c-Src kinase inhibitor, indicates that c-Src kinase is also an important regulator of such signaling (Song *et al.* 2002b, Santen *et al.* 2004). The pivotal role for increased activation of MAP kinase in determining the estrogen hypersensitive growth adopted by LTED has been confirmed by abrogation of their basal proliferation and hypersensitive responses to estrogen *in vivo* using the MAP kinase inhibitors, PD98059 or U0126 (Shim *et al.* 2000, Yue *et al.* 2002, Santen *et al.* 2004). As further proof, the addition of TGF α to parental MCF-7 cells *in vitro* in order to augment their MAP kinase activity promoted a 2-log increase in sensitivity to growth stimulation by estradiol (Yue *et al.* 2002, Santen *et al.* 2004). Associated growth end-points of the elevated non-genomic ER α /MAP kinase signaling pathway identified to date in LTED include phosphorylation of ELK-1, a regulator of proliferative activity, and cell membrane changes (Song *et al.* 2002a,b, Santen *et al.* 2003, 2004).

Interestingly, however, MAP kinase does not appear to be the only intracellular signaling pathway central to estrogen hypersensitive growth of LTED, since it was observed that MAP kinase inhibitors were unable fully to block this event. Hypersensitive cells *in vitro* also exhibited enhanced activation of Akt, p70 S6 kinase and eukaryotic initiation factor-4E binding protein (4E-BP1),

key effectors of the PI3K pathway (Yue *et al.* 2003, Santen *et al.* 2004). In other cell systems, estrogen has been shown to be capable of activating PI3K in a non-genomic manner, triggering association between ER α , c-Src and the p85 PI3K subunit, with this event converging on cell cycle progression (Castoria *et al.* 2001). In LTED, dual-inhibition of PI3K with LY294002 and MAP kinase with U0126 was able completely to reverse estrogen hypersensitivity (Yue *et al.* 2003, Santen *et al.* 2004). Thus, it appears that co-ordinated activity of PI3K and MAP kinase promotes adaptive hypersensitivity to estrogen deprivation and hence allows LTED growth. Interestingly, changes in MAP kinase and Akt have been associated with endocrine resistance and poorer prognosis in breast cancer samples from patients, suggesting clinical relevance of these signaling elements (Gee *et al.* 2001, Perez-Tenorio *et al.* 2002).

While rates of ER α -mediated gene transcription are certainly enhanced basally in LTED, global hypersensitivity to estradiol at the level of classically estrogen-regulated genes is not apparent (Jeng *et al.* 1998). Furthermore, MAP kinase inhibition is ineffective on such genes in LTED, an observation discordant with the growth inhibitory effects of this agent (Jeng *et al.* 2000). Thus, Santen's data suggest that it is an enhanced non-genomic estrogen/ER α mechanism that drives elevated MAP kinase/Akt signaling and thereby promotes growth in the presence of very low estrogen levels (Santen *et al.* 2004), rather than a predominant genomic pathway, where estrogens would classically interact with nuclear ER α to elevate gene transcription (for example, expression of growth factor ligands such as TGF α that could subsequently prime MAP kinase/Akt (Hutcheson *et al.* 2003)). However, additional studies from Santen's group suggest that some degree of undefined, synergistic interaction between non-genomic and genomic pathways does occur at the level of cell cycle regulators, such as E2F1, in the promotion of LTED growth (Yue *et al.* 2002, Santen *et al.* 2003, 2004).

Dominant 'genomic' ER α /growth factor signaling mechanism

By way of contrast, Johnston and Dowsett (2003) have suggested that enhanced ER α function and the estrogen hypersensitive state arises primarily as a result of increased cross-talk between various growth factor pathways at the level of nuclear ER α in long-term estrogen-deprived breast cancer cells. In their model, estrogen-deprived breast cancer cells initially pass through a stage of estrogen hypersensitivity (LTED-H; hypersensitivity profile similar to that described by Santen and colleagues). In accordance with many models (Katzenellenbogen *et al.* 1987, Welshons & Jordan 1987, Fernandez *et al.* 1998,

Santen *et al.* 2004), these cells again have elevated nuclear ER α . Interestingly, the ER α in LTED-H is highly phosphorylated on Ser118 residue in its AF-1 domain in the absence of estrogen, suggesting a key contribution to hypersensitive growth (Chan *et al.* 2002).

Surprisingly, however, after 80 weeks of culture under estrogen-deprived conditions, ER α appears to function independently of exogenous estradiol (LTED-I). This 'independent' state has similarly been reported in other prolonged estrogen deprivation studies (Darbre & Daly 1990, Daly *et al.* 1990). Dowsett's group have hypothesized that this phase of growth on long-term withdrawal has actually become 'super-sensitive' to residual estrogens (Chan *et al.* 2002, Martin *et al.* 2003). Critically, the steroid hormone-depleted culture conditions incorporated insulin into the growth medium for the development and maintenance of LTED-I. The group reported that exclusion of insulin resulted in an approximately 50% drop in basal growth rate of LTED-I cells and restoration of estrogen hypersensitivity (Martin *et al.* 2003). These data led the group to suggest that the supersensitive LTED-I cells are growing maximally in the insulin-supported media and therefore that exogenous estrogens can have no further growth-promoting action, explaining the apparent LTED-I state. Characterization of the LTED-I cells has revealed that they express elevated ER α with increased phosphorylation on Ser118. They also exhibit increased transcriptional activity at estrogen response elements (EREs) that are hypersensitive to estrogen stimulation, and their growth is inhibited by fulvestrant. Clearly in this model, therefore, cross-talk between ER α signaling pathways and insulin at the level of nuclear ER α /ERE-mediated transcription is likely to be pivotal to the adaptation of the cells to estrogen deprivation. Although the mechanics of the cross-talk leading to estrogen hypersensitivity and super-sensitivity are undoubtedly complex, comprehensive studies from the group have demonstrated, in addition to elevated ER α , a key involvement for increased IGF-1R and HER2 signaling in LTED-I. These promote activation of MAP kinase, ER α (and ER α co-activator) phosphorylation, ERE transcriptional regulation, LTED-I cell growth and supersensitivity to residual estrogens. Increases in IGF-1R have also been implicated in adaptation to long-term estrogen deprivation by Stephen *et al.* (2001), with some growth inhibitory effect of IGF-1R blockade using the IGF-1R specific antibody α IR3 noted in their model. Increases in HER2 signaling have also been noted on development of acquired resistance to estrogen deprivation in HER-2-transfected MCF-7 cells (Massarweh *et al.* 2003). Of note, the increased activation of MAP kinase in LTED-I cells was not triggered in a rapid manner by

estrogen, indicating that non-genomic ER α signaling is not a dominant contributor (Martin *et al.* 2003).

Interestingly, Dowsett's group showed that inhibition of MAP kinase activation only partially reduced basal ERE transcriptional activity and was ineffective in blocking Ser118 ER α phosphorylation. These data imply not only an involvement for additional interactive signaling elements in LTED-I, but also that enhancement of ER α transcriptional activity occurs via additional ER α phosphorylation sites (e.g. Ser167; Campbell *et al.* 2001) or perhaps via augmented co-activator activity (e.g. AIB1; Font de Mora & Brown 2000). In this regard, p90^{RSK} and PI3K signaling were shown to be additional contributory elements, data in total suggesting that a network of kinases is recruited on estrogen withdrawal to enhance growth sensitivity to residual estrogens via improvement of ER α transcriptional activation.

Adaptive hypersensitivity to the agonistic effects of tamoxifen and estrogen during antiestrogen treatment

The process of adaptive hypersensitivity, involving increases in growth factor signaling and its cross-talk with ER α , could feasibly be initiated not only by estrogen deprivation strategies, but also by interference with the estrogen/ER α response pathway using antiestrogens. This would be manifested by enhanced growth sensitivity not only to estrogens, but also to the agonistic effects of selective ER modulators (SERMs) such as tamoxifen, thereby allowing the emergence of tamoxifen-resistant growth. In agreement with this concept, MCF-7 breast cancer xenografts, while initially inhibited by tamoxifen, are growth-stimulated by this antiestrogen in the long-term and are also estrogen hypersensitive (Gottardis & Jordan *et al.* 1988, Osborne *et al.* 1991). Furthermore, in several acquired tamoxifen-stimulated xenograft MCF-7 and T47D models, very prolonged tamoxifen exposure is associated not only with tamoxifen stimulation but also tumoricidal effects of estrogen, a feature described above with excessive estradiol exposure of long-term estrogen-deprived cells (Jordan *et al.* 2003). Growth-enhancing effects of tamoxifen appear also to be a feature of some breast cancers clinically, as evidenced by antiestrogen withdrawal responses reported in ~20% of patients (Clarke *et al.* 2003). Moreover, enhanced tumor sensitivity to tamoxifen agonism may explain the initial superiority of AIs in the clinic, since these agents more effectively eliminate estrogens while exerting no agonistic effects (Mouridsen *et al.* 2001). A growth contribution for tamoxifen-bound ER α is similarly inferred by the frequency of second-line responses to either fulvestrant or AIs in the clinic (Howell *et al.* 1996, Buzdar *et al.* 1997).

Interestingly, the above described models for the development of adaptive estrogen hypersensitivity/super-sensitivity do show many phenotypic similarities with those generated for the study of ER α + SERM resistance. Supportive data have been derived both from *in vitro* and *in vivo* models of acquired resistance (reviewed in Nicholson *et al.* 2004), and are now beginning to emerge from clinical sample profiling at relapse (Gee *et al.* 2002, Dowsett *et al.* 2003). Schiff *et al.* (2003, 2004) provide an overview of experimental tamoxifen resistant data that are particularly complementary to the above-described mechanisms for adaptive hypersensitivity during estrogen deprivation. The group describe that both non-genomic (membrane, predominantly at the caveolar domains) and genomic (nuclear) ER α signaling influence, and are influenced by, growth factor signaling pathways resulting in enhanced agonistic activity of the tamoxifen-ER α complex in tamoxifen-resistant cells. In such cells, membrane ER α (perhaps even activated by tamoxifen; Figtree *et al.* 2000) rapidly activates cell surface tyrosine kinase receptors (such as EGFR (Razandi *et al.* 2003), HER2 (Chung *et al.* 2002) or IGF-1R (Kahlert *et al.* 2000)), leading to signaling through p21Ras/MAP kinase and PI3K/Akt. These protein kinases are able to phosphorylate nuclear ER α in its AF-1 domain, as well as phosphorylate other key components of the transcriptional machinery, notably co-activators (Bunone *et al.* 1996, Font de Mora & Brown 2000, Campbell *et al.* 2001). This results in re-activation of transcription by the tamoxifen-occupied ER α complex, increases in growth factor expression to re-enforce the signaling loop, and cell growth in the presence of antiestrogen (Schiff *et al.* 2003, 2004).

