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Endocrine resistance in breast cancer – an overview and update

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Abstract

Tumors that express detectable levels of the product of the ESR1 gene (estrogen receptor-α; ERα) represent the single largest molecular subtype of breast cancer. More women eventually die from ERα+ breast cancer than from either HER2+ disease (almost half of which also express ERα) and/or from triple negative breast cancer (ERα-negative, progesterone receptor-negative, and HER2-negative). Antiestrogens and aromatase inhibitors are largely indistinguishable from each other in their abilities to improve overall survival and almost 50% of ERα+ breast cancers will eventually fail one or more of these endocrine interventions. The precise reasons why these therapies fail in ERα+ breast cancer remain largely unknown. Pharmacogenetic explanations for Tamoxifen resistance are controversial. The role of ERα mutations in endocrine resistance remains unclear. Targeting the growth factors and oncogenes most strongly correlated with endocrine resistance has proven mostly disappointing in their abilities to improve overall survival substantially, particularly in the metastatic setting. Nonetheless, there are new concepts in endocrine resistance that integrate molecular signaling, cellular metabolism, and stress responses including endoplasmic reticulum stress and the unfolded protein response (UPR) that provide novel insights and suggest innovative therapeutic targets. Encouraging evidence that drug combinations with CDK4/CDK6 inhibitors can extend recurrence free survival may yet translate to improvements in overall survival. Whether the improvements seen with immunotherapy in other cancers can be achieved in breast cancer remains to be determined, particularly for ERα+ breast cancers. This review explores the basic mechanisms of resistance to endocrine therapies, concluding with some new insights from systems biology approaches further implicating autophagy and the UPR in detail, and a brief discussion of exciting new avenues and future prospects.

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1. Introduction

Breast cancer remains the most prevalent cancer diagnosed in women and the second most common cause of cancer mortality. It is estimated that almost 40,000 women die of breast cancer each year in the U.S. [1], a number that averages to approximately one death every 13 minutes. The largest single breast cancer subtype is defined by the expression of the proteins for estrogen receptor-alpha (ERα; ESR1) and/or the progesterone receptor (PR; PGR). The first molecularly target therapy for cancer, Tamoxifen (TAM), is still widely used and remains standard-of-care for ERα+ breast cancers in premenopausal women. TAM reduces the 10-year risk of recurrence by almost one-half and the risk of death by approximately one-third [2]. Aromatase inhibitors have broadly similar efficacy in postmenopausal women and they increase time to recurrence to a greater degree than TAM, although overall survival outcomes show very limited improvements over TAM [3–5]. Despite the favorable improvements in overall survival associated with endocrine therapies, more women die from ERα+ breast cancer than from any other breast cancer subtype. Moreover, the annual risks of recurrence and death, beyond the first five years after diagnosis, are generally higher for ERα+ breast cancer than for the other two subtypes [6]. ERα+ breast cancers can recur decades after diagnosis and apparently successful adjuvant interventions, evidence of emergence from dormancy in micrometastases likely already present at the time of initial diagnosis.

This overview explores some of the basic principles that have emerged in understanding how and why some breast cancers respond to endocrine therapies and others do not. The intent is to provide general insight, rather than an exhaustive review. To assist readers explore several aspects of endocrine resistance in more detail, citations to other reviews have been included liberally, rather than citations to all of the supporting primary materials.

1.1 Molecular subtypes and endocrine responsiveness

While many studies have attempted to define new molecular subtypes for breast cancer, most are not sufficiently reproducible for clinical use. Some classification schemes are no better predictors than random gene sets [7]. Even the widely cited luminal A,B,C, HER2 positive, basal, normal-like scheme [8] is not statistically robust [9]. In general, molecular classification schemes have two primary goals, (i) to estimate a patient's prognosis, and/or (ii) to determine what specific treatment a patient should receive. Some classifiers are built more to explore the molecular drivers of breast cancer and are not intended for clinical use. Despite some classification schemes being in widespread use, their limitations are often inadequately considered [7,10–13].

Molecular prognostic tools predict a patient's likely recurrence risk over a period of time, such as during the first 10 years post diagnosis; although, many patients with ERα+ breast cancer recur after this time point. Mammaprint (Agendia; based on the Amsterdam 70-gene breast cancer gene signature) is used mostly to predict the risk of distant recurrence and so can aid in the determination of which breast cancer patients may receive little or no benefit from chemotherapy. Prosigna (NanoString Technologies; based on the PAM50 score) and OncotypeDX (Genomic Health, Inc.) are focused on ERα+ breast cancers and also used mostly to determine who does not need to receive chemotherapy; most patients will still

receive an endocrine therapy. These tools do not determine which specific treatment should be used; for example they do not predict which chemotherapy to apply and generally do not influence whether or not a patient will receive endocrine therapy. Predictive markers determine which patients should receive which type of treatment. For clinical use in the selection of treatment type, the simple three gene classification scheme of ERα+ and/or PR+ (predicts for an ERα-targeted endocrine therapy of choice), HER2+ (predicts for a HER2-targeted therapy of choice), and triple negative breast cancer (TNBC; ERα−, PR−, HER2−; predicts for selection of a chemotherapy regimen of choice) remains widely used.

Tumors in the ERα+ and/or PR+ group, also called luminal breast tumors, appear to arise from within the luminal cells of the mammary duct and are candidates for an endocrine therapy such as surgical (ovariectomy) or chemical ablation (aromatase inhibitors, luteinizing hormone releasing hormone agonists), or chemical blockade of ERα function/expression (antiestrogens). Tumors in this molecular subtype account for approximately 70% of all breast cancers. A high proportion of these tumors respond to one or more endocrine therapies; approximately 50% of all patients with ERα+ breast cancer, and up to 75% if both ERα and PR are coexpressed, will benefit.

The HER2+ group represents approximately 15–20% of all breast cancers. These tumors are prime candidates for treatment with drugs that target HER2 or its signaling including Trastuzumab (Herceptin®; monoclonal antibody against HER2), Pertuzumab (Perjeta® a HER2 and HER3 dimerisation inhibitor) and Lapatinib (Tykerb®; tyrosine kinase inhibitor). A significant proportion of these tumors will respond to a HER2-targeted therapy. Almost one-half of the tumors in the HER2+ group will also express ERα and/or PR and may also receive endocrine therapy in addition to therapy that targets HER2. ERα+/HER2+ tumors generally respond to endocrine therapies, although the response rate may be lower, and the duration of response may be shorter, than ERα+/HER2− cancers [14].

The TNBC group, which comprises ~15% of all breast cancers, has no molecularly targeted therapies yet available. While often referred to as “basal-like” because they are thought to arise mostly in the basal cells of the mammary ducts, the TNBC group is molecularly diverse, and comprises at least three separate subgroups (basal, metaplastic, apocrine) [15]. Chemotherapy remains standard-of-care for these patients. Endocrine therapies are not usually administered because responses are rare in this group [2] and are generally thought to reflect false negative ERα and/or PR measurements. More recently, the role of antiandrogens as interventions for the TNBC subgroup that express androgen receptors has begun to attract attention and may offer clinical benefit to some patients [16,17].

1.2 Antiestrogens: SERMs and SERDs

Antiestrogens are drugs that act primarily at the receptor to block or compete with endogenous estrogens for activation of ERα. TAM was the first antiestrogen in clinical use [18] and it acts as a pharmacological partial agonist. Thus, TAM binds to the receptor and can exhibit both agonist and antagonist properties; these outcomes are both tissue and species specific [19]. The selectivity of responses to TAM led to it being described as a selective estrogen receptor modulator (SERM). Other examples of SERMs include raloxifene and toremifene. The agonist activity of TAM in the endometrium is thought to

partly explain the increased incidence of endometrial cancers in women receiving TAM [20]. Other SERMS do not necessarily have this agonist effect in the endometrium; raloxifene is a good example [21].

