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Gene network signaling in hormone responsiveness modifies apoptosis and autophagy in breast cancer cells☆

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ABSTRACT

Resistance to endocrine therapies, whether *de novo* or acquired, remains a major limitation in the ability to cure many tumors that express detectable levels of the estrogen receptor alpha protein (ER). While several resistance phenotypes have been described, endocrine unresponsiveness in the context of therapy-induced tumor growth appears to be the most prevalent. The signaling that regulates endocrine resistant phenotypes is poorly understood but it involves a complex signaling network with a topology that includes redundant and degenerative features. To be relevant to clinical outcomes, the most pertinent features of this network are those that ultimately affect the endocrine-regulated components of the cell fate and cell proliferation machineries. We show that autophagy, as supported by the endocrine regulation of monodansylcadaverine staining, increased LC3 cleavage, and reduced expression of p62/SQSTM1, plays an important role in breast cancer cells responding to endocrine therapy. We further show that the cell fate machinery includes both apoptotic and autophagic functions that are potentially regulated through integrated signaling that flows through key members of the BCL2 gene family and beclin-1 (BECN1). This signaling links cellular functions in mitochondria and endoplasmic reticulum, the latter as a consequence of induction of the unfolded protein response. We have taken a seed-gene approach to begin extracting critical nodes and edges that represent central signaling events in the endocrine regulation of apoptosis and autophagy. Three seed nodes were identified from global gene or protein expression analyses and supported by subsequent functional studies that established their abilities to affect cell fate. The seed nodes of nuclear factor kappa B (NFκB), interferon regulatory factor-1 (IRF1), and X-box binding protein-1 (XBP1) are linked by directional edges that support signal flow through a preliminary network that is grown to include key regulators of their individual function: NEMO/IKKγ, nucleophosmin and ER respectively. Signaling proceeds through BCL2 gene family members and BECN1 ultimately to regulate cell fate.

1. Introduction

Over 40,000 American women die of breast cancer each year [1]; incidence is broadly similar across the European Union when considered as a percentage of the population. In 2008, over 178,000 women will be diagnosed with invasive breast cancer in the U.S., almost 70% of which will be estrogen receptor-α positive (ER+; HUGO Gene Symbol = ESR1) [2,3]. The percentage of ER+ sporadic breast cancers increases linearly with age but even in premenopausal cases the proportion is high; 62% at age ≤35 and 72% by age 49 [2–4]. Data from randomized trials and meta-analyses clearly show that all breast cancer patients derive a statistically significant survival benefit from adjuvant chemotherapy, and that all hormone receptor positive breast cancer patients benefit from adjuvant endocrine therapy [5–9]. For postmenopausal women, the benefit from adjuvant Tamoxifen (TAM) is comparable to that seen for cytotoxic chemotherapy. While 5 years of adjuvant TAM produces a 26% proportional reduction in mortality [8], many ER+ tumors eventually recur [10]. Since advanced ER+ breast cancer largely remains an incurable disease, resistance to endocrine therapies is a significant clinical problem.

Endocrine therapy is administered as an antiestrogen (AE) like Tamoxifen (TAM) or Fulvestrant (FAS; Faslodex; ICI 182,780), or as an aromatase inhibitor (AI) such as Letrozole or Exemestane. It is less toxic and potentially more effective therapy in the management of hormone-dependent breast cancers. Antiestrogens, and TAM in

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particular, have been the "gold standard" first line endocrine therapy for over 30 years [11], clinical experience with this drug likely exceeding over 15 million patient years [10]. TAM increases both disease free and overall survival from early stage breast cancer, and it also reduces the incidence of invasive and noninvasive breast cancer in high-risk women [8,9]. Raloxifene, another antiestrogen, is effective in reducing the rate of postmenopausal bone loss from osteoporosis as well as the rate of invasive breast cancer [12]. Newer antiestrogens such as FAS show significant activity relative to TAM and some AIs [13,14]. Third generation AIs are now widely accepted as viable alternatives to AEs for first line endocrine therapy in postmenopausal women with metastatic disease; overall response rates are generally greater for AIs [15]. Importantly, Tamoxifen is the only single agent with demonstrated efficacy in both premenopausal and postmenopausal women with invasive breast cancer. Other AEs and all of the AIs require the complete cessation of ovarian function.

Of current interest is identification of the optimum choice and scheduling of AEs and AIs. Evidence clearly shows improvements in disease free survival for combined adjuvant therapy (an AI and an AE usually given sequentially) over single agent TAM [16–20]. However, the ability of AIs to induce a significant improvement in overall survival compared with 5 years of TAM alone is uncertain [15]. In terms of metastatic disease, recent data imply that response rates with an AI are either equivalent with or higher than with TAM [21,22]. Given the increasing number of endocrine treatment options, there is a clear need to optimize the selection and scheduling of agents for both early stage and advanced disease. Whichever way these controversies are eventually resolved, it is clear that both AIs and AEs will remain as key modalities in the management of ER+ breast cancers. Unfortunately, the inability of endocrine therapies to cure many women with ER+ disease will also remain.

1.1. Endocrine resistance: receptor phenotypes

Several resistance phenotypes are evident from both experimental models and clinical observations. The two primary receptor phenotypes are ER+ and ER−. These receptor-based phenotypes have been further stratified by addition of the estrogen-regulated receptor for progesterone (PGR; HUGO Gene Symbol = PGR). The degree of treatment benefit from endocrine therapy varies according to receptor phenotype. For example, approximately 75% of ER+/PGR+, 33% of ER+/PGR−, and 45% of ER−/PGR+ cases of metastatic breast cancer respond to TAM [10]. Endocrine responses in truly ER− tumors are probably relatively rare and of uncertain relevance, as they most likely reflect incorrect assessments of what may be very low ER and/or PGR expression values. Data from the Early Breast Cancer Trialists' Collaborative Group meta-analyses show that TAM therapy generates a non-significant 6% reduction in the 10-year risk of recurrence. A non-significant increase in the risk of death from any cause in patients with ER− breast cancer also was reported [8,9]. The real value of PGR, which is the only modification to this clinical prediction scheme for directing endocrine therapy to become routine in over 30 years (the value of directing endocrine therapy based on HER2 is still controversial), is largely limited to ER− tumors. It is general practice in the United States to treat all ER+ and/or PR+ invasive breast tumors with endocrine therapy. However, it remains impossible to predict whether an individual patient will receive benefit from treatment and the magnitude or duration of any benefit. Better predictors of each individual patient's endocrine responsiveness are clearly needed.

1.2. Endocrine resistance: pharmacological phenotypes

Several pharmacological phenotypes have been identified in experimental models of either human breast cancer cells growing *in vitro* or of xenografts in immune-deficient rodents [10]. These phenotypes include (i) estrogen-independent (which appears equivalent to AI resistance but is not so for antiestrogen resistance [23]—some breast cancers can become resistant to an AE but still respond to an AI and *vice versa*); (ii) estrogen-inhibited (recently identified in MCF-7 models [24]); (iii) TAM-stimulated (identified first in MCF-7 xenografts [25,26]); TAM-unresponsive but FAS sensitive [27] (identified first in MCF-7 models and subsequently observed in clinical trials [13]); TAM and FAS crossresistant [28] (perhaps this is truly antiestrogen crossresistant and it is seen both clinically in patients and experimentally in MCF-7 models [13,29]). Other variations on these phenotypes likely occur but are beyond the scope of our discussion.