Clearly, this ER α -growth factor signaling loop could be dominant under conditions where growth factor receptors were either elevated *de novo* or were promoted during antiestrogen challenge. HER2 overexpression could thus serve to augment this signaling loop *de novo*, markedly increasing MAP kinase activation and its target coactivator AIB1 to subsequently enhance nuclear ER α signaling and exaggerate the agonistic activity of the tamoxifen-ER α complex (Font de Mora & Brown 2000, Schiff *et al.* 2003, 2004). Certainly, in *de novo* tamoxifen-resistant MCF-7/HER2 cells, tamoxifen is agonistic yet these cells remain sensitive to estrogen deprivation (Massarweh *et al.* 2003). The concept is particularly exciting since clinical observations are indicating that HER2/AIB1-co-expressing tumors do fare poorly when treated with adjuvant tamoxifen (Osborne *et al.* 2003), while preliminary adjuvant and neoadjuvant studies have revealed that HER2+ or EGFR+ patients are relatively resistant to this antiestrogen *de novo*, yet retain sensitivity to the AIs, anastrozole or letrozole (Dowsett *et al.* 2001,

Figure 1 Regrowth of fulvestrant-treated tamoxifen-resistant cells in the presence of estradiol and tamoxifen. Tamoxifen-resistant MCF-7 cells were grown for 2 days in charcoal-stripped 5% FCS containing fulvestrant (10^{-7} M). The cells were then exposed to continuing fulvestrant (182), estradiol (E2; 10^{-7} M) or tamoxifen (10^{-7} M). At the time points indicated cells were subjected to FACS analysis and the proportion of cells in Go/G1 (A) and S-phase (B) of the cell cycle was recorded.

Ellis *et al.* 2001). Adaptive changes in growth factor signaling elements acquired during therapy may also promote tamoxifen agonistic activity. Adaptive increases in EGFR/HER2 signaling are certainly promoted in endocrine responsive cells during long-term tamoxifen challenge (Gee *et al.* 2003). Moreover, study of several *in vivo* and *in vitro* models has revealed that elevated growth factor signaling is subsequently maintained in the acquired resistant phenotype, is recruited to growth and, importantly, is highly interactive with ER α (reviewed in Nicholson *et al.* 2004). HER2 overexpression and its cross-talk with ER α appears to be a common feature of acquired tamoxifen-stimulated MCF-7 and T47D xenograft models (Schafer *et al.* 2003). In parallel, Berstein *et al.* (2003, 2004) have demonstrated that long-term tamoxifen treatment of MCF-7 breast cancer cells (LTTT) in a castrated *in vivo* xenograft model is associated with early adaptive increases in MAP kinase signaling. This event promotes hypersensitivity to the agonistic effects of this anti-hormone and subsequently to estradiol, where the critical role of such signaling is evidenced by marked growth inhibitory effects of a Ras antagonist.

In our own acquired tamoxifen-resistant *in vitro* model that emerged following prolonged exposure of MCF-7 to this antiestrogen, we have observed upwards of 20-fold increases in total and activated EGFR/HER2, with a corresponding increase in phosphorylation of MAP kinase, Akt and nuclear ER α on the AF-1 residues Ser118 and Ser167 (Nicholson *et al.* 2001b, 2003, 2004, Britton *et al.* 2002, 2003, Hutcheson *et al.* 2003, Knowlden *et al.* 2003b, Jordan *et al.* 2004). Importantly, we have established that phosphorylation of nuclear ER α and

ER α transcription in these cells is under the control of EGFR, where EGF-like ligands further enhance these ER α events and exposure to the EGFR-selective tyrosine kinase inhibitor, gefitinib, antagonises basal and EGF-primed ER α phosphorylation and transcription (Britton *et al.* 2002, Hutcheson *et al.* 2003, Nicholson *et al.* 2003, 2004). This enhanced ER α activity appears to result in increased expression of the EGFR ligands TGF α and amphiregulin to generate a self-propagating autocrine growth regulatory loop in the tamoxifen-resistant cells, essentially converting a growth inhibitory drug into one that readily facilitates tumor cell growth (Hutcheson *et al.* 2003). Additionally, IGF-1R appears to contribute by activating the EGFR and thus enhancing its impact on ER α signaling. An IGF-II challenge not only leads to the expected increase in the phosphorylation of IGF-1R, but also to a reproducible secondary increase in EGFR activation (Knowlden *et al.* 2003a, Hutcheson *et al.* 2004, Nicholson *et al.* 2004). These events, together with the phosphorylation of the ER α , can be blocked by inhibitors of IGF-1R signaling. The contribution of membrane-localized ER α to such signaling (if any) is currently being assessed by our group. Importantly, however, in this acquired tamoxifen-resistant model we have again noted that adaptive changes in growth factor pathways are associated both with tamoxifen agonism and enhanced sensitivity to estradiol. As may be seen from Fig. 1, following treatment of tamoxifen-resistant cells with fulvestrant to efficiently eliminate ER α , break the autocrine growth factor/ER α signaling loop and promote arrest of cells in Go/G1 (Fig. 1A), both estradiol and tamoxifen are able to induce substantial entry into the S-phase of the cell cycle (Fig. 1B) that translates out into

growth promotion of these cells. Our preliminary data indicate that activation of Akt and increases in cyclin E are components of this escape mechanism.

In total, the above data indicate that adaptive tamoxifen hypersensitivity, in common with adaptive estrogen hypersensitivity during estrogen deprivation, can result from multiple levels of interplay between growth factor signaling cascades and membrane and/or nuclear ER α . These interactions are likely to play a key synergistic role in the resultant acquired resistant growth. Frequent second-line responses to fulvestrant in these models, as well as to appropriate anti-growth-factor strategies, confirm the pivotal role played by ER α and growth factor signaling.

Increased growth factor signaling elements can interplay with ER α on estrogen deprivation of breast cancer cells in a manner not associated with adaptive hypersensitivity

It is clear from the above studies of Santen and Dowsett that their experimental conditions of estrogen deprivation are associated with substantial changes in sensitivity of the resultant resistant cells to estrogen, where the availability of exogenous growth factors appears to impact on the underlying molecular mechanism. In order to examine this further, our group has recently developed and characterized a further *in vitro* model of resistance to estrogen withdrawal (MCF-7X) where cells are routinely grown in charcoal-stripped, heat-inactivated serum and therefore subject to growth regulation primarily by autocrine growth factor pathways and residual steroid hormone. In our new model, although rapid tumor cell growth was established after a marked inhibitory phase in MCF-7 cells of approximately 6 months and the cells could be further stimulated by estrogens, we have failed to reveal any evidence of estrogen hypersensitivity. This is despite functional, phosphorylated ER α in MCF-7X cells and obvious growth inhibition by fulvestrant. Functional ER α and fulvestrant responses have similarly been reported in the MCF-7/S9 cell line that acquired resistance to serum-free culture conditions (Jensen *et al.* 2003). In addition, MCF-7X cells have readily detectable levels of activated MAP kinase and Akt. While we have failed, to date, to demonstrate a dominant role for MAP kinase signaling in these cells, we have observed that the PI3K inhibitor, LY294002, is effective, again suggesting an importance for the latter growth factor signaling element in promotion of estrogen deprivation.

Interestingly, in MCF-7X cells we have been unable to demonstrate a dominant growth factor-driven autocrine growth regulatory pathway, with for example, the cells

being only weakly EGFR/HER2 positive and showing only extremely modest growth inhibition (~10%) with gefitinib or the humanized HER2-directed antibody, herceptin. The resistant cells are similarly only very weakly positive for IGF-1R and virtually unresponsive to IGF-1R ligands or inhibitors. We have now profiled the effects of multiple growth factors and medium growth supplements in our quest to reveal pathways that may be contributing to growth of MCF-7X cells. In this respect, the most obvious mitogenic factor that we have discovered for MCF-7X to date, other than estrogen, is the iron transporter transferrin (Li & Qian 2002). Of note, MCF-7X cells express transferrin and show markedly increased levels of its receptor. Provocatively, PI3K signaling has been shown to interact with transferrin/transferrin receptor by regulating its trafficking (Jess *et al.* 1996). Hence our studies in MCF-7X may have revealed a previously unknown role for the PI3K enzyme in its promotion of estrogen-deprived states. Excitingly, both PI3K signaling and transferrin receptor have been linked with proliferative activity and disease progression in breast cancer samples, suggesting clinical relevance (Elliott *et al.* 1993, Perez-Tenorio *et al.* 2002). Detailed study of the networking between PI3K, transferrin and ER α in MCF-7X cells and its evolution during estrogen deprivation is obviously still required from our group. However, our data clearly demonstrate that ER α -dependent adaptive resistance to estrogen withdrawal can occur by mechanisms that do not always enhance sensitivity to residual estrogens or recruit classical growth factor receptors (notably EGFR/HER2/IGF-1R), but again involves PI3K as an essential signaling element.