Some antiestrogens affect ERα stability and cause downregulation of the receptor protein. Fulvestrant (Faslodex®; ICI 182780) is currently the most widely studied of this growing class of antiestrogens. Often referred to as a "pure" antiestrogen [22], essentially a pharmacological antagonist not partial agonist, Fulvestrant both inhibits ERα protein dimerization and targets the receptor for degradation [23–25]. The ability to downregulate ERα protein led to it being described as a selective estrogen receptor downregulator (SERD); occasionally the "D" is described as "degrader." New SERDS are already in clinical trials and include the orally active ARN-810/GDC-810; others are well advanced in preclinical testing.

Use of antiestrogens has begun to change in recent years. For example, while TAM was standard-of-care for decades, the improved disease free survival with the aromatase inhibitors has led to them often replacing TAM as a first line endocrine therapy for postmenopausal women. Fulvestrant (250 mg) is non-inferior to some aromatase inhibitors [26–28], and appears to be more effective at the higher dose of 500 mg [29]. In their recent meta analysis, Al-Mubarak *et al.* [30] implied superiority over aromatase inhibitors where Fulvestrant was used as first line therapy or where there was a smaller proportion of cases that had earlier adjuvant endocrine interventions. Confirmation of the superiority of Fulvestrant over aromatase inhibitors awaits the outcomes of ongoing randomized clinical trials [30]. Like aromatase inhibitors, its use is mostly restricted to postmenopausal women. Whether Fulvestrant or any of the newer SERDs will begin to displace aromatase inhibitors as the first line endocrine therapy of choice for postmenopausal women with ERα+ breast cancer remains to be seen.

1.3 Aromatase inhibitors

Steroids are derived initially from cholesterol through a biosynthetic pathway that produces the progestins, glucocorticoids, mineralocorticoids, androgens, and estrogens. Estrogens are synthesized from androgens by the action of the aromatase enzyme (CYP19). From the onset of puberty until menopause the primary site of estrogen production is within the ovaries. After menopause, ovarian estrogen production ceases but other tissues in the body continue to make estrogens including the brain, adipose tissue, and muscle. Independent of menopausal status, breast tumors contain high concentrations of primarily 17β-estradiol [31,32], which in part explains why both antiestrogens and aromatase inhibitors work well in ERα+ postmenopausal breast cancers. Drugs that target aromatase (aromatase inhibitors) include the reversible inhibitors letrozole and anastrozole, and the irreversible inhibitor exemestane. Most studies suggest that these drugs induce a longer disease free survival benefit compared with TAM. However, compelling evidence of a meaningful effect size showing a major improvement in overall survival relative to TAM has been somewhat elusive [3–5]. Because of their toxicity profiles and limited efficacy in women with functional ovaries, neither the aromatase inhibitors nor the SERD Fulvestrant have yet

replaced TAM as the primary endocrine therapy for ERα+ breast cancers that arise in premenopausal women.

1.4 Heterogeneity

Breast tumors are characteristically heterogeneous. Heterogeneity is evident within and among tumors [33,34] and even among circulating tumor cells [35,36]. Intratumoral heterogeneity can be seen in the mix of cell types present in many tumors (cellular heterogeneity) and also in the expression of key markers such as ERα protein (molecular heterogeneity). It is not unusual for immunohistochemical analysis of ERα expression to show differing staining of cells across a broad range of intensities. ERα− cells are often seen in what are otherwise classified as ERα+ tumors. For example, the Allred scoring for assessing ERα and PR expression measures both the percentage of cells that stain for the protein (scale 0–5) and the intensity of staining (scale 0–3) [37]. A recent study failed to find a high prevalence of ERα positivity among circulating tumor cells from metastatic patients with ERα+ primary tumors [35]. Phenotypic heterogeneity may reflect the patterns of inheritance of a series of genetically or epigenetically diverging clonal lineages over time [38] and/or the diverse cell-cell interactions occurring within the tumor microenvironment that can affect gene expression and phenotype in cells [39]. Increased genetic instability likely further complicates heterogeneity, even though not all newly acquired mutations may be important for drug resistance. The potential for heterogeneity of ERα expression to explain drug resistance is described briefly below (Section 1.6). However, since the measurements of expression are usually done at a single time point, but ERα expression is regulated and can fluctuate over time, some cells that stain negative at the time of measurement may be capable of re-expressing detectable levels of the protein at another time. Thus, heterogeneity has both spatial and temporal aspects that may be more dynamic than is currently understood. Whether resistance to endocrine therapies is driven only by ERα expression/function is unclear; resistance phenotypes can likely change dynamically in both the temporal and spatial dimensions.

1.5 Resistance and dormancy

Dormancy describes the late recurrence phenotype, a prevalent characteristic of ERα+ breast cancers. These cancers recur years to decades after the completion of what otherwise appears to have been successful adjuvant interventions(s). While late recurrences are documented for all breast cancer subtypes, the temporal patterns of recurrence differ between ERα+ versus ERα− tumors. ERα− tumors have a high recurrence risk within the first 3–5 years after diagnosis, the annual recurrence risk falls thereafter. ERα+ breast cancers have a relatively low recurrence risk that increases over the first 3–5 years, at which point the annual risk is at its greatest. Unlike ERα− breast cancers, the annual recurrence rate remains at this peak for the rest of a woman’s life. The annual risk of death for ERα− and ERα+ breast cancers follow closely the patterns of their respective annual recurrence risks [6]. Thus, the risk of experiencing a late recurrence, which can be thought of as emergence from dormancy, is more common for ERα+ than ERα− breast cancers after approximately 5 years from diagnosis.

Various mechanisms have been proposed to explain dormancy but none has yet been fully validated. Angiogenesis is probably the most widely studied trigger for emergence from dormancy [40] but the data are contradictory, partly because it is difficult to separate cause from effect [41–43], and anti-angiogenic therapies have been mostly disappointing in advanced breast cancer. Whatever the role of angiogenesis, it is needed before any tumor emerges from dormancy and grows in size beyond ~1–2 mm^3 [40,44]. A role for altered immune surveillance in dormancy has been proposed but the data also remain controversial (reviewed in [45,46]). Rather than being the primary drivers, inadequate immune surveillance may be mostly permissive for emergence from dormancy, and angiogenesis is required only for tumor volumes to reach a clinically detectable size. Endocrine therapies affect the immune system, although much of this work has focused more on cell mediated rather than humoral immunity [47–50].

For ERα+ breast cancers, dormancy may be induced by the very treatments applied to eliminate the disease. In experimental models, a profound growth inhibition [51], reflecting G_0/G_1 cell cycle arrest [52,53], is commonly seen in sensitive cells treated with an endocrine intervention. A reduction in expression of the proliferation marker Ki67 is reported in neoadjuvant studies [54], suggesting a similar inhibition of proliferation. Since these interventions have been previously administered for 5-years, and current recommendations are for 10-years of endocrine therapy [55], the drugs may drive cells into a growth arrest that then becomes epigenetically imprinted over time. An eventual change in this imprinting could partly explain the emergence from dormancy. Emerging cells could remain “hidden” from immune surveillance, as they have likely done for the intervening years, and induce angiogenesis only as required by the tumor’s increasing size.