1.3. Clinical evidence for the prevalence of pharmacological resistance phenotypes

Obtaining direct clinical evidence for the prevalence of each of the pharmacological resistance phenotypes is challenging. We have previously noted the utility of applying clinical responses to TAM withdrawal in metastatic breast cancer as one means to define, at least in broad terms, the likely relevance of a series of pharmacological phenotypes [29]. This approach is somewhat limited, as the number of cases across all studies is modest ($n = 241$). Furthermore, TAM withdrawal responses cannot readily distinguish between TAM-stimulation and estrogen-inhibition because each should predict for a clinical benefit. The latter would induce a benefit because many breast cancers contain significant concentrations of 17β-estradiol, independent of both menopausal and ER/PGR status [10], sufficient to produce the estrogen-inhibited phenotype [24]. Indeed, the superiority of AIs over TAM in inducing clinical response strongly implies that over 75% of ER+/PGR+, at least 50% of all ER+ breast cancers irrespective of PGR expression, and 45% or more of ER−/PGR+ breast tumors are probably driven by adequate access to estrogen.

In our prior assessment, almost 9% of patients received an overall clinical response to TAM withdrawal (partial responses + complete responses). When disease stabilizations were included we estimated that less than 20% of patients received clinical benefit [29], suggesting that the sum of TAM-stimulated plus estrogen-inhibited clinical phenotypes may not account for the majority of resistant phenotypes in women. Of course, given the number of ER+ breast cancers arising every year, these phenotypes are relevant to a notable number of women. The major response to TAM withdrawal was clinically detectable disease progression – greater than 80% of cases – strongly implicating unresponsiveness as the primary clinical resistance mechanism to TAM. Whether these breast cancers are fully crossresistant to all endocrine therapies, or retain sensitivity to AIs, cannot be determined from this simple analysis.

Nomura et al. [30] took a different approach and assessed the responsiveness to estrogen and TAM in short-term primary cell cultures of $n = 153$ ER+ breast cancer biopsies. This approach allowed the authors to separate the various pharmacological phenotypes; approximately 7% of ER+ primary cultures were stimulated by TAM and almost 3% were inhibited by physiological concentrations of estradiol—notably close to our estimate of 9% for the sum of these two clinical phenotypes.

It is important here to separate responses to physiological estrogens from those produced by pharmacological estrogen therapy. High dose estrogen therapy was used prior to the advent of TAM. As with all endocrine therapies, approximately 30% of all breast cancers (receptor status was not available when most of these studies were done) responded [31,32]. Side effects were unfavorable, probably explaining the switch to TAM that also induces responses in approximately 30% of all breast cancers (when receptor status is not considered). It is also likely that the mechanisms of action of pharmacological and physiological dose estrogens differ. Over 15 years

ago, we were the first to show that pharmacological concentrations of both estradiol and TAM induce changes in the membrane fluidity of breast cancer cells and that this correlates with changes in cell growth [33]. It is unlikely that membrane fluidity changes are major contributors to the action, either prosurvival or prodeath, of physiological estrogen exposures but they likely do contribute to the prodeath effects of pharmacological exposures.

2. Cell fate in the context of endocrine responsiveness

Therapeutic strategies for breast cancer generally aim to alter the balance between cell death and cell survival such that cancer cells (but ideally not normal cells) die. However, endocrine therapies consistently also induce a notable growth arrest in sensitive tumors. The relative importance of growth arrest and cell death remains unclear. To explore this issue, we will first discuss the forms of cell death and then compare the potential for cell death and cell growth arrest to contribute to endocrine responsiveness.

Cell death pathways include signaling to apoptosis, autophagy, mitotic catastrophe, necrosis, and senescence. Late events in cell death are reasonably well defined at the molecular (such as PARP cleavage) and cellular levels (including DNA disintegration). However, knowledge of the regulatory signaling upstream of these events, and how this signaling is integrated and processed, is now known to be incomplete. Mitochondrial function and integrity, regulated in part by BCL2 family members, are central to several forms of cell death [34–36].

2.1. Apoptosis

Apoptosis is a programmed cell death defined by morphological criteria related to organized chromatin condensation and fragmentation of the cell nucleus, accompanied by cleavage of DNA, formation of apoptotic bodies, cell shrinkage, and ruffling of the cell membrane [35,37,38]. Two major pathways are involved. The *intrinsic (mitochondrial) pathway* is regulated by the proapoptotic and antiapoptotic BCL2 family members; this pathway involves changes in mitochondrial membrane permeability (MMP), release of cytochrome c, exposure of phosphatidylserine on the outer leaflet of the plasma membrane, and the eventual loss of plasma membrane integrity [39]. The *extrinsic (cell surface receptor) pathway* is dependent upon extracellular signals including tissue necrosis factor-α (TNFα), Fas ligand, and TNF-related ligand TRAIL [37,38]. The intrinsic and extrinsic pathways activate caspases, the "executioners" of apoptosis, which cleave DNA and catabolize the cytoskeleton. Apoptosis is not a discrete process and occurs over time—early (4–18 h), middle (18–36 h), and late stages (≥36 h) are often described based largely on data from cell culture models. Changes in specific BCL2 family members (early events that can precede changes in MMP), changes in MMP, and the exposure of phosphatidylserine are generally interpreted as representing early-to-middle apoptosis. Cytoplasmic cytochrome c release from mitochondria, changes in propidium iodide staining, increased terminal transferase dUTP nick end labeling (TUNEL) and cleavage of the DNA repair enzyme PARP-1 are associated with late apoptosis or necrosis [35].

2.2. Autophagy

Autophagy is a lysosomal pathway where cytoplasmic contents are degraded by double/multi-membrane vacuoles or autophagosomes, normally resulting in the removal of defective or damaged organelles, *e.g.*, mitochondria. A better understanding of the regulation of autophagy has recently begun to emerge; key regulators are now known to include BCL2 family members [40,41] and their interacting proteins such as beclin-1/ATG6 (BECN1) [42]. BCL2 antiapoptotic proteins can block autophagy by inhibiting BECN1 [36]. Since monoallelic loss of the BECN1 locus is seen in >40% of breast cancers [43] (and in MCF-7 cells), modulating BCL2 may be an effective mechanism for regulating BECN1-activated autophagy. Autophagy can be identified by the absence of marginated nuclear chromatin, the presence of cytoplasmic vacuoles using transmission electron microscopy or monodansylcadaverine [44,45], cleavage of the LC3B protein [46,47], and regulation of the p62/SQSTM1 protein [48]. Early events in autophagy may be reversible; later events may (or appear to) share mechanisms with other cell death pathways. For example, cleavage of ATG5 by caplain [49] or upregulation of BID [41] can cause a switch from autophagy to apoptosis.