Increased growth factor signaling elements can contribute to resistance to estrogen deprivation and antiestrogens in the presence of reduced or absent ER α expression/signaling in breast cancer cells

Several studies have shown that increased growth factor signaling is capable of promoting proliferation of breast cancer cells under conditions of markedly reduced ER α expression and/or signaling. Thus, we have demonstrated that endocrine responsive MCF-7 cells that are significantly growth inhibited by the ER α down-regulator, fulvestrant, may be stimulated to proliferate in the presence of this agent by exogenous heregulin β 1 or IGF-I (Fig. 2A) despite their ER α being barely detectable. Under such conditions, such growth factors appear able considerably to up-regulate MAP kinase and Akt phosphorylation in a manner that is proportional to their ability to induce cell proliferation, and the cells are refractory to any endocrine manipulation (Tang *et al.* 1996, Atlas *et al.* 2003) (Fig. 2C).

Figure 2 Effect of growth factors on fulvestrant-treated breast cancer cells. Wild-type (A and C) and tamoxifen-resistant (B and D) cells were grown for 24 h in charcoal-stripped 5% FCS containing fulvestrant (Ful; 10^{-7} M). The cells were then maintained in fulvestrant in the presence or absence of the growth factors (100 ng/ml) heregulin β1 (Herβ1), insulin-like growth factor-I (IGF-1), epidermal growth factor (EGF) or transforming growth factor-α (TGFα). Cells were harvested for Western analysis of phosphorylation of AKT (p-AKT) and erk1/2 MAP kinase (p-ERK1/2) after 10 min or for cell counting after 7 days. Con, control – no growth factors.

Similarly, following inhibition of the EGFR/ERα auto-crine growth signaling loop in our acquired tamoxifen resistant sub-line using fulvestrant, re-activation of MAP kinase and Akt by exogenous heregulin β1 (Fig. 2D) appears sufficient to maintain high levels of cell growth in the presence of fulvestrant (Fig. 2B) where again the ERα protein is virtually absent and ERα/ERE activity is abrogated (Hutcheson *et al.* 2003, Nicholson *et al.* 2003, 2004). Since similar actions are produced by EGF and TGFα (Fig. 2B), extreme levels of growth factor signaling appear able to promote a ‘complete endocrine insensitive’ state.

Moreover, there is evidence that increased growth factor signaling can also act to reduce ERα expression and/or function. Growth factor dose-response and time-course studies suggest that while ERα activity and transcription can be activated as described in the section ‘Increased growth factor signaling elements contribute to

adaptive hypersensitivity in breast cancer cells by inter-play with ERα’ above, under certain extreme (as yet poorly defined) conditions blockade of these events is also a possible outcome. Since chronic ERα activation by estrogen can be associated with ERα downregulation (Pink & Jordan 1996), it is certainly feasible that constitutive/chronic activation of ERα by growth factor signaling could similarly result in a decline in ERα. Stoica and colleagues (Martin *et al.* 1995, Stoica *et al.* 1997, 2000a,b) systematically describe the capability of exogenous EGF, IGF-I, TGFβ and 12-o-tetradecanoyl phorbol-13-acetate (TPA) to down-regulate ERα mRNA and protein in MCF-7 cells via mechanisms involving increased growth factor signaling through EGFR, PI3K/Akt, phosphorylated (p) PKA and PKCs. In the case of prolonged treatment with the PKC activator TPA, ERα protein levels were reduced by 80% and there was a parallel decrease in ERα mRNA, ERα ligand binding,

Figure 3 ER expression in breast cancer cells. Wild-type MCF-7 cells (A) and short- (B) and long (C)-term fulvestrant (faslodex)-resistant cells were cultured for 7 days on coverslips. The cells were then fixed and processed for ER immunostaining.

ER α binding to DNA and ER α /ERE activity (Martin *et al.* 1995). TPA depletion of ER α appeared to be blocked by a PKC inhibitor (Saceda *et al.* 1991) and involved downstream activation of the AP-1 nuclear transcription factor complex (Doucas & Yaniv 1991). Further agents shown to depress ER α or its transcriptional activity include heregulin β 1 (Mueller *et al.* 1995, Tang *et al.* 1996) and retinoids (Demirpence *et al.* 1992).

At its extremes, sustained growth factor suppression of ER α expression/function may even promote ER α negativity. This condition appears achievable *in vitro* during extended challenge with endocrine strategies. Such cells are subject to the influences of prolonged, elevated growth factor signaling that evolves during treatment. For example, we have recently noted that our fulvestrant-resistant cells, whose growth is promoted primarily via increased EGFR/MAP kinase signaling in the presence of minimal ER α levels (McClelland *et al.* 2001), have now developed a fully ER α -phenotype following extended culture in this pure antiestrogen (Fig. 3). Interestingly, while early passages of the fulvestrant-resistant sub-line were able to regain full ER α signaling on removal of fulvestrant (McClelland *et al.* 2001), our recent studies have revealed that the long-term resistant cells are unresponsive to estrogens on antiestrogen removal and significant levels of ER α are not recovered even after several months withdrawal. Prolonged estrogen deprivation has similarly been associated with evolution of an ER α - phenotype in T47D cells (Murphy *et al.* 1989, 1990, Pink *et al.* 1996), as exemplified by the T47D:C4 sub-line that expresses TGF α and EGFR, as well as markedly elevated PKC α and AP-1 activity (Murphy *et al.* 1990, Tonetti *et al.* 2000, Chisamore *et al.* 2001).

Further supportive evidence for this transition can be drawn from several transfection studies where growth factor signaling elements introduced into ER α + breast cancer cells can act to deplete ER α and impair ER α function. In our own study, we have shown that constitutive up-regulation of MEK1 in MCF-7 cells

leads to a substantial increase in MAP kinase activation, decreased ER α levels and marked loss of progesterone receptor (PR) expression (R A McClelland, J M W Gee & R I Nicholson, unpublished observations). Similarly, El-Ashry and colleagues have noted precipitous falls in ER α mRNA and protein following transfection of constitutively active HER2, MEK (Δ mek), Raf1 (Δ raf) or ligand-stimulated EGFR into MCF-7 cells (Liu *et al.* 1995, Pietras *et al.* 1995, El-Ashry *et al.* 1997, Oh *et al.* 2001), with a parallel loss of estrogen-mediated induction of pS2 and PR and a marked suppression of activity of ERE-reporter gene constructs in transient transfection experiments that could not be overcome by estradiol treatment. Not surprisingly, the severe impact of elevated growth factor signaling on ER α expression and function also resulted in estrogen independence and antiestrogen resistance. Overexpression of Akt, PKC α and AP-1 components has similarly been linked with decreased ER α function, loss of ER α and endocrine-resistant states (Doucas & Yaniv 1991, Tzukerman *et al.* 1991, Smith *et al.* 1999).

In total, these data provide substantial evidence that while increased growth factor signaling can act to facilitate ER α function, at its extremes it may promote tumor cell growth in an apparently ER α -independent manner and may even encourage dislocation from ER α signaling and promote evolution of an ER α - phenotype. Interestingly, tumors with exaggerated EGFR signaling, whether ER α + or ER α -, can be endocrine insensitive on presentation (Nicholson *et al.* 1994, 2004, Gee *et al.* 2001, 2002), and while not examined in any depth, such a phenotype may also contribute to the significant cohort of patients who fail to respond to second-line endocrine challenge (Gee *et al.* 2002). The conditions under which this transition to ER α + complete endocrine insensitivity or ER α negativity occurs remains poorly defined; however, it is perhaps significant that our fulvestrant-resistant sub-line that shows markedly reduced ER α mRNA and ER α protein appears more susceptible to the generation

Figure 4 Model of growth factor-driven mechanisms of resistance to estrogen withdrawal. The black lines represent the experimental influences associated with increased growth factor signaling in breast cancer cells, while the red lines represent the potential reversal of such events by growth factor inhibitors.

of an ER α – phenotype than either our tamoxifen or estrogen-withdrawn variants.

Such data raise the intriguing possibility that sustained growth factor signaling may underlie clinical ER α negativity. About 30% of tumors are ER α – at diagnosis, while a proportion of ER α + tumors lack the receptor at the time of tamoxifen relapse in the adjuvant or metastatic setting. It is already established that the ER α gene is silenced via CpG island methylation or histone deacetylation in a proportion of clinical breast cancers that are ER α – on presentation (Parl 2003). Indeed, Yang *et al.* (2001) have shown that ER α negativity in the MDA-MB-231 and -435 cell lines can be partially reversed by the DNA methyl transferase 1 (DNMT1) inhibitor, aza-2-deoxycytidine (5-aza-dC) and by the histone deacetylase (HDAC) inhibitor, trichostatin (TSA), an event that restores some estrogen responsiveness from ERE reporter constructs and has obvious therapeutic implications for the future treatment of some ER α – patients. However, if the above described association between chronic growth factor signaling and ER α loss occurs clinically, it may also be feasible to manipulate growth factor signaling to reinstate the ER α + phenotype in a proportion of ER α – patients. In support of this

association, growth factor signaling elements do appear most extreme in ER α – breast cancer, as evidenced by the established inverse association between ER α and EGFR in clinical disease (Nicholson *et al.* 1994) and across multiple ER α – cell lines together with prominent ERK1/2 MAP kinase signaling (Biswas *et al.* 2000).

Therapeutic implications for the future treatment of resistant breast cancer

Many of the mechanisms that are emerging from laboratory studies, although complex, suggest that altered growth signaling cross-talking with ER α , as noted with antiestrogens, plays a central role in the development of acquired resistance to estrogen withdrawal. While detailed evaluation of these mechanisms is now clearly essential within clinical breast cancers from appropriate AI adjuvant trials and from examination of relapse material, model system data are already providing considerable rationale for designing anti-growth factor strategies, as well as improving anti-hormonal strategies, to treat or even prevent this disease state.