1.6 Acquired and de novo resistance

Resistance in cancer cells to a variety of drugs can be separated, largely in the context of response and time, into two basic patterns of drug failure. First, breast tumors that show no response to first line endocrine therapies represent *de novo* resistance. Second, tumors that show a good initial response but then regrow or recur reflect acquired resistance. It remains unclear how these two phenotypes are related, or if they are separate and unrelated. For example, many breast tumors are heterogeneous at both the cellular, molecular, and genetic/epigenetic levels. Tumors that have small fields of ERα+ cells amidst a background of predominately ERα− cells might be expected to respond poorly and for a relatively short duration. The interventions could eliminate the few ERα+ clones and the unresponsive ERα− clones could dominate over time. If the contribution of ERα+ clones in the primary tumor was small, there would be little detectable change in the clinical progress of the cancer. Such a tumor would exhibit *de novo* resistance even though it contained responsive cells. An association between lower ERα expression and a lesser extent and lower rate of response to endocrine interventions is well recognized [56].

The prevalence of cancers that lose all the ERα+ cells and become ERα− rapidly is uncertain but may reach only 10% [57]. Most sensitive ERα+ breast cancers that later recur remain ERα+, and responses to second and even third line therapies that target ERα are seen, although the frequency of response falls with increasing lines of treatment [58]. Since

many ERα+ breast cancers that do not respond do not become ERα− with treatment, then either the tumor ERα functionality was been lost, or cells have lost their dependence upon activation of ERα to drive proliferation and the presence of functional ERα is no longer a requirement for cell survival and proliferation.

The infrequency of ERα loss, and the persistence of some level of dependency on continued ERα expression, implies that tumor heterogeneity with loss of the ERα+ cells is not a frequent cause of acquired resistance. Responses to second and third line endocrine therapies also imply that selective growth of ERα− populations is not a common contributor to acquired resistance. Cancer cells appear highly adaptable in the face of stress. Most solid tumors emerge in a relatively hostile environment, where oxygen tension and nutrient availability is low, and immune cell infiltrates can potentially eliminate cells that are recognized as non-self. Since tumor cells survive, they have clearly adapted to this hostility. Some cancer cells adapt key aspects of their microenvironment to help them survive and grow. Adaptations that occur in cancer cells that allow survival and continued proliferation may also enable cells to resist various stressors, such as those induced by systemic therapies. In such an environment, cells that can adapt appropriately will survive and those cells that cannot will eventually die. Thus, unlike *de novo* resistance, which may already be “hard-wired” to be resistant to endocrine therapy by the time the tumor is diagnosed, acquired resistance may be an adaptive process that develops over time. We have recently reported that overexpression of MYC, which has been widely implicated as a driver in some ERα+ breast cancers, is found more frequently in acquired resistant rather than in sensitive or *de novo* resistant tumors [59].

2. Receptors and resistance

The primary role of endocrine therapies is to deprive the estrogen receptors of their endogenous activating ligands. Antiestrogens achieve this goal through competitive inhibition; aromatase inhibitors do so by blocking estrogen biosynthesis. While many compounds have been reported to activate ERα in addition to naturally occurring estrogens [60,61], the most potent natural ligand (17β-estradiol) is also the estrogen usually present in the highest concentration in breast tumors [31]. Since ERα is the main target for these drugs either directly (antiestrogens) or indirectly (aromatase inhibitors), the presence of ERα in a tumor is a primary indicator of the likelihood of eliciting a beneficial response with treatment. There are two estrogen receptor genes (ESR1/ERα and ESR2:ERβ). While both are expressed in breast cancer, ERα is the dominant form. The role of ERβ in breast cancer remains an area of investigation with much still left to be discovered [62,63]. Several isoforms of ERβ are known but not all are translated. Whether ERβ plays any role in determining endocrine responsiveness in ERα+ breast cancers is not clear [64]. The protein products of both estrogen receptor genes can heterodimerize and alter regulation of gene transcription. Thus, the ratio of ERα:ERβ could be important in some ERα+ breast cancers, particularly where the level of ERα protein is relatively low. A role for ERβ in ERα− breast cancers has been proposed and may yet prove to be more important than its role in ERα+ breast cancers [62].

2.1 Receptor phenotype: ERα+ vs ERα−

From a pharmacological perspective, there are two primary receptor phenotypes: ERα+ and ERα−. ERα rich cancers have a reasonable expectation of receiving clinical benefit from endocrine treatment, between 40% and 60%. However, not all ERα+ cancers are ERα rich. While widely used, ERα measurement alone is not a particularly strong indicator of response and most biomarkers that have a sensitivity of only ~50% would be discarded. The likelihood of a response is further increased if the cells also express progesterone receptor (PR), particularly in the metastatic setting [56]; ERα+/PR+ tumors have a response rate closer to 75%. The explanation usually given for this observation is that PR is an estrogen regulated gene and its presence is an indication of an active ERα, although it has been recently reported that PR can affect ERα activity [65]. However, with the high prevalence of ERα+ breast cancer, and the potential to reduce the risks of both recurrence and death with endocrine treatment, PR is not particularly useful in determining who will benefit in the presence of ERα. The absence of PR in ERα+ tumors is not sufficiently sensitive to warrant withholding treatment from ERα+/PR− cancers. PR may be useful in the absence of ERα (ERα−/PR+), with ~40% of these tumors responding to an endocrine therapy [66]. PR expression in ERα−/PR+ tumors is often taken to reflect what may be a false negative ERα measurement. However, as also noted for ERα, the predictive sensitivity of PR measurements is modest. The clearest value of ERα and PR measurements is their specificity when both are absent (ERα−/PR−). Tumors that express neither ERα nor PR, or have very low levels of expression, have a low probability (<10%) of responding to an endocrine therapy [67]. Loss of ERα expression is uncommon in the progression from endocrine sensitivity to resistance, with the majority of breast tumors at recurrence or progression retaining detectable ERα expression. The relatively low proportion that converts to ERα-negativity may be those where the ERα+ cells were eliminated by treatment and/or where they were only a small proportion of ERα+ cells in the primary tumors prior to treatment. ERα appears to remain a key driver of cell survival and proliferation in many patients progressing on endocrine treatment, since second and third line responses to endocrine therapies are well documented and sequential endocrine therapy is used widely in patients with ERα+ cancers. In experimental models, inhibiting ERα expression or function inhibits growth of endocrine resistant cells [68–70].

2.2 Coregulators

Coregulator proteins bind to the ERα protein and modify the effectiveness of the ERα protein complex in controlling gene expression. Coregulators that increase (coactivators) or reduce (corepressors) ERα activity are known and have been implicated in the endocrine resistant phenotype. A long and increasing list of such factors has emerged over time, with evidence of a role for coregulators for most steroid hormone receptors. These have been reviewed previously [66,71–73] and are only introduced briefly here. Some coregulators have been studied extensively including the coactivator AIB1 (SRC3) [74–77] and the corepressors N-COR and SMRT [78–80]. Interactions among coregulators and other signaling molecules are also well described. For example, AIB1 mediates the effects of insulin-like growth factor-I in some breast cancer cells [81]. Progressive loss of the recruitment of coregulators may contribute to the acquisition of endocrine resistance in some tumors [82]. While coregulators have been widely studied, none has yet been shown to have

sufficient predictive or prognostic power to be used in routine clinical practice or have been established as effective molecular targets for drug discovery.