Paradoxically, autophagy can act as a cell survival mechanism when extracellular nutrients or growth factors are limited, or as an alternative cell death pathway to apoptosis [50]. Prosurvival outcomes likely reflect an adequate adjustment to stress, with energy/nutrients recovered from the organelles "digested" in the autophagosomes. Prodeath outcomes may arise when the self-digestion of autophagy leads to such a loss of organelles that the cell can no longer survive. In cancer cells, autophagy induction can accelerate cell death [51–55] or promote cell survival [56–58], independently or in response to treatment with cytotoxic agents.

2.3. Mitotic catastrophe

Faulty DNA structure checkpoints, or the spindle assembly checkpoint, are key components of this form of cell death [59,60]. Disruption of the normal segregation of many chromosomes results in rapid cell death [59]. When this cell death does not occur, the cell can divide asymmetrically and produce aneuploid daughter cells [61] that can become neoplastic [59,61]. Thus, mitotic catastrophe is characterized by multinucleation.

2.4. Necrosis

Necrosis is a chaotic process marked by cellular edema, vacuolization of the cytoplasm, breakdown of the plasma membrane, and an associated inflammatory response caused by the release of cell contents into the surroundings. Increased permeability to trypan blue or other vital dyes, in the absence of organized chromatin condensation and DNA fragmentation, is characteristic of necrosis [44,62].

2.5. Senescence

Senescent cells are characteristically enlarged, flattened with vacuoles and a large nucleus, be come permanently cell cycle arrested and unresponsive to mitogenic stimuli and express β-galactosidase [45,63]. Normally, as telomerase activity falls over time, successive telomere shortening limits proliferation and leads to "cellular senescence" or "mortality stage 1 (M1)". Inactivation of p53 can by bypass M1 growth arrest, producing critically short telomeres and massive cell death called "mortality stage 2 (M2)" or "crisis" [64].

2.6. Endocrine-induced cell death in breast cancer

Precisely how breast cancer cells die following estrogen withdrawal (or AI treatment) or AE treatment is unclear. Senescence may not be the dominant mechanism, since this process frequently involves DNA damage and p53 activation [38,45] but breast cancer cells respond to AEs and to estrogen withdrawal even if they have mutated p53 [35,65]. While apoptosis is clearly implicated [65–68], some of the apoptosis endpoints in prior studies may not

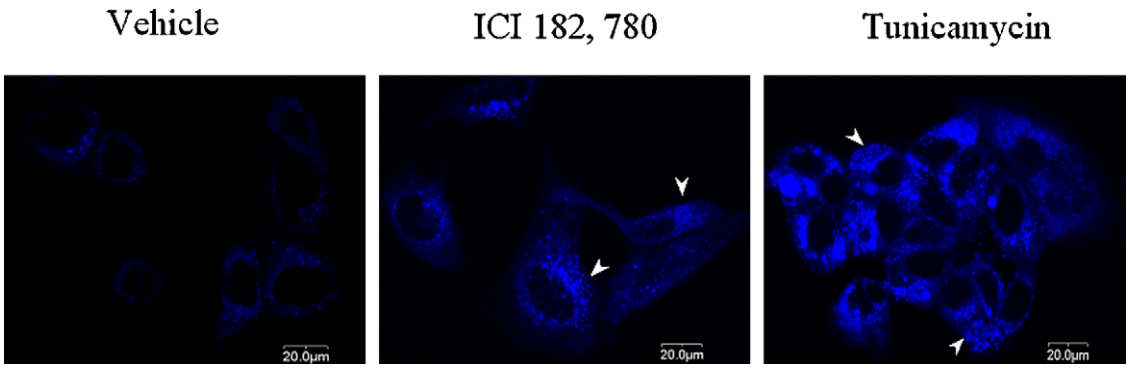

Fig. 1. Autophagy is enhanced upon FAS treatment in ER+ breast cancer cell lines. MCF-7 cells were treated with FAS (ICI 182,780), the endoplasmic reticulum stress and autophagy inducer tunicamycin (TUN), or ethanol control (vehicle) prior to staining with monodansylcadaverine (MDC). Increased MDC staining indicates that autophagy has been induced.

distinguish among earlier events more closely implicated with signaling initiated through autophagy. Autophagy has been implicated in response to endocrine therapy [69–71] and we also see the induction of significant autophagy associated with endocrine therapies.

Fig. 1 shows our ability to detect significant changes in the number of autophagosomes as measured by an increase in the presence of cytoplasmic vacuoles identified by monodansylcadaverine staining [44,45] (Fig. 1), increased cleavage of the LC3 protein [46,47], and reduced expression of p62/SQSTM1 [48,72–74] (Fig. 2). We have previously shown, as have others, that AE treatment and estrogen withdrawal are also accompanied by increases in the level of apoptosis and growth arrest in sensitive cells. Indeed, when restoring AE sensitivity in resistant cells we frequently see that sensitivity is reflected in the restoration of an ability of the antiestrogen (or estrogen withdrawal) to both increase apoptosis and reduce proliferation [75,76]. As shown in Figs. 1 and 2, and consistent with other reports [69–71], prodeath autophagy also is associated with the growth inhibitory effects of endocrine therapies in breast cancer cells. Thus in experimental models, cells responding to endocrine therapies concurrently experience an increase in cell growth arrest accompanied by both apoptosis and a prodeath autophagy.

2.7. Proliferation, cell death, and endocrine responsiveness

One of the most consistent observations in both experimental models *in vitro* and *in vivo* and in clinical specimens is the ability of endocrine therapies to induce a profound growth arrest in sensitive breast cancer cells. However, the relative importance of increased cell death compared with reduced proliferation is not entirely clear. In most endocrine sensitive experimental models, growth arrest and cell death concurrently occur and both clearly

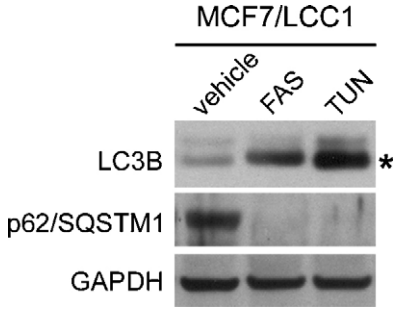

Fig. 2. Autophagy is enhanced upon FAS treatment in ER+ breast cancer cell lines. MCF7/LCC1 cells were treated with FAS, TUN, or vehicle prior to lysis and immunoblotting using standard procedures. Increased LC3BII (asterisk) and decreased p62/SQSTM1 expression both indicate that autophagy has been induced.

contribute to the ability of endocrine therapies to affect changes in anchorage-dependent cell number, anchorage-independent colony formation, or tumorigenesis over time [27,77,78]. Less clear is their relative contribution in driving clinical responses to endocrine therapies. Growth arrest appears to be readily detected in breast tumors responding to endocrine therapy. Less clear is the ability to detect robust changes in apoptosis. Some investigators do [79], and some do not [66], see an association of apoptosis or a molecular maker(s) of apoptosis with clinical response. The latter is in marked contrast to studies in experimental models. For some studies, response is related to molecular markers of apoptosis such as BCL2 [79] or the FasL:Fas ratio [80]. Notably, expression of the anti-apoptotic molecule BCL2 is reduced in responsive breast tumors by 3 months of TAM treatment [79], while in breast tumors that remain after TAM therapy BCL2 expression is elevated [81]. However, as noted above, BCL2 can affect both an apoptotic and autophagic cell death and its measurement alone is likely a poor predictor of any specific cell death mechanism.