Given the concept of adaptive hypersensitivity that has arisen from the above models, one might expect that

tamoxifen has only limited value in acquired resistance to estrogen deprivation since this drug could be perceived as an agonist (although surprisingly, there is model system data to suggest some efficacy may still be apparent; Long *et al.* 2002). However, it is feasible that continued optimization and improvement of estrogen deprivation strategies to further deplete estrogen levels may prove a fruitful avenue (Lonning 2001, Santen *et al.* 2004). Similarly, use of alternative AIs may be relevant for the development of resistance to initial therapy if they more potently reduce estrogen levels, since a lack of complete AI cross-resistance between the competitive inhibitors and inactivators has been reported (Lonning 2001). A unifying feature of all the acquired resistant models appears to be the value of near-maximally depleting ER α protein. Hence, the use of the pure antiestrogen, fulvestrant, to abrogate ER α /growth factor cross-talk may also be rational in acquired resistance. This agent is certainly effective following patient relapse on tamoxifen (Howell *et al.* 1996, Pritchard 2003, Santen *et al.* 2004), and preliminary data from acquired letrozole-resistant xenografts generated from aromatase-transfected MCF-7 cells (MCF-7-Ca) also indicate fulvestrant sensitivity (Long *et al.* 2002). Finally, since model system data reveal toxicity of estrogens at higher concentrations, patients becoming resistant to estrogen deprivation may benefit from estrogens administered at pharmacological doses (Lonning 2001, Jordan *et al.* 2003). Clearly, further research is needed to define the optimal type and sequence of endocrine agents in this setting (Pritchard 2003).

Excitingly, the above described model system and emerging clinical data also provide considerable impetus to consider anti-growth-factor therapies on the development of resistance to estrogen deprivation or indeed other endocrine strategies. Although it is currently unclear whether the transitions shown in Fig. 4 are linked or distinct, and what the determinants are for their establishment, it is certainly encouraging that our armoury of inhibitors for growth factor signaling elements is exponentially increasing and that many do appear to be tolerated in cancer patients (Johnston *et al.* 2003, Jones *et al.* 2004). In the future, therefore, it is highly probable that such inhibitors will be clinically examined in breast cancer as a novel means of (i) treating endocrine resistant and insensitive states, (ii) preventing/delaying emergence of endocrine resistance and (iii) regenerating endocrine responsiveness in *de novo*-resistant or acquired-resistant disease.

The successful application of several signal transduction inhibitors in the Santen and Dowsett adaptive hypersensitivity models and parallel acquired antiestrogen-resistant sub-lines are highly encouraging with regards to possible therapeutic strategies. Targeting growth factor

receptor expression/activation (e.g. EGFR, IGF-1R, HER2), elements interactive with ER α (e.g. c-src kinase), or key downstream signaling elements (e.g. PI3K/AKT; Ras/Raf/MAP kinase) can clearly be growth inhibitory in these acquired-resistant models. Our own group has shown that blockade of EGFR signaling in tamoxifen- or fulvestrant-resistant cells with gefitinib inhibits cell growth, motility and invasiveness, with responses lasting up to 6 months before gefitinib resistance emerges (McClelland *et al.* 2001, Knowlden *et al.* 2003b, Nicholson *et al.* 2003, 2004). This compares favorably with the time taken to develop antiestrogen resistance *in vitro* (~3 months; Knowlden *et al.* 2003b). Within our new ER α + dependent model of resistance to estrogen deprivation, sensitivity to PI3K blockade is still apparent, despite the cells being largely refractory to EGFR, HER2 or IGF-1R inhibition. Furthermore, given that multiple signaling elements are clearly synergistic in determining adaptive resistance to estrogen deprivation in several of the models, a particularly exciting approach may be to logically combine anti-growth factor strategies (e.g. PI3K/Akt and MAP kinase inhibitor combination). Alternatively, 'dirty' agents that hit multiple signaling pathways, such as the Hsp90 inhibitor, 17-allylamino-17-demethoxygeldanamycin (17AAG), known to promote degradation of multiple client proteins including tyrosine kinase receptors, signaling intermediates and steroid receptors and already demonstrated to be inhibitory in tamoxifen resistance, may prove valuable (Beliakoff *et al.* 2003).

There are also now several reports that logical combination of anti-hormonal and various anti-growth-factor strategies (e.g. anti-HER2, EGFR or Ras signaling) may be particularly powerful in preventing emergence of resistance, as well as restoring anti-hormone responsiveness in resistant cells (Wakeling *et al.* 2001, Johnston *et al.* 2003, Jones *et al.* 2004). Our own studies have demonstrated that when gefitinib is administered to anti-hormone-sensitive cells in combination with tamoxifen or fulvestrant (in anticipation of the development of EGFR signaling as a resistance mechanism), combination treatment promotes a much improved anti-proliferative and apoptotic effect than achieved with either drug alone (Gee *et al.* 2003, Nicholson *et al.* 2004). Indeed, apoptosis is so high using combination therapy that total cell kill is achieved and development of anti-hormone resistance is prevented (Gee *et al.* 2003). We hypothesize that since anti-hormones induce EGFR expression, early signaling from this receptor stimulates a survival pathway which reduces anti-hormone-mediated cell kill. Thus, the anti-hormone-treated cells, although initially growth inhibited, survive and eventually establish a resistant state driven by the EGFR pathway, an event abrogated by co-treatment with gefitinib (Gee *et al.* 2003). In a similar manner, we

have recently described that co-targeting of EGFR and IGF-1R in our acquired tamoxifen-resistant cells is superior to inhibition of either pathway alone, where IGF-1R signaling clearly forms the survival and resistance mechanism recruited during treatment with gefitinib alone (Nicholson *et al.* 2004). Moreover, combination studies from Massarweh *et al.* (2002) have demonstrated that gefitinib delays resistance to estrogen deprivation and improves tamoxifen's anti-tumor activity in their *de novo* tamoxifen-resistant HER-2 transfected MCF-7 cells.

This article has also presented the available clinical and experimental data implicating growth factor signaling in ER α + *de novo* resistance. Increased HER2/EGFR signaling is a feature of ER α + *de novo* tamoxifen-resistant clinical material, phenotypic data implying that anti-growth factor strategies would be worthy of consideration in such tumors. Interestingly, emerging clinical data indicate that such ER α + patients still respond to estrogen deprivation. The underlying growth mechanism for such tumors may therefore involve elevated EGFR/HER2 signaling cross-talking with ER α , as described for acquired tamoxifen resistance. Addition of anti-HER2 or anti-EGFR strategies together with AIs could thus have potential to improve response and inhibit a proportion of such ER α + *de novo* tamoxifen-resistant disease. Disappointingly, however, the recently published clinical trial of fulvestrant versus tamoxifen (Howell *et al.* 2004) shows that in the first-line setting tamoxifen is equivalent to fulvestrant, the data shedding doubt on the proposed ER α cross-talk with HER2/EGFR signaling in *de novo* resistance. There remains some concern as to whether an appropriate dose of fulvestrant was achieved in this trial, and certainly earlier studies with fulvestrant did not achieve complete ER α down-regulation, an event believed to discriminate its actions from SERMs. Nevertheless, continued study of appropriate *de novo* resistant models and further clinical trial material, notably AI-resistant samples, clearly remains essential if we are fully to understand *de novo* resistance. Indeed, diverse cellular mechanisms outside the remit of this article, including aberrations in cell cycle components and tumor suppressor genes, have all been linked previously with *de novo* resistance, data suggesting that growth regulation of this state is not simply an extrapolation of acquired resistance mechanisms.

Finally, experimental growth factor challenge and transfection studies suggest that growth factor signaling elements aberrantly expressed may be able to promote *de novo* resistance under conditions of reduced ER α , and even in an ER α -independent manner. Extreme or chronic, sustained growth factor signaling, arising during therapy or apparently *de novo*, can diminish ER α expression/function, dislocate cells from ER α signaling promoting

endocrine insensitivity, and generate ER negativity in models. Treatment of these insensitive states with appropriate anti-growth factors may again be possible, while such agents may also reverse this transition and re-instate the anti-hormone responsive phenotype. Excitingly, *in vitro* blockade of constitutive EGFR, HER2, or MAP kinase with MEK inhibitors or dominant negative constructs can re-instate ER α expression in *de novo*-resistant cells (Oh *et al.* 2001), and preliminary studies indicate that reversion of the ER α - phenotype and re-instatement of endocrine responsiveness is initiated in a proportion of advanced HER2+ breast cancer patients by use of herceptin to inhibit such growth factor signaling (Munzone *et al.* 2003).

This article, in total, establishes a proof of principle that blocking growth factor signaling cascades could be therapeutically effective (particularly in combination with anti-hormonal strategies) and it is certainly encouraging that preliminary data associated with the use of gefitinib in breast cancer patients resistant to tamoxifen are demonstrating clinical benefit, especially in ER α + acquired resistant disease (Robertson *et al.* 2003). We eagerly anticipate the results of various ongoing and proposed trials examining the efficacy of a spectrum of such agents in *de novo* and acquired endocrine resistance, in particular those aiming to treat or prevent resistance to estrogen deprivation.