2.3 ERα mutations

One question that has remained difficult to address fully is why so many breast cancers that express ERα exhibit either *de novo* resistance or develop acquired resistance to ERα-targeted therapies. Mutations in the ERα gene that produce a constitutively active protein could explain resistance to aromatase inhibitors. To explain antiestrogen resistance, the translated mutant proteins would also need to be mechanistically insensitive to the presence of an antiestrogen. While mutations in ERα have been known for many years, mutations in ERβ are relatively uncommon although exon deleted variants of both ERα and ERβ have been reported [83]. Amplification of either the ERα or ERβ gene appears to be rare in breast cancer.

The presence and functional relevance of some ERα mutations and isoforms has been known for some time [83–86]. Several studies have recently reported the presence of ERα mutations, almost exclusively in metastatic lesions and with varying prevalence [87–90] (see Table 1). Evidence of these mutations has also been seen in a small number of patient derived xenografts (PDX; n=4/6) [91]. The PDX data are not included in Table 1 because estimating prevalence from this study could be confounded if cells with ERα mutations are more aggressive or have a different take rate than cells with wild type ERα [92] and/or if substantial selection for small clonal populations occurs with this technology [93,94] and makes the detection of ERα mutations more likely. ERα mutations have also been inconsistently described in circulating tumor cells [35,94], perhaps reflecting the effects of the technology used for their selection and/or subsequent *ex vivo* propagation. Consequently, data from these studies also are not included in Table 1.

Recent reports of ERα mutations being found primarily in metastatic lesions from patients are not surprising. Cell lines in which ERα mutations were first reported were derived from metastases and yet the prevalence of the ERα mutants in metastatic lesions was not initially explored widely. Moreover, these earlier studies did not have the advantages of the throughput or sensitivity of next generation sequencing. Evidence for the presence of ERα mutations in primary lesions is available; these are uncommon and the role of these mutations has been well reviewed elsewhere [95].

Whether the presence of ERα mutations will prove to be of clinical importance requires further study, and several key issues remain to be determined. For example, given the notable intratumor and intertumor heterogeneity of breast cancer [33,34], it is unclear if the prevalence of these mutations is high among the cells in any given metastasis (intratumor heterogeneity), and/or in all metastases in an individual patient (intertumor heterogeneity). The data from Table 1 suggests that perhaps up to 80% of metastases in patients with ERα+ breast cancers may not have detectable mutant ERα. If many metastases arise from circulating tumor cells, the heterogeneity in ERα expression among these cells [35,94] could add enough noise to invalidate any ability to use the presence of an ERα mutant protein in a single metastasis as a predictor of a patient's overall response to subsequent endocrine therapy.

Some mutant ERα proteins are also likely to heterodimerize with other ERα forms present in the tumors including wild type receptors. At least one patient derived xenograft has shown the presence of both wild type and mutant ERα [91]. Thus, the relative rates of transcription and translation of the wildtype and mutant ERαs proteins could be important. Also relevant will be the respective affinities of each ERα form for each other, the affinities of the various ERα homo- or hetero-dimers for binding the available coregulator proteins, and the potency for transcriptional regulation of the various ERα mutant-containing complexes formed at the regulatory elements of genes that drive cell fate outcomes. If the mutant ERαs represent a small proportion of the ERα proteins present in the sampled metastasis, or if they are not functionally dominant, of if the bulk of the metastatic burden in patients expresses primarily wild type ERα, then the mutated ERαs may have limited or unpredictable impact upon the biology or clinical course of the disease and any response to endocrine therapies.

Since many of the mutant ERαs identified are present mostly in metastases, there will likely also be an association with poor outcome; metastatic breast cancers are usually clinically aggressive and appear less responsive to treatment than primary disease. Where cancers with mutated ERαs are associated with a worse outcome than other ERα+ metastatic breast cancers treated with the same drugs, it will be important to identify whether this reflects cause or effect. Evidence beyond a correlation is required to demonstrate that the mutant proteins are functionally responsible for this association, rather than acting as a biomarker of a more aggressive phenotype. For example, acquisition of these mutations may reflect the greater genetic instability of drug resistant advanced breast cancers. Many of the patients studied are likely to have received primary endocrine therapy, and second and third line endocrine therapies generally induce lower response rates and shorter durations of response. As these drugs are increasingly ineffective in suppressing proliferation, ERα mutations may occur together with a range of other mutations in resistant cells that continue to replicate aberrantly (see below). Whether diminishing response rates to second and third line therapies are a consequence of ERα mutations is thus unclear, since responses to cytotoxic drugs can also diminish with sequential interventions over time in the metastatic setting. A general, perhaps non-specific, acquisition of a more aggressive phenotype may produce the appearance of multiple drug/hormone resistance independent of the presence of ERα mutations.

The functions of most mutant ERα proteins have been explored [88–90,95]. Some mutants may shift the dose response curve to SERMs and SERDs towards lower responsiveness. It remains unclear whether those studies that have used only *in vitro* data are pharmacologically relevant. Most of these studies have used a range of drug concentrations usually seen in serum. However, drugs like TAM are known to accumulate in tumors and to attain much higher intratumor concentrations than serum concentrations, although bioavailability within neoplastic tissues is uncertain. While the intratumor concentrations previously estimated for TAM and some of its metabolites are very rough approximations [31], if they are correct or even within an order of magnitude then it seems unlikely that mutant ERαs will functionally drive the failure to respond even if their expression is correlated with clinical outcome. It seems more likely that any mutant proteins, if relevant,

will have an important role in driving acquired resistance to aromatase inhibitors. Constitutively active ERαs would continue to drive the tumors in the absence of ligand, and so confer resistance to aromatase inhibitors. Thus, it may be that the presence of specific ERα mutations will prove sufficient to select among the available endocrine therapies for directing or sequencing specific treatments to individual patients. An antiestrogen may be preferred over an aromatase inhibitor in patients harboring at least one metastasis with detectable levels of a mutant ERα gene. Thus, more effective sequencing of endocrine therapies may yet emerge from the study of ERα mutations, despite current uncertainty about their overall clinical value.

2.4 Modeling the ERα as a control mechanism

Mutation is not the only mechanism by which the ERα can become constitutively activate. Several growth factors signal through kinases that can phosphorylate ERα and activate these receptors in the absence of ligand [97,98]. ERα activation can also produce a reciprocal activation of growth factors and their receptors [99]. Thus, ERα can exist in several interchangeable states where the ligand binding site either is occupied by ligand or is unoccupied. The receptors can be occupied and phosphorylated/active, unoccupied and non-phosphorylated/inactive, or unoccupied but phosphorylated/active (possibly several states depending on ERα location and extent/site(s) of phosphorylation). There will be some selectivity for the site of phosphorylation, since not all phosphorylation sites generate constitutively active receptors. Unlike mutated ERα, which is not a functionally reversible state, phosphorylated sites are generally reversible through loss/gain of ligand or loss gain of growth factor mediated phosphorylation. The clinical relevance of phosphorylated ERα has been reviewed by Murphy *et al.* [100].

Chen *et al.* recently modeled the dynamics of ERα activation in two studies. The first study showed that the ERα might act as a bistable switch, where it can persist in either of two states (active or inactive) and can switch freely between these states. However, the model indicated that the barrier between the states is not equal: it is lower for the state transition from inactive to active than for the transition from active to inactive. Thus, ERα may "prefer" to be active rather than inactive [101]. ln the second study this team created a mathematical model of the ERα "landscape", which represents the probability of transitions between states of different ERα sensitivity. This model suggested that intermittent endocrine therapy might provide a better response than sustained or sequential endocrine therapy [102]. While there is support for intermittent cancer therapy with some chemotherapy regimens [103], it remains to be determined if intermittent treatment will improve outcomes with endocrine therapies, as the model predicts.