If cell death does not occur in clinical breast cancer this observation clearly requires explanation. Several possible explanations exist—in the absence of compelling experimental/clinical data supporting or eliminating these explanations we make no assessment at this time on their relative merits. Firstly, it should be noted that measures of apoptosis are usually the primary endpoints for assessing rates of cell death. Our previously published results, the data in Figs. 1 and 2, and the work of others [69–71] show that estrogen withdrawal or antiestrogens increase both the rates of apoptosis and autophagy in breast cancer models responding to treatment. We interpret this as a prodeath autophagy in sensitive cells, consistent with other reports [69–71]. It remains unclear whether autophagy or apoptosis dominates as the cell death mechanism or whether this varies among different breast cancer cells. Measuring apoptosis may be the wrong measure of cell death in tumors, or it may be an inadequate measure if it represents only some proportion of cells that die through this process. Secondly, apoptosis is often considered to comprise early, mid and late stages, and an irreversible commitment to cell death may not be robustly associated with endpoints other than those definitively reflecting late stage apoptosis. A measure of apoptosis that is not robustly associated with ultimate cell death could provide an incomplete assessment of the rate or extent of cell death. Thirdly, if the timing of apoptosis is as fast in patient tumors as it is *in vitro*, measurements taken before 24–36 h and/or after 36–48 h could miss many of the key events. The most sensitive cells would have been through apoptosis and be already dead and gone, and the rate of apoptosis could have returned to the basal level. Fourthly, duration of the apoptotic response may differ between basal apoptosis and drug-induced apoptosis. If drug-induced apoptosis leads to a more rapid death, the number of cells processing though apoptosis could increase

without any detectable change across time in the apparent rate of apoptosis.

Finally, a reduction in cell proliferation alone could be sufficient to account for some shrinkage of tumor size, as the rate of cell replacement might no longer be sufficient to account for cell loss from either a basal rate of cell death and/or loss to migration and metastasis. However, unless almost all growth arrested cells also undergo some form of cell death, it is unclear why growth arrest alone should lead to large and relatively rapid reductions in tumor size (over several weeks compared with often many years of presumably much longer growth prior to clinical detection and treatment). Growth arrest alone may be sufficient to account for good responses in some tumors, particularly where there is a high basal rate of cell death. However, it is not immediately clear how this applies to tumors with an inherently low rate of proliferation, whether because the growth fraction is large but cycling slowly or the growth fraction is small but proliferating rapidly. This is an area where mathematical modeling could be particularly useful, since it could compare the effect sizes needed for relative changes in proliferation and cell death to affect predicted overall tumor size over time.

While there is currently no definitive understanding of the primary cell death mechanisms in either experimental models or in breast tumors in women, or of the relative importance of endocrine therapy-induced changes in proliferation compared with cell death, there are potentially important implications for the underlying biology of the cancer cells. If the primary driver of response as seen in tumor shrinkage is a reduction in proliferation, this will leave many cells alive and still metabolically active. Surviving cells have the ability to adapt to the endocrine-induced stress and eventually overcome the proliferative blockade and grow—they will become resistant. This process seems unlikely to occur in many of those women who receive the clear long term benefit of a significant reduction in the risk of death [8,9].

Whether it is the growth arrested but surviving cells that eventually become resistant is unknown but it is certainly an intuitively satisfying hypothesis. Moreover, this hypothesis is supported by the ability to take sensitive cells in culture, expose them for prolonged periods to either estrogen withdrawal or AE treatment, and eventually induce an acquired resistant phenotype [27,28,77,82]. This process is accompanied by a profound and prolonged period of growth arrest prior to the emergence of resistant cells, a pattern consistent with the clinical progress of the disease in tumors that initially respond to therapy but that eventually recur—often a decade or more after the initiation of TAM treatment.

3. Molecular signaling and resistance

The precise mechanisms of resistance to an AE and/or an AI remain unclear, reflecting an incomplete understanding of the signaling affecting cell proliferation, survival, and death and their hormonal regulation in breast cancer. We have previously reviewed the mechanisms of resistance to AEs and to estrogen deprivation elsewhere in some detail [10,23,29], so we focus here on the molecular signaling aspects of resistance and how these may be integrated and explored using emerging technologies. We will focus primarily on signaling to cell death—signaling to regulate proliferation in the context of endocrine responsiveness will be the subject of a separate review.

The primary technologies that have matured sufficiently to enable global approaches to network modeling include gene expression microarrays, ChIP-on-chip, SNP chips, high-throughput DNA sequencing, and array CGH. Each of these technologies has reached a high level of maturity, and each is characterized by the generation of very high dimensional data on each sample whether the read-out be genomic or transcriptomic data; this also is true of the emerging high-throughput proteomic technologies. The remarkable volume of data, and the diversity of biological information that informs the interpretation of these data, has begun to transform the fields of biostatistics, computer science, and bioinformatics. However, the properties of these datasets are often not fully understood nor are the challenges these properties provide for data analysis and network modeling. Readers interested in exploring some of these challenges can read recent reviews [83,84]. Here we will address briefly several approaches to the use of these data for network modeling.

3.1. A network signaling hypothesis of endocrine responsiveness

Estrogen-independence and AE resistance are complex phenotypes and both genomic and non-genomic activities are implicated [10,33,85]. We consider it unlikely that endocrine resistance in ER+ tumors is driven by a single gene/signaling pathway. Unlike many previous single gene/pathway studies, our central hypothesis invokes a gene network that confers diversity and redundancy in signaling [10,86]. The cell death/survival network incorporates specific signaling as affected by estrogen and AE modification of ERα function. Thus, AEs regulate this network differently than other agents such as cytotoxic drugs.

Signaling leads first to the reversible initiation of several cell death/survival signaling pathways within the network. The irreversible machinery of cell destruction is activated at some later point. This machinery may induce common outcomes – such as activation of effector caspases and DNA/plasma membrane disintegration – independent of the early specific initiating signals. Hence, we envision multiple concurrent signals processing through this network, some prosurvival and some prodeath, with cell fate reflecting the dominant signaling. In endocrine resistant cells, endocrine regulation and/or function of components of this network are changed and prodeath signals are either no longer induced or dominant.