References

- Abdul-Wahab K, Corcoran D, Perachiotti A & Darbre PD 1999 Overexpression of insulin-like growth factor II (IGFII) in ZR-75-71 human breast cancer cells: higher threshold levels of receptor (IGFIR) are required for a proliferative response than for effects on specific gene expression. *Cell Proliferation* **32** 271–287.
- van Agthoven T, van Agthoven TL, Portengen H, Foekens JA & Dorssers LC 1992 Ectopic expression of epidermal growth factor receptors induces hormone independence in ZR-75-71 human breast cancer cells. *Cancer Research* **52** 5082–5088.
- van Agthoven T, van Agthoven TL, Dekker A, Foekens JA & Dorssers LC 1994 Induction of estrogen independence of ZR-75-71 human breast cancer cells by epigenetic alterations. *Molecular Endocrinology* **8** 1474–1483.
- Akimoto T, Hunter NR, Buchmiller L, Mason K, Ang KK & Milas L 1999 Inverse relationship between epidermal growth factor receptor expression and radiocurability of murine carcinomas. *Clinical Cancer Research* **5** 2884–2890.
- Arteaga CL, Carty-Dugger T, Moses HL, Hurd SD & Pietenpol JA 1993 Transforming growth factor beta 1 can induce estrogen-independent tumorigenicity of human breast cancer cells in athymic mice. *Cell Growth and Differentiation* **4** 193–201.
- Atlas E, Cardillo M, Mehmi I, Zahedkargaran H, Tang C & Lupu R 2003 Heregulin is sufficient for the promotion of

- tumorigenicity and metastasis of breast cancer cells *in vivo*. *Molecular Cancer Research* **1** 165–175.
- Bates NP & Hurst HC 1997 An intron 1 enhancer element mediates estrogen-induced suppression of ERBB2 expression. *Oncogene* **15** 473–481.
- Baum M, Buzdar A, Cuzick J, Forbes J, Houghton J, Howell A & Sahmoud T; The ATAC (Arimidex, Tamoxifen Alone or in Combination) Trialists' Group 2003 Anastrozole alone or in combination with tamoxifen versus tamoxifen alone for adjuvant treatment of postmenopausal women with early-stage breast cancer: results of the ATAC (Arimidex, Tamoxifen Alone or in Combination) trial efficacy and safety update analyses. *Cancer* **98** 1802–1810.
- Beliakoff J, Bagatell R, Paine-Murrieta G, Taylor CW, Lykkesfeldt AE & Whitesell L 2003 Hormone-refractory breast cancer remains sensitive to the antitumor activity of heat shock protein 90 inhibitors. *Clinical Cancer Research* **9** 4961–4971.
- Benz CC, Scott GK, Sarup JC, Johnson RM, Tripathy D, Coronado E, Shepard HM & Osborne CK 1993 Estrogen-dependent, tamoxifen-resistant tumorigenic growth of MCF-7 cells transfected with HER2/neu. *Breast Cancer Research and Treatment* **24** 85–95.
- van den Berg HW, Claffie D, Boylan M, McKillen J, Lynch M & McKibben B 1996 Expression of receptors for epidermal growth factor and insulin-like growth factor I by ZR-75-71 human breast cancer cell variants is inversely related: the effect of steroid hormones on insulin-like growth factor I receptor expression. *British Journal of Cancer* **73** 477–481.
- Berstein LM, Zheng H, Yue W, Wang JP, Lykkesfeldt AE, Naftolin F, Harada H, Shanabrough M & Santen RJ 2003 New approaches to the understanding of tamoxifen action and resistance. *Endocrine-Related Cancer* **10** 267–277.
- Berstein LM, Wang JP, Zheng H, Yue W, Conaway M & Santen RJ 2004 Long-term exposure to tamoxifen induces hypersensitivity to estradiol. *Clinical Cancer Research* **10** 1530–1534.
- Biswas DK, Cruz AP, Gansberger E & Pardee AB 2000 Epidermal growth factor-induced nuclear factor kappa B activation: a major pathway of cell-cycle progression in estrogen-receptor negative breast cancer cells. *PNAS* **97** 8542–8547.
- Britton D, Hutcheson IR, Barrow D, Gee JMW & Nicholson RI 2002 Increased estrogen receptor phosphorylation at serine 118 in tamoxifen resistant MCF-7 breast cancer cells. *Biochemical Society Transactions* **31** P114.
- Britton D, Hutcheson IR, Barrow D, McClelland RA & Nicholson RI 2003 Estrogen receptor phosphorylation in hormone sensitive and anti-hormone resistant breast cancer cells. *Breast Cancer Research and Treatment* **82** S61.
- Bunone G, Briand PA, Miksicek RJ & Picard D 1996 Activation of the unliganded estrogen receptor by EGF involves the MAP kinase pathway and direct phosphorylation. *EMBO Journal* **15** 2174–2183.
- Buzdar AU, Jonat W, Howell A & Plourde PV 1997 ARIMIDEX: a potent and selective aromatase inhibitor for the treatment of advanced breast cancer. *Journal of Steroid Biochemistry and Molecular Biology* **61** 145–149.
- Campbell RA, Bhat-Nakshatri P, Patel NM, Constantinidou D, Ali S & Nakshatri H 2001 Phosphatidylinositol 3-kinase/AKT-mediated activation of estrogen receptor alpha: a new model for anti-estrogen resistance. *Journal of Biological Chemistry* **276** 9817–9824.
- Castoria G, Migliaccio A, Bilancio A, Di Domenico M, de Falco A, Lombardi M, Fiorentino R, Varricchio L, Barone MV & Auricchio F 2001 PI3-kinase in concert with Src promotes the S-phase entry of oestradiol-stimulated MCF-7 cells. *EMBO Journal* **20** 6050–6059.
- Chan CM, Martin LA, Johnston SR, Ali S & Dowsett M 2002 Molecular changes associated with the acquisition of estrogen hypersensitivity in MCF-7 breast cancer cells on long-term estrogen deprivation. *Journal of Steroid Biochemistry and Molecular Biology* **81** 333–341.
- Chen Z, Ke LD, Yuan XH & Adler-Storthz K 2000 Correlation of cisplatin sensitivity with differential alteration of EGFR expression in head and neck cancer cells. *Anticancer Research* **20** 899–902.
- Chisamore MJ, Ahmed Y, Bentrem DJ, Jordan VC & Tonetti DA 2001 Novel antitumor effect of estradiol in athymic mice injected with a T47D breast cancer cell line overexpressing protein kinase C alpha. *Clinical Cancer Research* **7** 3156–3165.
- Chrysogelos SA, Yarden RI, Lauber AH & Murphy JM 1994 Mechanisms of EGF receptor regulation in breast cancer cells. *Breast Cancer Research and Treatment* **31** 227–236.
- Chung YL, Sheu ML, Yang SC, Lin CH & Yen SH 2002 Resistance to tamoxifen-induced apoptosis is associated with direct interaction between Her2/neu and cell membrane estrogen receptor in breast cancer. *International Journal of Cancer* **97** 306–312.
- Ciardiello F & Tortora G 2001 A novel approach in the treatment of cancer: targeting the epidermal growth factor receptor. *Clinical Cancer Research* **7** 2958–2970.
- Clarke R, Liu MC, Bouker KB, Gu Z, Lee RY, Zhu Y, Skaar TC, Gomez B, O'Brien K, Wang Y & Hilakivi-Clarke LA 2003 Antiestrogen resistance in breast cancer and the role of estrogen receptor signaling. *Oncogene* **22** 7316–7339.
- Coutts AS & Murphy LC 1998 Elevated mitogen-activated protein kinase activity in estrogen-nonresponsive human breast cancer cells. *Cancer Research* **58** 4071–4074.
- Daly RJ, King RJ & Darbre PD 1990 Interaction of growth factors during progression towards steroid independence in T-47-D human breast cancer cells. *Journal of Cell Biochemistry* **43** 199–211.
- Daly RJ, Harris WH, Wang DY & Darbre PD 1991 Autocrine production of insulin-like growth factor II using an inducible expression system results in reduced estrogen sensitivity of MCF-7 human breast cancer cells. *Cell Growth and Differentiation* **2** 457–464.
- Darbre PD & Daly RJ 1990 Transition of human breast cancer cells from an estrogen responsive to unresponsive state. *Journal of Steroid Biochemistry and Molecular Biology* **37** 753–763.
- Demirpence E, Pons M, Balaguer P & Gagne D 1992 Study of an antiestrogenic effect of retinoic acid in MCF-7 cells. *Biochemical and Biophysical Research Communications* **183** 100–106.