3. Pharmacology, pharmacogenetics and resistance

Changes in the pharmacokinetics or pharmacodynamics of a drug can alter its potency. TAM is a highly effective drug in part because it has excellent accessibility to breast tumor tissues. While the metabolism of TAM is complex and includes the production of both antiestrogenic and estrogenic drug metabolites [31,104], a simple estimate suggests that the cumulative intratumor concentrations of antiestrogenic metabolites dominates and is generally well in excess of the intratumor concentrations of the primary competing agonist

ligand 17β-estradiol [31]. Given the very favorable biodistribution of TAM and its metabolites, it is not clear how large a change in its metabolite profile would be required to reduce its efficacy and confer resistance. Increasing the presence of estrogenic compounds such as the soy isoflavone genistein, can reduce responsiveness to endocrine therapies in animal models [105,106].

While TAM, and perhaps other endocrine therapies, can have problematic side effects that affect compliance, many but not all women manage to complete their full course of treatment [107]. It is not immediately clear if the vasodilator effects that exacerbate hot flashes and renders the treatment intolerable for some women will occur with new ERα-targeted therapies. The osteogenic benefits of TAM reflect its agonist activity in bone and are not seen with the aromatase inhibitors or SERDs. The increased risk of endometrial cancer associated with long term TAM therapy reflects its agonist effects and remains problematic, whereas this risk is not apparent with Fulvestrant, raloxifene, or the aromatase inhibitors. In addition to menopausal status, differences in toxicity profiles and their respective tolerability for specific endocrine agents all affect the treatment choices for each woman [108].

3.1 CYP2D6

Altered metabolism of TAM has been widely studied as a possible explanation for the diversity of responses seen in patients. A primary focus, and an area that has generated significant controversy, has been on the role of different forms of the CYP2D6 gene, which are often present in liver and both normal and neoplastic breast tissue [109,110]. The product of the CYP2D6 gene metabolizes the parent drug TAM to endoxifen (4-hydroxy-N-desmethyltamoxifen), one of its major metabolites [111]. In addition to the parent drug and endoxifen, other metabolites that are often present in relatively high serum/tissue concentrations include 4-hydroxytamoxifen and N-desmethyltamoxifen [31]. The relative antiestrogenic potencies of 4-hydroxytamoxifen and endoxifen are comparable, but the plasma concentrations of endoxifen are up to 10-fold higher in patients with functional CYP2D6. Three metabolizer groups have been identified based on their CYP2D6 allele profile: PM (poor metabolizers), IM (intermediate metabolizers), and EM (extensive metabolizers). Thus, there have been several studies to determine whether the CYP2D6 genotype can adequately predict TAM responsiveness in patients, such that individuals with a PM genotype might be at higher risk of experiencing a suboptimal benefit from standard TAM therapy (20 mg/day). A further complication is the potential for adverse drug interactions between TAM and selective serotonin reuptake inhibitors (SSRIs) and other drugs that inhibit CYP2D6 activity [112,113]. Much of the controversy comes from conflicting evidence of the correlation between CYP2D6 genotype and responsiveness to TAM in a somewhat diverse series of mostly retrospective clinical studies. Where outcomes suggest that PM patients have a poor outcome to TAM therapy, investigators have proposed that genotyping could be used to direct endocrine therapy and to avoid use of CY2D6 inhibitors such as SSRIs [114,115]. Others interpret the inconsistent outcomes across the same diverse studies as indicating that it is premature to take such actions [116,117].

While the hypothesis that altered metabolism could affect response is intuitively rational, it is evident that the drivers that determine responsiveness to TAM are multifactorial. For example, as noted above in the discussion of the contributions of mutant ERα, tissue rather than plasma concentrations of the profile of all TAM metabolites are likely to be most important. Changes in the serum concentrations of one major metabolite, such as endoxifen, may not be sufficient to affect substantially overall TAM responsiveness. If changes in endoxifen concentrations, as regulated by CYP2D6, are important this could be relevant in only a subset of patients that are variably present in different patient cohorts. While this observation might explain the inconsistent associations across studies, any variation in patient cohorts, should it exist, is unclear.

The International Tamoxifen Pharmacogenetics Consortium (https://www.pharmgkb.org/page/itpc) was formed in part to address the controversy surrounding the role of CYP2D6 genotype and TAM responses by collecting and analyzing genetic and clinical data from appropriate international studies. While the work is not yet completed, Province *et al.* [118] have suggested that current evidence is most consistent with CYP2D6 being one of several factors contributing to TAM responsiveness.

4. Growth factors, growth factor receptors, and oncogenes

A role for growth factors and signaling from their receptors has been widely implicated in affecting the responses to endocrine therapies in breast cancer [119–121]. These potential interactions will not be reviewed here in detail. Some data suggest that VEGF may play a role, perhaps mediated by paracrine signaling in the tumor microenvironment [122]. Alterations in FGFR and related signaling have been also implicated in resistance [123]. Two growth factor families have received substantial attention for their potential roles in driving endocrine independence and resistance to endocrine therapies: the EGFR superfamily and insulin/IGFs. The ability of growth factor receptors to activate ERα in the absence of ligand is one of the most commonly implied mechanisms of action, although not all growth factors in this family are implicated in endocrine resistance [124]. The broader effects of growth factor receptor activation are central to the modeling of ERα action. Several downstream signals from activated growth factor receptors converge on kinases, particularly in the MAPK family, that can activate ERα proteins by phosphorylation. This resistance mechanism might be effective for aromatase inhibitors and perhaps some SERMs but it is less clear whether this mechanism is a primary driver of resistance to SERDs, since these drugs will likely degrade the receptor even if activated by growth factor regulated signaling.

Growth factors and other signaling could affect drug responsiveness through their ability to stimulate the survival and proliferation of cells with stem-like properties [125]. Since mitogenesis is a primary response to many growth factors, a change in the balance between cell growth and cell death/arrest could also create the appearance of drug resistance. For example, rapidly proliferating cells could be highly sensitive to inhibition by drugs but simply regrow so quickly that the population appears to be pharmacologically resistant [11,126]. Since there has been consistent evidence from both experimental models and correlative data from clinical studies implying interactions between growth factor signaling

and endocrine responsiveness, it is not surprising that the clinical utility of combining inhibitors of growth factors, their receptors, and signaling, has been explored. Unfortunately, evidence that using the many inhibitors of EGFR/insulin/IGF action could be clinically useful in endocrine resistant disease has been mostly disappointing. Most clinical studies have shown little value for inhibiting EGFR or associated tyrosine kinase activity [127–130] or insulin/IGF receptors [131] in patients with ERα+ breast cancers particularly with respect to significant improvements in overall survival.

The apparent disconnect between correlative and mechanistic studies implying a central role for growth factors in endocrine resistance, and the relatively disappointing outcomes from clinical studies, requires explanation. It seems likely that concurrent targeting of multiple growth factor signaling pathways will be needed [131]. Overexpression of EGFR or HER2 generally downregulates ERα expression in experimental models [132], and HER2 overexpression is often associated with a clinically meaningful but less robust response to endocrine therapies [133]. Studies in the subgroup of ERα+/HER2+ breast cancers, comprising approximately 10% of all ERα+ breast cancers, suggest a modest potential benefit in combining endocrine therapies with inhibition of HER2 or its signaling for these patients [134–136]. Whether strategies that combine EGFR and endocrine interventions will have an advantage in the chemopreventive setting also remains unclear [137]. The failure to translate data from some studies in laboratory models of endocrine resistance into clinically meaningful advances may reflect the inability of such models to fully replicate the heterogeneity or the microenvironment within tumors in patients.