This cell fate signaling network hypothesis is intuitively logical and certainly testable. Evidence that cells induce prosurvival signaling in an attempt to circumvent stressors implies that some cells are successful and ultimately survive whereas others are unsuccessful and die. Thus, the balance between prosurvival and prodeath signaling is likely the final arbiter of cells fate [83]. While this remains an area of active investigation, we first discuss the basic principles of network modeling and then provide an example of a seed-gene network of endocrine-regulated signaling in endocrine responsiveness.

3.2. Basic concepts of gene networks

Cellular signaling occurs more in the context of interactive networks than through linear pathways [83]. The basic topology of a network is defined by nodes (genes/proteins) and their interconnections (edges). Interconnections are multi-faceted and include one-to-one, one-to-many, or many-to-one relationships, and feed-forward or feed-back loops. The dynamic activity of a network is constrained by the various forms of interactions, and the network behaves only in certain ways and controlled manners in response to changing cellular conditions or external stimuli [87]. While often built solely from gene expression microarray data, these data are high dimensional and contain spurious correlations that can confound simple solutions for network building [83,84]. Relevant events also occur in the genome and proteome, some of which can affect the transcriptome. For example, a transcription factor (TF) may be activated by phosphorylation and bind to responsive elements in the genome but the regulation of its downstream targets is seen in the transcriptome [83]. An example of this relationship is

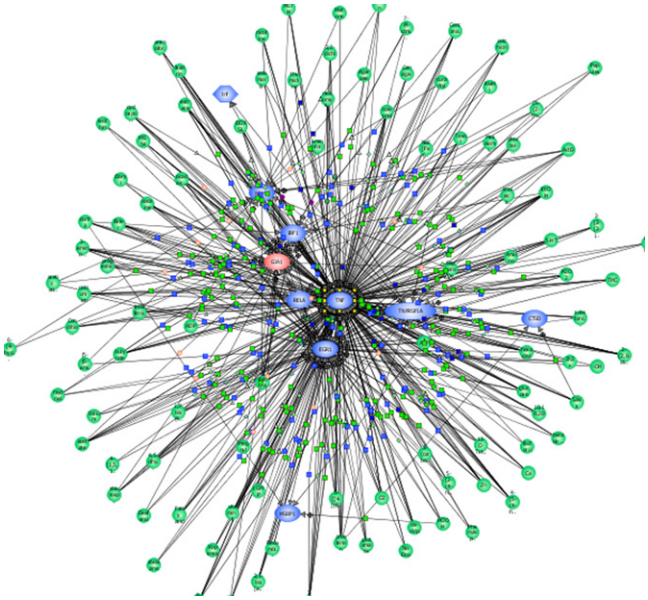

Fig. 3. Illustration of the complex and challenging nature of pathway analysis. Genes identified as being differentially expressed in resistant MCF7/LCC9 cells by SAGE and gene expression microarray were analyzed by Pathway Architect (Stratagene) to identify relationships *in silico*.

the ligand-independent activation of ERα following its phosphorylation on SER118 by MAPK [88].

Simplistically, there are two basic approaches to network modeling of high dimensional data: top-down and bottom-up. The former is probably the most widely used approach as several accessible commercial software packages are available that make this an easy task to perform without the need for training in biostatistics or bioinformatics. These packages often apply various implementations of gene ontologic and semantic search algorithms that identify cellular functions and pathways to which individual nodes are assigned; these data are then graphically represented.

The solutions produced by several popular top-down algorithms are often characterized by representations of tens-to-hundreds of nodes linked by hundreds-to-thousands of edges, making interpretation challenging (Fig. 3). Whether the algorithms address the confounding properties of high dimensional spaces, such as the curse of dimensionality or the confound of multimodality, or incorporate the critical aspects of cellular context and alleviate the trap of self-fulfilling prophesy, is not clear [83]. Among the additional challenges are the incompleteness of relevant biological knowledge and the annotation error rate in the source databases searched by these algorithms [83]. Nonetheless, these approaches can be useful when carefully applied and their limitations fully understood, and when experts from both the biological and mathematics domains combine expertise to assess the validity of the solutions. Currently, such approaches probably have most to offer in the area of hypothesis generation, rather than in the construction of truly biologically meaningful signal transduction networks.

3.3. The "seed-gene" approach to network modeling

The bottom-up approach is generally referred to as the "seed-gene approach" to network modeling [89]. This approach requires the extraction of a small number of seed genes from within the primary data; these genes are then used to grow the network in several ways. We will not address all the various approaches in this review but provide a few brief examples. Various modeling methods can be applied to find and link adjacent nodes, growing the network *de novo*. Local subnetworks can be identified and overlaid or linked to the initial seed genes. A simple approach is the incorporation of a canonical pathway (which may be a subnetwork in what would be a final and much broader network) when it is known to be relevant in the cellular context under study and where incorporating the nodes and edges of the canonical pathway members is consistent with statistical properties of the growing model topology.

Knowledge of how a gene (node) affects the expression/function of another node provides directional connectivity information that can be applied to the interacting nodes. Transcription networks can be grown (or transcriptional edges between nodes in a network that incorporates other biological knowledge) by linking TFs to their downstream targets. These targets can be predicted using specific algorithms [90–93]; where possible it is preferable to incorporate functional data such as that obtained from ChIP-on-chip arrays [91]. Thus, interacting nodes can be identified along with the directionality of their edges as the seed-gene network is grown.

The most labor intensive approach is to derive experimentally nodes and edges, growing the network using definitive laboratory-derived knowledge. Where additional high-throughput data are already available, such as ChIP-on-chip, this is preferable. Currently, functional data is probably more often obtained one gene at a time, using standard molecular methods such as gene knock-down and overexpression. This laborious approach is becoming supplanted with the emerging functional genomic methods such as siRNA, ribozyme, or antisense libraries that can test experimentally the contribution of hundreds to thousands of genes. These methods enable investigators to extract concurrently nodes that experimentally generate biologically appropriate changes in the phenotype under investigation.

Once seeds and their edges are identified, and functional biological metadata obtained, interactive models can be grown using neural network and other machine learning tools. Several models have been proposed to reveal the behaviors of regulatory networks from gene expression data [22,23] including Boolean networks [24–26], Bayesian networks [27–30], linear additive regulation models [31,32], state-space models (SSMs) [33,34], and recurrent neural networks (RNN) [35,36]. However, these methods use only mRNA expression data to infer networks.

Integrated approaches have been recently proposed to learn transcriptional regulation from various data sources [27,30,37–43]. An iterative search on mRNA expression and ChIP-on-chip data [37], or the incorporation of expression profiles, ChIP-on-chip, and motif data [41] have each been used in yeast to discover transcriptional networks. Several linear models or matrix decomposition methods have also been proposed [43–46]. Network component analysis (NCA) is a notably powerful approach [45] but NCA and these other methods cannot easily infer regulatory networks in biological systems more complex than yeast.

Other limitations exist in network modeling. Complete biological knowledge for topology estimation (node–node edges and directionality), such as high-throughput ChIP-on-chip data or functional data from laboratory experiments, are often not (or only partially) available for human cells. When heterogeneous data sources are integrated for computational inference, the consistency of different data sources is often inadequate or unknown. Topological knowledge also comes from biological experiments, which often contains false positives/negatives that can lead to incorrect network inference.