- Dickstein BM, Wosikowski K & Bates SE 1995 Increased resistance to cytotoxic agents in ZR75B human breast cancer cells transfected with epidermal growth factor receptor. *Molecular and Cellular Endocrinology* **110** 205–211.
- Donovan JC, Milic A & Slingerland JM 2001 Constitutive MEK/MAPK activation leads to p27(Kip1) deregulation and antiestrogen resistance in human breast cancer cells. *Journal of Biological Chemistry* **276** 40888–40895.
- Doucas V & Yaniv M 1991 Functional interaction between estrogen receptor and proto-oncogene products c-Jun and c-Fos. *Comptes Rendus des Seances de la Societe de Biologie et de ses Filiales* **185** 464–474.
- Dowsett M, Harper-Wynne C, Boeddinghaus I, Salter J, Hills M, Dixon M, Ebbs S, Gui G, Sacks N & Smith I 2001 HER-2 amplification impedes the antiproliferative effects of hormone therapy in estrogen receptor-positive primary breast cancer. *Cancer Research* **61** 8452–8458.
- Dowsett MC, Gutierrez S, Mohsin R, Schiff S, Detre S, Johnston & Osborne CK 2003 Molecular changes in tamoxifen-relapsed breast cancer: relationship between ER, HER2 and P38-MAP-kinase. *Proceedings for the American Society of Clinical Oncology* **22** 3 (Abstract 7).
- Dunn SE, Hardman RA, Kari FW & Barrett JC 1997 Insulin-like growth factor I (IGF-I) alters drug sensitivity of HBL100 human breast cancer cells by inhibition of apoptosis induced by diverse anticancer drugs. *Cancer Research* **57** 2687–2693.
- El-Ashry D, Miller DL, Kharbanda S, Lippman ME & Kern FG 1997 Constitutive Raf-1 kinase activity in breast cancer cells induces both estrogen-independent growth and apoptosis. *Oncogene* **15** 423–435.
- Elliott RL, Elliott MC, Wang F & Head JF 1993 Breast carcinoma and the role of iron metabolism. A cytochemical, tissue culture, and ultrastructural study. *Annals of the New York Academy of Sciences* **698** 159–166.
- Ellis MJ, Coop A, Singh B, Mauriac L, Llombert-Cussac A, Janicke F, Miller WR, Evans DB, Dugan M, Brady C, Quebe-Fehling E & Borgs M. 2001 Letrozole is more effective neoadjuvant endocrine therapy than tamoxifen for ErbB-1- and/or ErbB-2-positive, estrogen receptor-positive primary breast cancer: evidence from a phase III randomized trial. *Journal of Clinical Oncology* **19** 3808–3816.
- de Fazio A, Chiew YE, McEvoy M, Watts CK & Sutherland RL 1997 Antisense estrogen receptor RNA expression increases epidermal growth factor receptor gene expression in breast cancer cells. *Cell Growth and Differentiation* **8** 903–911.
- Fernandez P, Wilson C, Hoivik D & Safe SH 1998 Altered phenotypic characteristics of T47d human breast cancer cells after prolonged growth in estrogen-deficient medium. *Cell Biology International* **22** 623–633.
- Figtree GA, Webb CM & Collins P 2000 Tamoxifen acutely relaxes coronary arteries by an endothelium-, nitric oxide-, and estrogen receptor-dependent mechanism. *Journal of Pharmacology and Experimental Therapeutics* **295** 519–523.
- Font de Mora J & Brown M 2000 AIB1 is a conduit for kinase-mediated growth factor signaling to the estrogen receptor. *Molecular and Cellular Biology* **20** 5041–5047.
- Fuqua SA, Wiltschke C, Zhang QX, Borg A, Castles CG, Friedrichs WE, Hopp T, Hilsenbeck S, Mohsin S, O'Connell P & Allred DC 2000 A hypersensitive estrogen receptor-alpha mutation in premalignant breast lesions. *Cancer Research* **60** 4026–4029.
- Gee JM & Nicholson RI 2003 Expanding the therapeutic repertoire of epidermal growth factor receptor blockade: radiosensitization. *Breast Cancer Research* **5** 126–129. Epub 2003 Feb 20.
- Gee JM, Willsher PC, Kenny FS, Robertson JF, Pinder SE, Ellis IO & Nicholson RI 1999 Endocrine response and resistance in breast cancer: a role for the transcription factor Fos. *International Journal of Cancer* **84** 54–61.
- Gee JM, Robertson JF, Ellis IO & Nicholson RI 2001 Phosphorylation of ERK1/2 mitogen-activated protein kinase is associated with poor response to anti-hormonal therapy and decreased patient survival in clinical breast cancer. *International Journal of Cancer* **95** 247–254.
- Gee JMW, Madden TA, Robertson JFR & Nicholson RI 2002 Clinical response and resistance to SERMS. In *Endocrine Therapy in Breast Cancer*, pp 155–190. Eds JFR Robertson, RI Nicholson & DF Hayes. London: Martin Dunitz Ltd.
- Gee JM, Harper ME, Hutcheson IR, Madden TA, Barrow D, Knowlden JM, McClelland RA, Jordan N, Wakeling AE & Nicholson RI 2003 The antiepidermal growth factor receptor agent gefitinib (ZD1839/Iressa) improves antihormone response and prevents development of resistance in breast cancer *in vitro*. *Endocrinology* **144** 5105–5117. Epub 2003 Aug 07.
- Gooch JL, Van Den Berg CL & Yee D 1999 Insulin-like growth factor (IGF)-I rescues breast cancer cells from chemotherapy induced cell death — proliferative and anti-apoptotic effects. *Breast Cancer Research and Treatment* **56** 1–10.
- Gottardis MM & Jordan VC 1988 Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term antiestrogen administration. *Cancer Research* **48** 5183–5187.
- Gross JM & Yee D 2003 The type-1 insulin-like growth factor receptor tyrosine kinase and breast cancer: biology and therapeutic relevance. *Cancer and Metastasis Review* **22** 327–336.
- Guvakova MA & Surmacz E 1997 Overexpressed IGF-I receptors reduce estrogen growth requirements, enhance survival, and promote E-cadherin-mediated cell-cell adhesion in human breast cancer cells. *Experimental Cell Research* **231** 149–162.
- Herman ME & Katzenellenbogen BS 1994 Alterations in transforming growth factor-alpha and -beta production and cell responsiveness during the progression of MCF-7 human breast cancer cells to estrogen-autonomous growth. *Cancer Research* **54** 5867–5874.
- Howell A, DeFriend DJ, Robertson JF, Blamey RW, Anderson L, Anderson E, Sutcliffe FA & Walton P 1996 Pharmacokinetics, pharmacological and anti-tumor effects of the specific anti-estrogen ICI 162,780 in women with advanced breast cancer. *British Journal of Cancer* **74** 300–308.
- Howell A, Robertson JF, Abram P, Lichinitser MR, Elledge R, Bajetta E, Watanabe T, Morris C, Webster A, Dimery I & Osborne CK 2004 Comparison of fulvestrant versus tamoxifen for the treatment of advanced breast cancer in postmenopausal women previously untreated with endocrine

- therapy: a multinational, double-blind, randomized trial. *Journal of Clinical Oncology* **22** 1605–1613.
- Hutcheson IR, Knowlden JM, Madden TA, Barrow D, Gee JM, Wakeling AE & Nicholson RI 2003 Estrogen receptor-mediated modulation of the EGFR/MAPK pathway in tamoxifen-resistant MCF-7 cells. *Breast Cancer Research and Treatment* **81** 81–93.
- Hutcheson IR, Knowlden JM, Barrow D & Nicholson RI 2004 IGF-1R/EGFR cross-talk in tamoxifen resistant MCF-7 breast cancer cells. *AACR 2004 Proceedings* **45** 5189.
- Jeng MH, Shupnik MA, Bender TP, Westin EH, Bandyopadhyay D, Kumar R, Masamura S & Santen RJ 1998 Estrogen receptor expression and function in long-term estrogen-deprived human breast cancer cells. *Endocrinology* **139** 4164–4174.
- Jeng MH, Yue W, Eischeid A, Wang JP & Santen RJ 2000 Role of MAP kinase in the enhanced cell proliferation of long term estrogen deprived human breast cancer cells. *Breast Cancer Research and Treatment* **62** 167–175.
- Jensen J, Kitlen JW, Briand P, Labrie F & Lykkesfeldt AE 2003 Effect of antiestrogens and aromatase inhibitor on basal growth of the human breast cancer cell line MCF-7 in serum-free medium. *Journal of Steroid Biochemistry and Molecular Biology* **84** 469–478.
- Jess TJ, Belham CM, Thomson FJ, Scott PH, Plevin RJ & Gould GW 1996 Phosphatidylinositol 3'-kinase, but not p70 ribosomal S6 kinase, is involved in membrane protein recycling: wortmannin inhibits glucose transport and downregulates cell-surface transferrin receptor numbers independently of any effect on fluid-phase endocytosis in fibroblasts. *Cellular Signaling* **8** 297–304.
- Johnston SR & Dowsett M 2003 Aromatase inhibitors for breast cancer: lessons from the laboratory. *Nature Reviews Cancer* **3** 821–831.
- Johnston SR, Lu B, Scott GK, Kushner PJ, Smith IE, Dowsett M & Benz CC 1999 Increased activator protein-1 DNA binding and c-Jun NH2-terminal kinase activity in human breast tumors with acquired tamoxifen resistance. *Clinical Cancer Research* **5** 251–256.
- Johnston SR, Head J, Pancholi S, Detre S, Martin LA, Smith IE & Dowsett M 2003 Integration of signal transduction inhibitors with endocrine therapy: an approach to overcoming hormone resistance in breast cancer. *Clinical Cancer Research* **9** 524S–532S.
- Jones HE, Nicholson RI, Hutcheson IR, Knowlden JM, Harper ME, Jordan N, Hiscox SE, Barrow D & Gee JM 2004 Endocrine resistance in breast cancer: therapeutic intervention using signal transduction inhibitors. *Advances in Breast Cancer* **1** 3–9.
- de Jong PC, Blankenstein MA, Nortier JW, Slee PH, van de Ven J, van Gorp JM, Elbers JR, Schipper ME, Blijham GH, Thijssen JH, Lu Q, Jelovac D & Brodie AM 2003 The relationship between aromatase in primary breast tumors and response to treatment with aromatase inhibitors in advanced disease. *Journal of Steroid Biochemistry and Molecular Biology* **87** 149–155.
- Jordan VC, Osipo C, Schafer JM, Fox JE, Cheng D & Liu H 2003 Changing role of the estrogen receptor in the life and death of breast cancer cells. *Breast* **12** 432–441.
- Jordan NJ, Gee JMW, Barrow D, Wakeling AE & Nicholson RI 2004 Increased constitutive activity of PKB/Akt in tamoxifen resistant breast cancer MCF-7 cells. *Breast Cancer Research and Treatment* **87** 167–180.
- Kahlert S, Nuedling S, van Eickels M, Vetter H, Meyer R & Grohe C 2000 Estrogen receptor alpha rapidly activates the IGF-I receptor pathway. *Biological Chemistry* **275** 18447–18453.
- Karnik PS, Kulkarni S, Liu XP, Budd GT & Bukowski RM 1994 Estrogen receptor mutations in tamoxifen-resistant breast cancer. *Cancer Research* **54** 349–353.
- Katzenellenbogen BS, Kendra KL, Norman MJ & Berthois Y 1987 Proliferation, hormonal responsiveness, and estrogen receptor content of MCF-7 human breast cancer cells grown in the short-term and long-term absence of estrogens. *Cancer Research* **47** 4355–4360.
- Knowlden JM, Hutcheson IR, Barrow D & Nicholson RI 2003a IGF-1R and EGFR crosstalk in tamoxifen resistant breast cancer cells. *Breast Cancer Research and Treatment* **82** S171.
- Knowlden JM, Hutcheson IR, Jones HE, Madden T, Gee JMW, Harper ME, Barrow D, Wakeling AE & Nicholson RI 2003b Elevated levels of epidermal growth factor receptor/c-erbB2 heterodimers mediate an autocrine growth regulatory pathway in tamoxifen-resistant MCF-7 cells. *Endocrinology* **144** 1032–1044.
- Kurokawa H & Arteaga CL 2003 ErbB (HER) receptors can abrogate antiestrogen action in human breast cancer by multiple signaling mechanisms. *Clinical Cancer Research* **9** 511S–515S.
- Li H & Qian ZM 2002 Transferrin/transferrin receptor-mediated drug delivery. *Medicinal Research Reviews* **22** 225–250.
- Liu Y, El-Ashry D, Chen D, Ding IY & Kern FG 1995 MCF-7 breast cancer cells overexpressing transfected c-erbB-2 have an *in vitro* growth advantage in estrogen-depleted conditions and reduced estrogen dependence and tamoxifen-sensitivity *in vivo*. *Breast Cancer Research and Treatment* **34** 97–117.
- Long B, McKibben BM, Lynch M & van den Berg HW 1992 Changes in epidermal growth factor receptor expression and response to ligand associated with acquired tamoxifen resistance or estrogen independence in the ZR-75-71 human breast cancer cell line. *British Journal of Cancer* **65** 865–869.
- Long BJ, Jelovac D, Thiantanawat A & Brodie AM 2002 The effect of second-line antiestrogen therapy on breast tumor growth after first-line treatment with the aromatase inhibitor letrozole: long-term studies using the intratumoral aromatase postmenopausal breast cancer model. *Clinical Cancer Research* **8** 2378–2388.
- Lonning PE 2001 Stepwise estrogen suppression manipulating the estrostat. *Journal of Steroid Biochemistry and Molecular Biology* **79** 127–132.
- Luftner D, Jung A, Schmid P, Geppert R, Kienle E, Wernecke KD & Possinger K 2003 Takeda Adjuvant Breast Cancer Study with Leuprorelin Study Group. Upregulation of HER-2/neu by ovarian ablation: results of a randomized trial comparing leuprorelin to CMF as adjuvant therapy in node-positive breast cancer patients. *Breast Cancer Research and Treatment* **80** 245–255.
- McClelland RA, Barrow D, Madden TA, Dutkowski CM, Pamment J, Knowlden JM, Gee JM & Nicholson RI 2001