One of the more widely studied signaling pathways is PI3K/AKT/mTOR, which can regulate cell survival and proliferation, and likely also energy metabolism. The mitogenic signaling downstream of PI3K is driven, at least partly, by regulation of MEK, ERK, and/or JNK. There is notable crosstalk among genes within this pathway, and external crosstalk with ERα activation/signaling [99]. Components of this pathway are frequently mutated in breast cancer [138], although these are not always associated with clinical outcomes in an intuitive manner. For example, PIK3CA mutations are associated with a good outcome in patients with ERα+ breast cancer [139]. Nonetheless, PI3K/AKT/mTOR pathway activation is associated with lower levels of ERα expression [140] and with growth in an *in vitro* model that mimics aspects of aromatase inhibitor resistance [141].

Drugs that target some of the key drivers in the PI3K/AKT/mTOR pathway are already in clinical trials. For example, TORC1 inhibitors such as everolimus have shown evidence of improved outcomes when combined with exemestane or TAM in several studies in patients with advanced ERα+ breast cancer (see [142] for a recent listing of these studies). The U.S. Federal Drug Administration has approved a combination of exemestane and everolimus for the treatment of ERα+ breast cancers that have progressed on either anastrozole or letrozole. Nonetheless, current approaches are likely suboptimal because of feedback events within the pathways. Inhibiting TORC1 removes some of the inhibitory functions acting on PI3K/AKT [143,144] and inhibition of either AKT or PI3K feeds back to activate prosurvival receptor tyrosine kinases [145,146]. Determining the correct drug combinations and sequencing of drugs to optimize efficacy and limit toxicity will likely be challenging but has the potential to improve outcomes significantly for patients.

5. New insights from a systems biology view

A systems biology approach views the central question of what drives endocrine resistance by studying the problem as an integrated and interacting network of molecules that initiate, coordinate, control, and execute cell fate decisions [126,147,148]. From our perspective, we have chosen to focus on the cell fate decisions of survival/death and proliferation/growth arrest [126]. The molecules of interest/relevance can come from within the cells, the tumor microenvironment, or beyond because the system is not simply the cancer cell but all of the intrinsic and extrinsic factors that affect its cell fate decisions and the processes through which these decisions are executed. In this context, it is not unusual to integrate information from the genome, transcriptome, proteome, and metabolome with information about cell phenotype, responsiveness to drugs, and other data from cell lines, animal models, and from human specimens and populations. This level of data integration and analysis often involves some aspect of multiscale modeling, incorporating time and dose scaling in addition to scaling across the other domains (-omics, phenotype, clinical, other). There is rarely a single solution to problems like endocrine resistance, since part of the solution can exist in each dimension of the problem. Rather, it is the integration of knowledge from partial solutions in different domains that is not only the most challenging aspect of seeking a new solution, but also the direction from whence new insights are most likely to arise. Data integration requires the use of mathematical and computational modeling. Most studies explore signaling genes and proteins and overlay the outcomes onto known canonical signaling pathways. Despite the appeal and utility of this approach, it limits the scope of investigations to create new knowledge of signaling topology because it forces new data onto networks defined by current knowledge of different and often unrelated cellular contexts. Relatively few studies into endocrine resistance have taken a systems biology approach to discover new mechanisms.

5.1 Endoplasmic reticulum stress and the unfolded protein response in ERα+ breast cancer

Endoplasmic reticulum stress arises from the build-up of inappropriately misfolded or unfolded folded proteins within the lumen of the endoplasmic reticulum. Increased activation of the unfolded protein response (UPR), an ancient stress response network, frequently follows. This “canonical” UPR is activated in response to sensing insufficient energy to fold a cell’s new proteins, by damage to new or existing proteins from reactive oxygen species, hypoxia/HIF1 [149,150], and by other stressors experienced by cells in the tumor microenvironment [151,152]. Some level of basal UPR, or activity of select UPR components, may always be present in most cells, allowing cells to monitor and regulate ongoing protein folding and maintain energy balance. Prior to a significant increase in protein production, a cell’s metabolism and its machinery for managing any newly synthesized proteins (primarily the UPR and its components) generally increase [153]. For example, signals that will increase protein production, such as growth factors, can signal through AKT/mTOR to activate UPR in the absence of detectable evidence of endoplasmic reticulum stress (this has been described as a “non-canonical” or “anticipatory” UPR) [154,155]. Estrogen, which also induces significant protein production in breast cancer cells, elicits a similar response using this “non-canonical” signaling [156]. Consistent with our

earlier studies in breast cancer, antiestrogens and estrogen withdrawal activate UPR components [59,148,157–160] including regulation through AMPK/mTOR signaling [161].

Rapid UPR responses to ligand changes on ERα ("non-canonical") and long term regulation in response to endocrine therapy-induced UPR ("canonical") occur. However, from a systems biology perspective, this separation into "canonical" (with endoplasmic reticulum stress; UPR induction) and "non-canonical" (without endoplasmic reticulum stress) is unnecessary. Both signaling routes are representations of the same overall UPR network topology, differentially regulated to create an appropriate response (time, specific UPR functions, magnitude of responses) to the timing and nature of the cell's need. The primary UPR effectors that are activated, and the cellular outcomes ultimately regulated, are similar. The network features that are regulated in the "non-canonical" scenario may rapidly initiate the signaling to allow time for adaptation of the broader network to maintain the response over hours, days, and even months or years ("canonical" UPR signaling). As we have previously described, the fundamental outcome of UPR network activation in ERα+ breast cancer cells is to regulate the balance between apoptosis and autophagy in a manner that determines the cell fate decisions that drive clinical responses (see [147,148] for reviews and section 5.2 below for more detail).

The "canonical" UPR is usually described as being regulated by glucose regulated protein 78 (GRP78) [162], a protein chaperone that binds to unfolded or misfolded proteins so that they can be either repaired or degraded. GRP78 is kept inactive by binding to three components of the UPR: PERK, ATF6, and IRE1. The UPR is activated once GRP78 is released from these three components. Since the production of properly folded proteins is critical for cell survival, UPR-regulated reductions in the rates of transcription and translation reduce the load of improperly folded proteins within the endoplasmic reticulum.

Both cell death and cell survival signaling can be activated by the UPR, enabling the cell either to execute a program of cell death or to survive and proliferate, as appropriate, depending on how well the cell can manage the stress [163]. Cell death outcomes are frequently mediated by PERK-CHOP signaling, whereas cell survival can be directed through IRE1-XBP1 signaling. Key effectors can be regulated by signaling that also appears to be external to the UPR. For example, XBP1 can be regulated by ERα and can, in turn, regulate the effectiveness of transcriptional regulation by ERα [164–166] including in a manner independent of ligand [167]. Upregulation of several UPR features in antiestrogen resistant cells has been known for some time and includes XBP1, HSP27, BCL2, and NFκB [157].