4. Seed-gene model for cell signaling and the regulation of cell fate

While we continue to develop new methods for network modeling, we have yet to report our modeling approaches to our own expanding data sets. Hence, we will here describe our initial studies

on the use of seed genes and experimental data to construct a simple wiring-diagram of our initial seed-gene network. The inability to induce signaling to irreversible cell death is a central component of drug resistance [94]. Thus, we propose that cells possess a common cell death/survival regulatory decision network of integrated and/or interacting pathways (see above).

Prior to building network models, it is necessary to extract initial nodes (seed genes) from which a network can be built [89]. Since ER is a TF and regulates other functionally relevant TFs that influence endocrine responsiveness and cell fate, selecting a small number of TFs as seed genes is reasonable for network modeling. The full list of *relevant* ER-regulated TFs that may affect cell fate is unknown. Nonetheless, our published data support the central hypothesis that that IRF1 [65,95–97], XBP1 [76,95] and NFκB (RELA) [75,95] are key regulatory nodes or control key modules in this network. Moreover, our experimental data in endocrine sensitive and resistant breast human cancer cells now allow us to map their edges and directionality, in an appropriate cellular context, with some confidence.

4.1. X-box binding protein-1 (XBP1) and the unfolded protein response (UPR)

UPR is a central component of the endoplasmic stress response [98], an adaptive signaling pathway that allows cells to survive the accumulation of unfolded proteins in the endoplasmic reticulum lumen [99]. Initially a compensatory mechanism allowing cells to recover normal endoplasmic reticulum function, a prolonged UPR may induce cell death. UPR, which can be induced by cellular stressors such as hypoxia, is activated by each of three molecular sensors: IRE1α, ATF6, PERK [100]. XBP1's *unconventional* splicing (occurs in the cytosol) by IRE1α is an obligate component in both IRE1α- and ATF6-induced UPR [100,101]. The UPR (initiated by XBP1 splicing by IRE1α) can activate autophagy [102]. Whether this is a pro-survival or prodeath form of autophagy is unknown, since UPR activation also can induce both prodeath and prosurvival outcomes [103].

XBP1 is a transcription factor that belongs to the basic region/leucine zipper (bZIP) family [104,105]. The unspliced form, XBP1(U), has a molecular weight of ~33 kDa and acts as a dominant negative of spliced XBP1 [106,107]. The spliced form, XBP1(S), has a molecular weight of ~54 kDa; splicing removes a 26 bp intron and creates a translational frame-shift. Regulation of transcription by XBP1(S) is a consequence of its homodimers activating specific cAMP response elements (CREs) with a conserved ACGT core sequence GATG**ACGT**G(T/G) NNN(A/T)T—sometimes called the UPR element [103,104,108]. XBP1(S), which is implicated in affecting plasma cell differentiation [109], is essential for fetal survival, neurological development, bone growth, immune system activation, and liver development [110,111]. XBP1 is also rapidly induced in response to estrogen-stimulation [112,113]. Consistent with the work of others [108], we have shown that XBP1(S) can bind to and activate ERα in a ligand-independent manner (Fig. 4).

We have recently shown that XBP1(S) confers E2-independence (effectively an AI resistant phenotype) and AE crossresistance (TAM and FAS crossresistance) in both MCF-7 and T47D human breast cancer cells [76]. This activity appears to be driven primarily by XBP1(S), as introduction of the full-length XBP1 cDNA in either MCF-7 or T47D cells generates predominately the XBP1(S) protein. This observation suggests that the basal activity of IRE1α is already adequate and that XBP1(S) is the rate limiting protein. XBP1 is the only known substrate for the IRE1α endonuclease and only IRE1α can splice mammalian XBP1. Since XBP1 splicing is thought to function primarily within the UPR, breast cancer cells may be primed to respond to multiple stressors by activating a prosurvival induction of UPR.

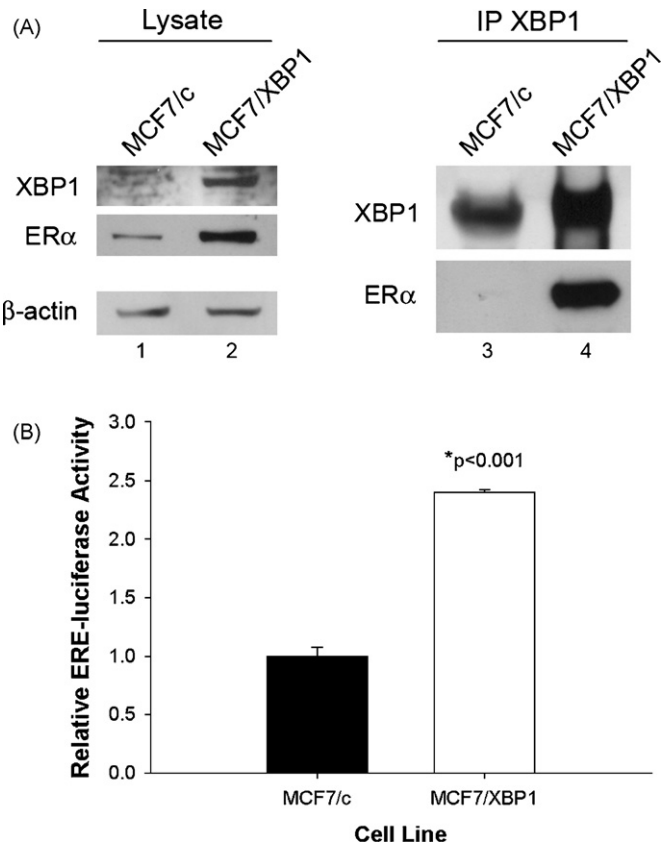

Fig. 4. Physical association of XBP1 and ERα is accompanied by robust ERE-driven transcriptional activity in MCF7/XBP1 cells. (A) MCF-7 cells stably expressing XBP1 cDNA or the empty vector control (c) were treated with FAS or ethanol control (ctrl.) vehicle prior to lysis and immunoblotting (lanes 1 and 2) or co-immunoprecipitation of XBP1 and ERα (lanes 3 and 4) using standard procedures. (B) MCF7/c and MCF7/XBP1 cells were transiently co-transfected with plasmids encoding 3xERE-luciferase and phRLSV40-Renilla for 24 h prior to lysis and promoter–reporter luciferase assay by standard methods. Data are presented as mean relative ERE-luciferase activity ±SE for a representative experiment performed in triplicate, $^{*}p < 0.001$.