- Enhanced epidermal growth factor receptor signaling in MCF7 breast cancer cells after long-term culture in the presence of the pure antiestrogen ICI 182,780 (Faslodex). *Endocrinology* **142** 2776–2788.
- McLeskey SW, Kurebayashi J, Honig SF, Zwiebel J, Lippman ME, Dickson RB & Kern FG 1993 Fibroblast growth factor 4 transfection of MCF-7 cells produces cell lines that are tumorigenic and metastatic in ovariectomized or tamoxifen-treated athymic nude mice. *Cancer Research* **53** 2168–2177.
- Martin MB, Garcia-Morales P, Stoica A, Solomon HB, Pierce M, Katz D, Zhang S, Danielsen M & Saceda M 1995 Effects of 12-O-tetradecanoylphorbol-13-acetate on estrogen receptor activity in MCF-7 cells. *Journal of Biological Chemistry* **270** 25244–25251.
- Martin LA, Farmer I, Johnston SR, Ali S, Marshall C & Dowsett M. 2003 Enhanced estrogen receptor (ER) alpha, ERBB2, and MAPK signal transduction pathways operate during the adaptation of MCF-7 cells to long term estrogen deprivation. *Journal of Biological Chemistry* **278** 30458–30468. Epub 2003 May 29.
- Masamura S, Santner SJ, Heitjan DF & Santen RJ 1995 Estrogen deprivation causes estradiol hypersensitivity in human breast cancer cells. *Journal of Clinical Endocrinology and Metabolism* **80** 2918–2925.
- Massarweh S, Shou J, Mohsin SK, Ge M, Wakeling AE, Osborne CK & Schiff R 2002 Inhibition of epidermal growth factor/HER2 receptor signaling using ZD1839 ('Iressa') restores tamoxifen sensitivity and delays resistance to estrogen deprivation in HER2-overexpressing breast tumors. *Proceedings for the American Society of Clinical Oncology* **21** 33 (Abstract).
- Massarweh SA, Jiang S, Mohsin SK, DiPietro M, Wakeling AE, Osborne CK & Schiff R 2003 Resistance to endocrine therapy in a xenograft model of HER-2 overexpressing breast cancer is accompanied by increased HER-2 but loss of IGF-1 receptor expression. *Proceedings for the American Society of Clinical Oncology*, 26th Annual Meeting, San Antonio, TX, USA. Abstract 1007.
- Miller DL, El-Ashry D, Cheville AL, Liu Y, McLeskey SW & Kern FG 1994 Emergence of MCF-7 cells overexpressing a transfected epidermal growth factor receptor (EGFR) under estrogen-depleted conditions: evidence for a role of EGFR in breast cancer growth and progression. *Cell Growth and Differentiation* **5** 1263–1274.
- Mouridsen H, Gershonovich M, Sun Y, Perez-Carrion R, Boni C, Monnier A, Apffelstaedt J, Smith R, Sleeboom HP, Janicke F, Pluzanska A, Dank M, Becquart D, Bapsy PP, Salminen E, Snyder R, Lassus M, Verbeek JA, Staffler B, Chaudri-Ross HA & Dugan M 2001 Superior efficacy of letrozole versus tamoxifen as first-line therapy for postmenopausal women with advanced breast cancer: results of a phase III study of the International Letrozole Breast Cancer Group. *Clinical Oncology* **19** 2596–2606.
- Mueller H, Kueng W, Schoumacher F, Herzer S, & Eppenberger U 1995 Selective regulation of steroid receptor expression in MCF-7 breast cancer cells by a novel member of the heregulin family. *Biochemical and Biophysical Research Communications* **217** 1271–1278.
- Munzone EF, Nole G, Renne A, Balduzzi G, Sanna L, Corsetto A & Goldhirsch 2003 Reverting estrogen receptor (ER) negative phenotype in advanced breast cancer patients (pts) over-expressing HER2 after treatment with trastuzumab plus chemotherapy. *Proceedings for the American Society of Clinical Oncology* **22** 848 (Abstract 3409).
- Murphy CS, Meisner LF, Wu SQ & Jordan VC 1989 Short- and long-term estrogen deprivation of T47D human breast cancer cells in culture. *European Journal of Cancer and Clinical Oncology* **25** 1777–1788.
- Murphy CS, Pink JJ & Jordan VC 1990 Characterization of a receptor-negative, hormone-nonresponsive clone derived from a T47D human breast cancer cell line kept under estrogen-free conditions. *Cancer Research* **50** 7285–7292.
- Nicholson RI & Gee JM 2000 Estrogen and growth factor cross-talk and endocrine insensitivity and acquired resistance in breast cancer. *British Journal of Cancer* **82** 501–513.
- Nicholson RI, McClelland RA, Gee JM, Manning DL, Cannon P, Robertson JF, Ellis IO & Blamey RW 1994 Epidermal growth factor receptor expression in breast cancer: association with response to endocrine therapy. *Breast Cancer Research and Treatment* **29** 117–125.
- Nicholson RI, Gee JM & Harper ME 2001a EGFR and cancer prognosis. *European Journal of Cancer* **37** (Suppl 4) S9–S15.
- Nicholson RI, Hutcheson IR, Harper ME, Knowlden JM, Barrow D, McClelland RA, Jones HE, Wakeling AE & Gee JM 2001b Modulation of epidermal growth factor receptor in endocrine resistant, estrogen receptor-positive breast cancer. *Endocrine-Related Cancer* **8** 175–182.
- Nicholson RI, Gee JM, Knowlden J, McClelland R, Madden TA, Barrow D & Hutcheson I 2003 The biology of antihormone failure in breast cancer. *Breast Cancer Research and Treatment* **80** (Suppl 1) S29–S34; discussion S35.
- Nicholson RI, Hutcheson IR, Knowlden JM, Jones HE, Harper ME, Jordan N, Hiscox SE, Barrow D & Gee JM 2004 Nonendocrine pathways and endocrine resistance: observations with antiestrogens and signal transduction inhibitors in combination. *Clinical Cancer Research* **10** 346S–354S.
- Oh AS, Lorient LA, Holloway JN, Miller DL, Kern FG & El-Ashry D 2001 Hyperactivation of MAPK induces loss of ERalpha expression in breast cancer cells. *Molecular Endocrinology* **15** 1344–1359.
- Osborne CK, Coronado E, Allred DC, Wiebe V & DeGregorio M 1991 Acquired tamoxifen resistance: correlation with reduced breast tumor levels of tamoxifen and isomerization of trans-4-hydroxytamoxifen. *Journal of the National Cancer Institute* **83** 1477–1482.
- Osborne CK, Bardou V, Hopp TA, Chamness GC, Hilsenbeck SG, Fuqua SA, Wong J, Allred DC, Clark GM & Schiff R 2003 Role of the estrogen receptor coactivator AIB1 (SRC-3) and HER-2/neu in tamoxifen resistance in breast cancer. *Journal of the National Cancer Institute* **95** 353–361.
- Parl FF 2003 Multiple mechanisms of estrogen receptor gene repression contribute to ER-negative breast cancer. *Pharmacogenomics Journal* **3** 251–253.
- Pellicci G, Lanfrancione L, Salcini AE, Romano A, Mele S, Grazia Borrello M, Segatto O, Di Fiore PP & Pellicci PG 1995