UPR activation and/or XBP1 expression has been widely reported in both ERα+ and ERα− breast tumors [168–172]. XBP1 expression correlates with other genes implicated in driving cell fate decisions including IRF1 and NFκB [168], and with GRP78 [170]. The primary prosurvival form of XBP1 (spliced; XBP1s) is upregulated in antiestrogen resistant breast cancer cells and tumors [157,158,168] and it acts at least partly through its ability to regulate NFκB [157,165,173,174], BCL2 family members [158,174,175], and autophagy [163,176]. XBP1 over-expression in sensitive cells confers both estrogen independence (analogous to resistance to aromatase inhibitors) and crossresistance to TAM and Fulvestrant (analogous

to a multiple hormone resistant phenotype) [158,177,178]. Increased expression of XBP1 mRNA is associated with a poor response to TAM in ERα+ breast cancers [169]. Regulation of XBP1 and the UPR is also implicated in the estradiol-inhibited phenotype [179]. Targeting IRE1 and XBP1 may offer new approaches for preventing and/or reversing endocrine resistance in patients [177]. More recently, it has been shown that both the spliced and unspliced forms of XBP1 can drive endocrine resistance [165]. Expression of XBP1 and NFκB are correlated in breast cancer [168], and the prosurvival actions of XBP1 likely require its ability to activate NFκB [165]. As the most upstream regulator of canonical UPR signaling, the role of GRP78 in endocrine resistance has been strongly supported [59,70,161,180] and an initial mathematical model of its signaling has been described [160]. Importantly, GRP78 has been reported as a therapeutic target in several cancers [181,182].

5.2 Autophagy and apoptosis

Autophagy is a natural process through which cells recycle damaged or unnecessary subcellular organelles (macroautophagy) or proteins (microautophagy). Herein, “autophagy” refers to macroautophagy unless otherwise specified. Depending on the nature of the stressor, some cancer cells may appear autophagy dependent or independent. The response to autophagy inhibition may also depend on the presence or absence of other stressors [183]. A key role for autophagy in affecting responsiveness to endocrine therapies has been described [70,163,175,176,184] and blocking autophagy can increase responsiveness to antiestrogens [50,161,185]. Clearly the UPR must be a key regulator of the balance between autophagy and apoptosis because the UPR senses and attempts to correct energy imbalance to ensure adequate protein folding, and autophagy is a source of energy – or can conserve energy – through its role in organelle and protein recycling. The primary UPR regulator, GRP78, manages the balance between prosurvival autophagy and prodeath apoptosis and confers endocrine resistance *in vitro* and *in vivo* [70,161]. In the context of endocrine resistance, we have recently reported that estrogen withdrawal and antiestrogens alter glucose (GLU) and glutamine (GLN) uptake and affect cellular energy levels to activate the UPR [59]. Integration of cellular responses to endocrine therapies includes GRP78-medidated signaling where multiple pathways can inhibit apoptosis and activate autophagy in an attempt to survive the stress. These pathways include TSC2/AMPK-mediated mTOR inhibition that may be independent of BECN1. When autophagy’s prosurvival role is inhibited, GRP78 overexpression no longer protects cells, and an inhibition of caspases is released. Signaling redundancy is likely because concurrent knockdown of both BECN1 and GRP78 is synergistic, implying that they may function independently to restore antiestrogen sensitivity in resistant cells. BECN1 is known to control directly the onset of autophagy, and GRP78 can affect BECN1 indirectly through the regulation of BCL2 following XBP1 activation [147,158,175]. GRP78 also affects autophagy through its regulation of AMPK-mediated mTOR inhibition of the ULK1 complex, a key component in the onset of autophagy [161].

BCL2 family members are well known to be key determinants of breast cancer survival, although the relationship is complex. For example, overexpression of BCL2 in primary tumors (often measured pretreatment) is paradoxically associated with better response to endocrine therapy [186]. However, BCL2 expression is reduced in breast tumors responding

to TAM treatment [187], whereas BCL2 expression is increased in those breast tumors that survive TAM therapy [188]. Prosurvival BCL2 family members can influence autophagy through their abilities to bind to and effectively sequester BECN1, perhaps explaining their ability to protect cells from endocrine therapies. Resistant cells exhibit increased BCL2 expression and are more sensitive to growth inhibition by small molecule inhibitors of BCL2 [173,175,189]. There are several prosurvival members of the BCL2 family and this redundancy likely complicates data interpretation. For example, greater effects in the reversal of antiestrogen resistance are seen when more than one prosurvival BCL2 family member is inhibited [175]. Given the number and complexity of interactions that can occur, further confounded by cell context, a full understanding of the role of all members of the extended BCL2 family in determining cellular outcomes in response to endocrine therapies will likely require much additional study.

Addressing the complexity of these signaling pathways is currently the subject of our mathematical modeling. From a systems perspective, dynamical modeling of signaling networks enables us to assess the relative importance of interactions among signaling components *in silico*, and this knowledge helps us to integrate experimental results from a variety of sources [126,148,190] and to design new laboratory experiments that provide a better understanding of these stress responses [126,148]. In principle, this body of work implies that antiestrogens and estrogen withdrawal reduce GLN and GLU uptake, and consequently cellular energy levels fall. Energy levels may also fall due to ATP depletion from prolonged opening of the inositol-3-phosphate receptor calcium channel in the endoplasmic reticulum [156]. Because cells no longer have sufficient resources to fold their proteins, this change in GLU uptake may be sensed by GRP78 to activate the UPR. Activated UPR initially inhibits apoptosis, arrests cells in G_0/G_1, and increases autophagy (and likely also scavenging of external nutrients), in an attempt to reestablish metabolic homeostasis. Concurrently, a decrease in the ATP:AMP ratio (from reduced GLU/GLN metabolism and/or altered intracellular calcium flux) can be sensed by AMPK, which reinforces the autophagy response [161], an example of crosstalk and degeneracy of signaling modules. When these integrated and adaptive responses are successful, cells resist the stress and survive; when unsuccessful, the cells die [147]. Work to further understand these integrated events, and to find other responses that lie outside the UPR/autophagy/apoptosis axis, are currently underway.

5.3 Antiestrogens and tumor nutrient deprivation: endocrine therapies as antimetabolites

Cancer cell metabolism and its regulation differ in many respects from “normal” cells. For example, the addiction of cancer cells to GLU and GLN is well-known. The Warburg effect in cancer cells, a form of aerobic glycolysis that produces only 2 ATP molecules when glucose is fermented to lactic acid compared with 36 ATP molecules if glucose is fully oxidized to CO_2 through the Krebs cycle, has been known for over 50 years [8]. The M2-PK form of pyruvate kinase, present mostly in cancer cells, may explain how they consume GLU at an accelerated rate [9,10]. Several oncogenes affect cellular metabolism as reviewed in [11]. Cancer cells use different approaches to scavenge nutrients, including selective use of the solute carrier gene group. Cancer cells also may rely on macroautophagy more often than normal cells, recycling the products of autolysosomal degradation into intermediary

metabolism [6]. Scavenging and autophagy may alter responses to nutrient deprivation in ways that are cancer specific. Thus, cancer cells may differ from each other in how they manage their addiction to GLU and GLN, and how they respond to limitations in nutrient supply.

Cells will rarely enter the cell cycle unless they have sufficient energy and resources to complete a full cycle [191]; those deprived of nutrients while cycling generally arrest in G_0/G_1 phase until adequate resources become available [192]. ERα+ breast cancer cells subjected to estrogen withdrawal or treated with an antiestrogen also arrest in G_0/G_1 phase. Antiestrogens reduce GLU and GLN uptake and total cellular ATP production [59]. Aromatase inhibitors are enzyme inhibitors that create estrogen withdrawal and its consequent metabolic effects [59]. Thus, endocrine therapies may act like antimetabolites, resulting in changes in cellular nutrient balance and inducing a form of nutrient deprivation that leads initially to cell cycle arrest and eventually to cell death. For example, the antimetabolite methotrexate inhibits dihydrofolate reductase to block one-carbon metabolism and inhibit DNA synthesis producing subsequent S-phase arrest and cell death [193].