4.2. Interferon regulatory factor-1 (IRF1)

RFLP linkage analysis assigned the IRF1 gene to 5q23-31; more definitive studies identified the locus as 5q31.1 [114]. IRF1 was initially identified because of its transcriptional activation of type I interferon (IFN) genes. We first showed the ability of interferons to sensitize breast cancer cells to TAM over 20 years ago [115]. More recently, IRF1 was implicated in T-cell development [116], and it is now known also to coordinate expression of the immunoproteasome [117], to regulate human telomerase activity [118,119], and to regulate key aspects of DNA damage repair [120,121]. Loss of IRF1 increases tumorigenicity in mouse models driven by ras or loss of p53 [122]. These activities may reflect IRF1's ability to signal to apoptosis [123], which can occur in a p53-dependent or -independent manner [120,124], with or without induction of $p21^{cip1}$ [124] or $p27^{kip1}$ [125], and through caspase-1 [120], caspase-3 [96], caspase-7 [96,126], caspase-8 [96,127], and/or FasL [128].

Following our initial observations of IRF1's likely role in breast cancer [129–131] and antiestrogen resistance [129], we confirmed its functional involvement using a dominant negative approach (dnIRF1) [65]. IRF1 and dnIRF1 induce opposing effects on proliferation *in vitro* and tumorigenesis *in vivo* through regulation of caspases-3/7 and caspase-8 activities [96]. These observations are consistent with the effects of inoculating an adenoviral vec-

tor containing IRF1 directly into mouse mammary tumors [132]. While p53-dependent apoptosis occurs in the breast [133], T47D cells express mutant p53 and our data show that intact p53 is not required for the proapoptotic actions of IRF1 [65,96]. In AE sensitive breast cancer cells, inhibition of AE-induced IRF1 activity by dnIRF1 is accompanied by reduced proapoptotic activity [65]. These observations on IRF1 and AE responsiveness have been confirmed and extended by others in both normal [134] and other neoplastic breast cell culture models [135,136]. IRF1, which can signal through both p53-dependent and -independent mechanisms [120,124], provides a new and potentially important signaling molecule for integrating and regulating breast cancer cell survival in response to AEs.

4.3. Nuclear factor kappa B (NFκB)

The NFκB p50/p65 heterodimer complex comprises two homologous proteins; the p50 product of its p105 precursor (NFκB1; chromosome 4q24) and the p65 (RELA; 11q13). NFκB is maintained in the cytosol in an inactive state, bound with members of the IκB family that inhibit nuclear transport or block NFκB's nuclear translocation signal [137]. Activation usually proceeds by the IKK kinase complex phosphorylating IκB, resulting in IκB ubiquitination and degradation [138]. NFκB (RELA/NFκB1) is implicated in several critical cellular functions [139]. Reflecting its regulation by both estrogen and growth factors [140,141] that are involved in endocrine resistance [10,142], normal mammary gland development is dependent upon NFκB [143]. Increased NFκB activity arises during neoplastic transformation in the rat [144] and mouse mammary gland [145]. Upregulation of NFκB is associated with E2-independence [140,143]. The predominant NFκB form in breast cancer cell lines is RELA/NFκB1; the p52 family member also is expressed in some breast cancers [146].

We have shown that NFκB can confer estrogen-independence and AE crossresistance [75,95,147]. Estrogen-independent growth *in vitro* and *in vivo* is supported by increases in both NFκB DNA binding activity and expression of BCL3 [147]. This study highlights the functional implications of NFκB in AI resistance. Expression of IκBα (NFκB repressor) in estrogen-independent LCC1 cells (LCC1 cells are derived from MCF-7 and are estrogen-independent but sensitive to AEs [148]), which have increased NFκB activation relative to estrogen-dependent MCF-7 cells, eliminates their estrogen-independence *in vivo*.

LCC9 cells (TAM and FAS crossresistant variant of LCC1 [28]) exhibit a further increase in NFκB expression and activation relative to LCC1 cells, apparently driven by increased expression of NEMO (IKKγ) [75]. These observations imply that the level of activity in LCC1 cells is adequate for estrogen-independence but not AE resistance. Increased activation of NFκB [95] and loss of its antiestrogenic regulation in LCC9 cells [75] suggest that these cells might be dependent upon NFκB for survival/growth. Thus, we compared the growth response of LCC1 and LCC9 cells to vehicle or parthenolide (300 and 600 nM), a small molecule inhibitor of NFκB [149]. Parthenolide produces a dose-dependent inhibition of MCF7/LCC9 cells with an apparent IC_{50} of approximately 600 nM ($p < 0.01$ at both 300 and 600 nM parthenolide). In marked contrast, parthenolide does not affect growth of LCC1 cells at either of these concentrations [75]. We next asked if parthenolide can re-sensitize LCC9 cells to FAS-mediated apoptosis. FAS and parthenolide synergize to induce LCC9 cell death [75]. Since FAS alone is inactive [28], this synergism reflects at least a partial reversal of the FAS resistance component of the LCC9 cell phenotype and implicates NFκB as a key determinant [75]. Thus, AE crossresistant cells exhibit a greater reliance upon NFκB signaling for proliferation, and inhibition of NFκB restores their sensitivity to apoptosis induced by FAS [95].

4.4. Expression of ER, PGR, XBP1, NFκB and IRF1 in breast tumors

Using gene expression microarrays, we previously compared the global structures of the transcriptomes of three ER+ human breast cancer cell lines (MCF-7, T47D, ZR75-1) and 13 human breast tumors (11 ER+; 2 ER−) and showed these to be notably similar to ER+ breast tumors from patients [150]. The striking similarities between cell lines and tumors are supported by a report that the estrogen-regulated genes in these cell lines are similarly regulated in breast tumors [151]. These data show that ER+ breast cancer cell lines and ER+ breast tumors in women share global similarities in the structures of their respective transcriptomes [150], and that these cell lines are appropriate models in which to identify clinically relevant endocrine-regulated molecular events [150,151]. Nonetheless, it is necessary to show that the seed genes we have selected are likely to be relevant to the biology of ER+ breast tumors.

To begin to explore the possible clinical relevance of these functional studies, we first asked if we could detect XBP1, NFκB, and IRF1 in breast tumors. We then asked whether any of these proteins were coexpressed in patterns consistent with the experimental data from cell lines. Using a series of breast cancer tissue arrays comprising 480 cores from 54 breast carcinomas (mostly ER+ tumors), we applied immunohistochemistry to explore the expression of the seed genes [152]. Pairwise correlation analyses cannot account for the possibility that unknown associations among proteins may confound each other, so we applied a novel use of partial correlation coefficient analysis. Partial correlation analysis allows an estimate of the correlation between two variables while controlling for a third, fourth and/or fifth and is particularly useful in the analysis of small signaling networks of 3–5 variables [153].

We confirmed the well established coexpression of ERα and PgR, implying that the samples are representative of most ER+ breast cancers. XBP1, NFκB, and IRF1 are each found in a high proportion of breast tumors [152]. Total XBP1 was measured, as XBP1(S) antibodies were not then available. XBP1 staining is variable but detectable in 79% of breast tumors. A very recent study has reported a significant association between XBP1(S) mRNA and poor response to endocrine therapy [154]—entirely consistent with our studies in breast cancer cell lines [76]. 57% of the tumors express detectable RELA in their neoplastic cells, similar to a prior study of $n = 17$ breast tumors [146].