- Constitutive phosphorylation of Shc proteins in human tumors. *Oncogene* **11** 899–907.
- Perez-Tenorio G, Stal O & Southeast Sweden Breast Cancer Group 2002 Activation of AKT/PKB in breast cancer predicts a worse outcome among endocrine treated patients. *British Journal of Cancer* **86** 540–545.
- Pietras RJ, Arboleda J, Reese DM, Wongvipat N, Pegram MD, Ramos L, Gorman CM, Parker MG, Sliwkowski MX & Slamon DJ 1995 HER-2 tyrosine kinase pathway targets estrogen receptor and promotes hormone-independent growth in human breast cancer cells. *Oncogene* **10** 2435–2446.
- Pink JJ & Jordan VC 1996 Models of estrogen receptor regulation by estrogens and antiestrogens in breast cancer cell lines. *Cancer Research* **56** 2321–2330.
- Pink JJ, Bilimoria MM, Assikis J & Jordan VC 1996 Irreversible loss of the estrogen receptor in T47D breast cancer cells following prolonged estrogen deprivation. *British Journal of Cancer* **74** 1227–1236.
- Pritchard KI 2003 Endocrine therapy of advanced disease: analysis and implications of the existing data. *Clinical Cancer Research* **9** 460S–467S.
- Razandi M, Pedram A, Park ST & Levin ER 2002 Proximal events in signaling by plasma membrane estrogen receptors. *Journal of Biological Chemistry* **278** 2701–2712.
- Robertson JFR, Gutteridge E, Cheung KL, Owers R, Koehler M, Hamilton L, Gee J & Nicholson RI 2003 Gefitinib (ZD1839) is active in acquired tamoxifen (TAM)-resistant estrogen receptor (ER)-positive and ER-negative breast cancer: results from a phase II study. *Proceedings for the American Society of Clinical Oncology* **22** 7 (Abstract 23).
- Russell KS & Hung MC 1992 Transcriptional repression of the neu protooncogene by estrogen stimulated estrogen receptor. *Cancer Research* **52** 6624–6629.
- Saceda M, Knabbe C, Dickson RB, Lippman ME, Bronzert D, Lindsey RK, Gottardis MM & Martin MB 1991 Post-transcriptional destabilization of estrogen receptor mRNA in MCF-7 cells by 12-O-tetradecanoylphorbol-13-acetate. *Journal of Biological Chemistry* **266** 17809–17814.
- Sachdev D & Yee D 2001 The IGF system and breast cancer. *Endocrine-Related Cancer* **8** 197–209.
- Salomon DS, Brandt R, Ciardiello F & Normanno N 1995 Epidermal growth factor-related peptides and their receptors in human malignancies. *Critical Reviews in Oncology/Haematology* **19** 183–232.
- Santen RJ 2003 Inhibition of aromatase: insights from recent studies. *Steroids* **68** 559–567.
- Santen R, Jeng MH, Wang JP, Song R, Masamura S, McPherson R, Santner S, Yue W & Shim WS 2001 Adaptive hypersensitivity to estradiol: potential mechanism for secondary hormonal responses in breast cancer patients. *Journal of Steroid Biochemistry and Molecular Biology* **79** 115–125.
- Santen RJ, Song RX, Zhang Z, Kumar R, Jeng MH, Masamura S, Yue W & Bernstein L 2003 Adaptive hypersensitivity to estrogen: mechanism for superiority of aromatase inhibitors over selective estrogen receptor modulators for breast cancer treatment and prevention. *Endocrine-Related Cancer* **10** 111–130.
- Santen RJ, Song RX, Zhang Z, Yue W & Kumar R 2004 Adaptive hypersensitivity to estrogen: mechanism for sequential responses to hormonal therapy in breast cancer. *Clinical Cancer Research* **10** 337S–345S.
- Schafer JM, Bentrem DJ, Takei H, Gajdos C, Badve S & Jordan VC 2002 A mechanism of drug resistance to tamoxifen in breast cancer. *Journal of Steroid Biochemistry and Molecular Biology* **83** 75–83.
- Schiff R, Massarweh S, Shou J & Osborne CK 2003 Breast cancer endocrine resistance: how growth factor signaling and estrogen receptor coregulators modulate response. *Clinical Cancer Research* **9** 447S–454S.
- Schiff R, Massarweh SA, Shou J, Bharwani L, Mohsin SK & Osborne CK 2004 Cross-talk between estrogen receptor and growth factor pathways as a molecular target for overcoming endocrine resistance. *Clinical Cancer Research* **10** 331S–336S.
- Shim WS, Conaway M, Masamura S, Yue W, Wang JP, Kmar R & Santen RJ 2000 Estradiol hypersensitivity and mitogen-activated protein kinase expression in long-term estrogen deprived human breast cancer cells *in vivo*. *Endocrinology* **141** 396–405.
- Smith LM, Wise SC, Hendricks DT, Sabichi AL, Bos T, Reddy P, Brown PH & Birrer MJ 1999 cJun overexpression in MCF-7 breast cancer cells produces a tumorigenic, invasive and hormone resistant phenotype. *Oncogene* **18** 6063–6070.
- Song RX & Santen RJ 2003 Apoptotic action of estrogen. *Apoptosis* **8** 55–60.
- Song RX, Mor G, Naftolin F, McPherson RA, Song J, Zhang Z, Yue W, Wang J & Santen RJ 2001 Effect of long-term estrogen deprivation on apoptotic responses of breast cancer cells to 17 β -estradiol. *Journal of the National Cancer Institute* **93** 1714–1723.
- Song RX, Santen RJ, Kumar R, Adam L, Jeng MH, Masamura S & Yue W 2002a Adaptive mechanisms induced by long-term estrogen deprivation in breast cancer cells. *Molecular and Cellular Endocrinology* **193** 29–42.
- Song RX, McPherson RA, Adam L, Bao Y, Shupnik M, Kumar R & Santen RJ 2002b Linkage of rapid estrogen action to MAPK activation by ER α -Shc association and Shc pathway activation. *Molecular Endocrinology* **16** 116–127.
- Song RX, Barnes CJ, Zhang Z, Bao Y, Kumar R & Santen RJ 2004 The role of Shc and insulin-like growth factor 1 receptor in mediating the translocation of estrogen receptor α to the plasma membrane. *PNAS* **101** 2076–2081. Epub 2004 Feb 05.
- Stephen RL, Shaw LE, Larsen C, Corcoran D & Darbre PD 2001 Insulin-like growth factor receptor levels are regulated by cell density and by long term estrogen deprivation in MCF7 human breast cancer cells. *Biological Chemistry* **276** 40080–40086. Epub 2001 Jul 16.
- Stoica A, Saceda M, Fakhro A, Solomon HB, Fenster BD & Martin MB 1997 The role of transforming growth factor- β in the regulation of estrogen receptor expression in the MCF-7 breast cancer cell line. *Endocrinology* **138** 1498–1505.
- Stoica A, Saceda M, Doraiswamy VL, Coleman C & Martin MB 2000a Regulation of estrogen receptor- α gene expression by epidermal growth factor. *Journal of Endocrinology* **165** 371–378.

- Stoica A, Saceda M, Fakhro A, Joyner M & Martin MB 2000b Role of insulin-like growth factor-I in regulating estrogen receptor- α gene expression. *Journal of Cellular Biochemistry* **76** 605–614.
- Tang CK, Perez C, Grunt T, Waibel C, Cho C & Lupu R 1996 Involvement of heregulin- β 2 in the acquisition of the hormone-independent phenotype of breast cancer cells. *Cancer Research* **56** 3350–3358.
- Tonetti DA, Chisamore MJ, Grdina W, Schurz H & Jordan VC 2000 Stable transfection of protein kinase C α cDNA in hormone-dependent breast cancer cell lines. *British Journal of Cancer* **83** 782–791.
- Tsuda H 2001 Prognostic and predictive value of c-erbB-2 (HER-2/neu) gene amplification in human breast cancer. *Breast Cancer* **8** 38–44.
- Tzukerman M, Zhang XK & Pfahl M 1991 Inhibition of estrogen receptor activity by the tumor promoter 12-O-tetradecanoylphorbol-13-acetate: a molecular analysis. *Molecular Endocrinology* **5** 1983–1992.
- Vickers PJ, Dickson RB, Shoemaker R & Cowan KH 1988 A multidrug-resistant MCF-7 human breast cancer cell line which exhibits cross-resistance to antiestrogens and hormone-independent tumor growth *in vivo*. *Molecular Endocrinology* **2** 886–892.
- Wakeling AE, Nicholson RI & Gee JM 2001 Prospects for combining hormonal and nonhormonal growth factor inhibition. *Clinical Cancer Research* **7** (Suppl 12) 4350s–4355s; discussion 4411s–4412s.
- Wells A 1999 EGF receptor. *International Journal of Biochemistry and Cellular Biology* **31** 637–643.
- Welshons WV & Jordan VC 1987 Adaptation of estrogen-dependent MCF-7 cells to low estrogen (phenol red-free) culture. *European Journal of Cancer and Clinical Oncology* **23** 1935–1939.
- Wilson MA & Chrysogelos SA 2002 Identification and characterisation of a negative regulatory element within the epidermal growth factor receptor gene first intron in hormone-dependent breast cancer cells. *Journal of Cellular Biochemistry* **85** 601–614.
- Winer EP, Hudis C, Burstein HJ, Byrant J, Chlebowski RT, Ingle JN, Edge SB, Mamounas EP, Gelber R, Gralow J, Goldstein LJ, Pritchard KI, Braun S, Colbleigh MA, Langer AS, Perotti J, Powles TJ, Whelan TJ & Browman GP 2003 ASCO technology assessment working group update: use of aromatase inhibitors in the adjuvant setting. *Journal of Clinical Oncology* **21** 2597–2599.
- Wong ZW & Ellis MJ 2004 First-line endocrine treatment of breast cancer: aromatase inhibitor or antiestrogen? *British Journal of Cancer* **90** 20–25.
- Yang X, Phillips DL, Ferguson AT, Nelson WG, Herman JG & Davidson NE 2001 Synergistic activation of functional estrogen receptor (ER)- α by DNA methyltransferase and histone deacetylase inhibition in human ER- α -negative breast cancer cells. *Cancer Research* **61** 7025–7029.
- Yarden RI, Wilson MA & Chrysogelos SA 2001 Estrogen suppression of EGFR expression in breast cancer cells: a possible mechanism to modulate growth. *Journal of Cellular Biochemistry* **81** 232–246.
- Yu Y & Feig LA 2002 Involvement of R-Ras and Ral GTPases in estrogen-independent proliferation of breast cancer cells. *Oncogene* **21** 7557–7568.
- Yue W, Berstein LM, Wang JP, Clark GM, Hamilton CJ, Demers LM & Santen RJ 2001 The potential role of estrogen in aromatase regulation in the breast. *Journal of Steroid Biochemistry and Molecular Biology* **79** 157–164.
- Yue W, Wang JP, Conaway M, Masamura S, Li Y & Santen RJ 2002 Activation of the MAPK pathway enhances sensitivity of MCF-7 breast cancer cells to the mitogenic effect of estradiol. *Endocrinology* **143** 3221–3229.
- Yue W, Wang JP, Conaway MR, Li Y & Santen RJ 2003 Adaptive hypersensitivity following long-term estrogen deprivation: involvement of multiple signal pathways. *Journal of Steroid Biochemistry and Molecular Biology* **86** 265–274.