How antiestrogens regulate GLU and GLN transport and metabolism remains an area of interest. Over expression of MYC has been associated with antiestrogen resistance [194], and MYC can regulate the glucose transporter GLUT1 in antiestrogen resistant cells. Moreover, antiestrogen resistant cells are more sensitive to GLUT1 inhibition by the MYC inhibitor, STF31 [59]. As described above, glucose regulated proteins sense changes in intracellular GLU concentrations [162] and antiestrogen-induced GLU deprivation activates the UPR, an effect driven by increased GRP78 activation/expression [147,161,162]. Overexpression of GRP78 induces autophagy and confers antiestrogen resistance, likely due in part to the ability of autophagy to provide an alternative source of intermediate metabolites in the presence of low GLU and GLN [59,147]. Thus, the metabolic effects of antiestrogens, and the ability of cells to bypass this blockade, appear to be fundamental, but heretofore underappreciated, principles in how endocrine therapies regulate breast cancer growth. Understanding the aspects of metabolic regulation that are unique to ERα+ cells may lead to new interventions to enhance existing therapies and provide novel ways to target newly identified vulnerabilities in the regulation of cell metabolism in ERα+ cells.

6. Future directions

Current third generation aromatase inhibitors are remarkably effective in modulating the enzyme activity of their target. Thus, it seems unlikely that future developments in new aromatase inhibitors will offer significant therapeutic advantages. Patient outcomes for interventions using aromatase inhibitors are more likely to be improved by finding ways to reduce toxicity and improve compliance and perhaps by novel sequencing or combinations with other drugs. Targeting other enzymes in the estrogen biosynthetic pathway has not been explored effectively. Such targets have been suggested, mostly the enzymes that affect the bioavailability and/or ERα affinity of the natural ligand, such as 17β-hydroxysteroid dehydrogenases [195] or the steroid sulfatase [196,197]. Directly targeting ERα through the development of new SERMs and SERDs offers greater opportunities for drug development.

The optimal dosing of Fulvestrant is still unclear. It has yet to be established whether doses higher than 500 mg can be administered or achieved and also offer sufficiently increased clinical benefit. Fulvestrant's high lipophilicity is also problematic, and likely limits its intracellular accessibility to ERα proteins and explains its inability to fully eliminate ERα proteins in all cells. Among the challenges with Fulvestant use in patients are its lack of oral bioavailability such that intramuscular administration can be painful for some women (although this has the advantage of improved assessments of compliance), and toxicity can limit its use as a single agent in some postmenopausal women (a limitation shared with aromatase inhibitors). Combination therapies with drugs that suppress ovarian function show promise for both aromatase inhibitors and Fulvestrant for premenopausal patients [198,199]. The problem of oral bioavailability may have been adequately solved with some of the new SERDs such as GDC-0810 (ARN-810) [200]. The true potential of new classes of SERDs will await more detailed investigations of their safety, tolerability, and efficacy relative to existing endocrine interventions.

In sensitive cells, endocrine therapies induce substantial growth arrest. Other agents also target proliferation and show significant promise, particularly the CDK inhibitors such as the CDK4/6 inhibitor Palbociclib. By inhibiting cell cycle progression, these agents may offer novel opportunities, in combination with endocrine therapies, to arrest those cells that are less affected by endocrine interventions alone. Early reports are suggestive of improvements in response rates for these combinations in ERα+ breast cancers [201,202]. Whether better suppression of growth arrest will produce improvements in both response and disease free survival, and translate also into improved overall survival, will take some time to establish. Nonetheless, without an increase in cell killing, improved growth arrest alone may only lead to meaningful improvements in disease free survival. Major improvements in overall survival relative to existing endocrine interventions may prove as elusive for the CDK4/6 inhibitors as they have been (relative to TAM) for the aromatase inhibitors.

A resurgence in the field of cancer immunology has produced remarkable responses in some solid cancers and includes the use of T-cell check point inhibitors such as programmed cell death receptor ligand 1 (PDL1) antagonists like MPDL3280A in lung cancer [203] or inhibitors of its receptor programmed cell death 1 (PD-1) like ipilumumab in melanoma [204] or pembrolizumab in non-small-cell lung cancer [205]. These approaches have so far shown more limited responses in breast cancers but may be useful in TNBCs that tends to overexpress PDL1 [206]. ERα+ breast cancers may be less immunogenic, with inadequate activation of immune effector cells and/or other adaptations in the tumor microenvironments that suppress antigenicity and/or suppress activation of the adaptive or innate immune effector systems. Studies to determine how to reestablish immune-based host elimination of tumors offer potential, particularly for the eradication of the many small foci of growth arrested but surviving cells that remain during treatment with endocrine therapies and are the source of distant recurrences in ERα+ cancers.

The potential that endocrine therapies act like antimetabolites raises the possibility that new combinations of drugs that target specific aspects of cellular metabolism may be particularly effective. Blocking autophagic recycling using chloroquine is effective in animal and cell culture models [50]. Combining UPR inhibition with autophagy inhibitors and endocrine

therapies could make metabolic reprogramming difficult for many ERα+ cells and delay or reverse resistance, emergence from dormancy, and/or cell survival.

Studies have shown that unless endocrine therapy switches off proliferation within 14 days of start of treatment response is unlikely [207]. With aberrant proliferation occurring under the stress of drug treatment, mutations in DNA are more likely and mutations in some cells could result in a clone of endocrine resistant cells acquiring a greater mutational load and a more aggressive phenotype. Acquisition of these new mutations could further reduce responses to second or third line endocrine therapies, and limit responses to additional chemotherapy based interventions. Further work on the role of mutations as contributors to endocrine resistance and malignant progression is clearly needed.

Systems approaches offer an effective means to address the multiscale nature of cellular responses to the stresses of endocrine based interventions. Increased understanding of the degeneracy and redundancy in the integrated nature of stress responses is required if we are to learn how to block metabolic reprogramming effectively and drive cell fate decisions to cell death. Clearly, the UPR and autophagy signaling modules offer innovative new targets for intervention, and drugs that are active are already available, such as the use of chloroquine to inhibit autophagy. While prolonged growth arrest can lead to significant clinical benefit, until we can eradicate all breast cancer cells, or drive them into a permanently dormant state, we may continue to see greater improvements in disease free survival than in overall survival rates.

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Highlights

- Reviews the use of SERMs, SERDs, and AIs in breast cancer treatment
- Discusses tumor heterogeneity and dormancy in ERα+ breast tumors
- Summarizes the prevalence of ERα mutations in endocrine resistance
- Describes the mechanisms of acquired endocrine resistance
- Details the upregulation of the unfolded protein response and resistance to autophagy in antiestrogen resistance
- Discusses the role of glucose and glutamine metabolism and MYC overexpression in endocrine resistance

Table 1

Prevalence of any ERα mutation from recent studies. The study by Toy *et al.*, included data from two different patient populations.

Study	Prevalence
Merenbakh-Lamin *et al.* [96]	5/13 (38%)
Toy *et al.* [88]	9/36 (25%)
Toy *et al.* [88]	5/44 (11%)
Robinson *et al.* [89]	6/11 (55%)
Jeselsohn *et al.* [90]	9/76 (12%)
Total	34/180 (19%)