Expression of several of the proteins is correlated in breast tumors. IRF1 correlates with ER and PGR, and also with RELA and XBP1. While, these correlations depend on the subcellular localization of IRF1 and some are direct and others inverse correlations, they are fully consistent with the interpretation that these expression patterns reflect functionally relevant signaling links. For example, we might predict that IRF1 sequestered in the cytosol, unlike that in the nucleus, cannot act as a proapoptotic TF (the full coexpression patterns are described detail in the report by Zhu et al. [152]). We also find coexpression of XBP1 and RELA, consistent with the observation that XBP1 may be downstream of NFκB [109]. When each of the significant correlations is examined in the partial correlation coefficient models, the IRF1, NFκB, and XBP correlations remain [152]. These data are consistent with these three reflecting some component of a larger signaling network active in some ER+ breast cancers and further support their selection as seed genes from which to grow this network and understand its topology and function. Moreover, the functional data from our experimental models implies that this network links signaling and function through two key subcellular components—mitochondria and the endoplasmic reticulum.

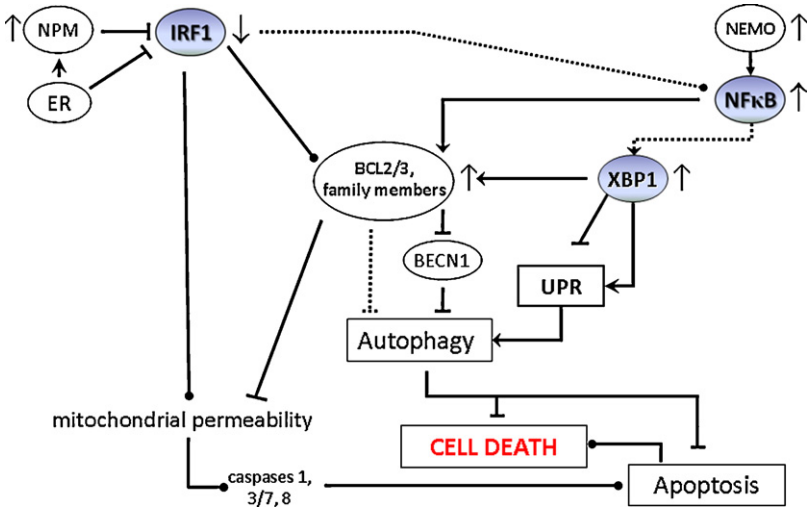

Fig. 5. Endocrine resistance seed-gene network. Simple representation of a seed-gene network of XBP1, NFκB and IRF1 based on functional data obtained from an appropriate cellular context (resistant MCF7/LCC9 cells).

4.5. Simple representation of a seed-gene network of XBP1, NFκB and IRF1 based on functional data obtained from an appropriate cellular context

The experimental data supporting the wiring-diagram representation of the network model shown in Fig. 5 are discussed the preceding sections. Here we discuss how the signals may flow through this network. The three primary seed genes of IRF1, XBP1, and NFκB are evident as previously proposed [95]. IRF1 expression is repressed in resistant cells [95] but induced by antiestrogens in sensitive cells [65]. A dominant negative IRF1 confers an antiestrogen resistant phenotype, implying that IRF1-driven prodeath signaling is key to the regulation of cell fate [65].

In addition to changes in the expression of IRF1, the upregulation of NPM expression [95,155] could also affect IRF1 action. Both NPM and IRF1 are estrogen-regulated genes in MCF-7 cells, IRF1 expression being suppressed, whereas NPM is induced [129,155]. Since NPM inhibits the transcription regulatory activities of IRF1 [156], the increase in NPM expression could bind remaining IRF1 and inhibit its ability to initiate an apoptotic caspase cascade. We also cannot exclude the possibility that NPM has activities independent of blocking IRF1, since NPM overexpression is sufficient to transform NIH 3T3 cells in a standard oncogenesis assay [156]. Increased levels of serum autoantibodies to NPM predict recurrence on TAM 6-months prior to clinical detection [157].

IRF1 and NFκB are known to form heterodimers and to regulate directly gene expression [158,159] including that of the inducible nitric oxide synthase promoter [158]. Since we do not know if it is primarily the gene regulatory effects of these heterodimers, or if their subcellular location is key (they act by preferentially sequestering one or the other so that transcriptional regulation does not occur), this is shown as a dotted line. We would predict, based on the inverse expression between NFκB and IRF1 in LCC9 cells [95] and in some breast cancers [152], that either the prodeath effects of any remaining IRF1 are being sequestered by NFκB in resistant cells and/or that the overexpression and activation of NFκB leads to a dominance of its prosurvival activities. The increased sensitivity of resistant cells to parthenolide is consistent with the functional relevance of at least the latter signaling outcome [75].

We have previously shown that the upregulation of NFκB in antiestrogen resistant cells [95] is likely driven in part by increased NEMO/IKKγ activity [75]. The prosurvival activities of NFκB are well documented [160]. Precisely how NFκB regulates cell survival remains to be fully established but activation of prosurvival members of the BCL2 gene family are involved in both acquired estrogen-independence [147] and antiestrogen resistance [75,76]. While NFκB is predicted to induce transcription of XBP1 [109], we have yet to report this direct regulation in breast cancer cells (studies are in progress). Whether or not this occurs, XBP1 is clearly upregulated in resistant cells [95] and this activity is sufficient to confer both estrogen-independence and antiestrogen resistance [76]. More recently, increased XBP1 mRNA expression has been show to predict for a poor response to TAM in breast cancer patients [154].

The central role of XBP1 within the UPR clearly implicates UPR activation in responsiveness to both estrogen-withdrawal and antiestrogen treatment [76]. UPR also is known to induce autophagy [102], although whether this is a prosurvival or prodeath autophagy remains unclear in the context of determining endocrine responsiveness. Autophagy is regulated, at least in part, by the action of BECN1. BECN1 activity is regulated by BCL2, which binds BECN1 and can block BECN1-mediated autophagy [36].

The regulation of BCL2 family members (BCL2, BCL3, and probably others) whether by IRF1, NFκB, and/or XBP1, can affect both autophagy and the intrinsic apoptosis pathway. The intersection of their signaling at BCL2 family members, as shown in Fig. 5, is one location within the broader network where the balance between prodeath and prosurvival signaling, and whether prodeath is autophagic or apoptotic, is determined. This intersection also links signaling through the UPR and endoplasmic reticulum to the mitochondria with the cell fate decision mechanisms—at least in the context of determining cell fate in the context of endocrine responsiveness in breast cancer. The signaling depicted in Fig. 5 represents only a small component of this broader network. Nevertheless, this initial wiring-diagram is consistent with a body of functional data in experimental models and it provides sufficient seed genes, their edges, and the directionality of these edges, to begin a more detailed exploration of this central network. Understanding this network's topology and function will lead to better candidates for drug discovery and to better algorithms to predict how individual tumors will respond to specific endocrine therapies.

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