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Tamoxifen Resistant Breast Cancer and Autophagy

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1. Introduction

1.1 Tamoxifen for the treatment of breast cancer

Tamoxifen was originally developed with the hope of becoming a "morning after" contraceptive. During the 1960s, studies showed that tamoxifen and other anti-estrogens have a profound impact on the fertility of laboratory rats and it was believed that tamoxifen could elicit the same effects in humans. Coincidently, in humans, anti-estrogens were found to improve fertility by inducing ovulation (Jordan, 2006).

The use of tamoxifen for the treatment of breast cancer became apparent when early *in vitro* and *in vivo* studies found that tamoxifen inhibited estradiol (E2) binding to estrogen receptors (ERs) in breast tissue (Jordan, 1976; Jordan & Dowse, 1976; Jordan & Koerner, 1975; Nicholson & Golder, 1975). The inhibition of ERs proved to be a significant finding as the estrogen-stimulated growth and the ovarian dependence of some breast cancers had been known since the later part of the eighteenth century (Beatson, 1896). Prior to the 1970s, and the successful development of drug-based endocrine therapies, attempts to reduce estrogen-stimulated growth of breast cancer include the surgical removal of the ovaries and/or pituitary and adrenal glands (Kennedy, 1965). Unfortunately, for years it was not known which breast tumors would respond favorably to ablative surgery, where only 30% of patients would receive a benefit (Jensen *et al.*, 1971; Kennedy, 1965). By examining proteins from breast tumor biopsies, Jensen *et al.* (1971) showed that if the estrogen receptor is present, tumors will respond to ablative surgical treatment, but if absent, the tumors fail to respond.

Fortunately today, endocrine-based surgical procedures for breast cancer are not necessary for most women as drug-based endocrine therapies have advanced with the use of estrogen antagonists and aromatase inhibitors. Estrogen antagonists, such as tamoxifen or fulvestrant competitively inhibit estrogen binding to ERs. Aromatase inhibitors prevent estrogen production, by inhibiting the aromatase class of enzymes, impeding the conversion of androgens to estrogens and thus reducing the bioavailability of estrogen hormones (Jordan, 1994; Mokbel, 2002).

Currently tamoxifen is typically used as an adjuvant treatment option for early and advanced ER-positive (ER+) breast cancer in pre- and post-menopausal women (Jordan, 1994). Adjuvant treatment with tamoxifen, has significantly improved disease-free survival and reduced the number of deaths from breast cancer (Early Breast Cancer Trialists' Collaborative, 2005). Tamoxifen may also be used in the neoadjuvant setting and as a

preventative agent for women at high risk of developing the disease, although some patients may feel that when used as a preventative agent the risks may outweigh the benefits (Cuzick *et al.*, 2003; Waters *et al.*, 2010). The most adverse effect of tamoxifen is the increased propensity to develop endometrial cancer that occurs predominantly in post-menopausal women. To subside this effect, tamoxifen, in the adjuvant setting, is administered for at most 5 years. If longer therapy is required, patients are typically switched to aromatase inhibitors or a different anti-estrogen, such as fulvestrant (Perez, 2007).

In breast tissue, tamoxifen acts as an antagonist, binding to ERα, competitively inhibiting E2, and preventing the expression of mitogen, angiogenic, and apoptotic factors. In other tissues such as the endometrium, tamoxifen acts as an agonist and elicits a response similar to estrogen when bound to ERs, potentially leading to the development of endometrial cancer. 'Pure' anti-estrogens, like fulvestrant bind the ER competitively inhibiting estrogen but have no estrogen like effects. In some women, tamoxifen's agonistic actions have been found to have favorable effects on serum cholesterol and protection against bone loss and cardiovascular disease (Hoskins *et al.*, 2009; Osborne, 1998). The tissue dependent agonist and antagonist actions of tamoxifen classify the drug as a selective estrogen receptor modulator (SERM). SERMs modulate the ER signal transduction pathway in ER target tissues through complex and not completely understood mechanisms (Jordan, 2006), although the agonist and antagonist functions of tamoxifen are thought to be mediated through two distinct transactivation domains of ERα, Activating Function-1 (AF-1) close to the N-terminus, and AF-2, in the ligand binding site. In breast tissue, tamoxifen inhibits AF-2 activation and functions as an antagonist of genes that rely on AF-2 transactivation. In genes where AF-2 function is redundant, tamoxifen may function as an agonist and transcription may be driven solely by the AF-1 domain (McDonnell *et al.*, 1995; Ring & Dowsett, 2004; Tzukerman *et al.*, 1994).

1.2 Estrogen receptors

The primary target of tamoxifen is the ER. The ER is a ligand-activated transcription factor that is a critical regulator of breast epithelial cell proliferation, differentiation, and apoptosis. There are two ER isoforms, ERα and ERβ (Dahlman-Wright *et al.*, 2006). The receptors differ in their cellular function, tissue distribution, as well as ligand binding properties. Both ERα and ERβ are expressed in normal and neoplastic tissues. ERα is expressed in 15-30% of the luminal epithelial cells while ERβ is more ubiquitously expressed throughout the mammary tissue. Estrogen dependent proliferation in non-neoplastic breast epithelial cells is thought to occur through paracrine mechanisms where ERα cells promote the proliferation of adjacent ER negative cells (Riggins *et al.*, 2007). In neoplastic breast tissue, ERα is expressed in a greater proportion of cells and growth is thought to occur through both autocrine and paracrine mechanisms (Riggins *et al.*, 2007). The roll of ERβ in breast cancer development is not well understood. ERβ is often down regulated in malignant cells and its presence is often correlated with a better prognosis (Saji *et al.*, 2005; Sugiura *et al.*, 2007). *In vivo* studies in mice have revealed that ERβ expression prevents tumor formation and angiogenesis (Behrens *et al.*, 2007; Paruthiyil *et al.*, 2004). Indeed ERβ may contribute to the pregnancy/lactation-associated protection from breast cancer by disrupting the formation of tight junctions and altering the expression of β-cantenin, traits that have been associated with malignant phenotypes (Riggins *et al.*, 2007). Moreover, ERβ is a marker of improved

response to tamoxifen (Hopp *et al.*, 2004). Thus most endocrine therapies target the actions of ERα.

ERα-positive (ERα⁺) breast cancers account for at least 75% of the breast cancer patient population. The presence of ERα, assessed immunohistologically, serves as a biomarker for a patient's response to endocrine therapy (Harvey *et al.*, 1999). The majority, 65%, of ERα⁺ breast cancers also express the progesterone receptor (PR) (Hoskins *et al.*, 2009). The PR, like the ER, is a ligand activated transcription factor that is involved in a wide range of physiological functions, contributing to cell homeostasis and differentiation. A small number of ER-negative but PR-positive tumors also respond favorably to tamoxifen treatment, but this is likely due to a small presence of ERα below the limit of detection (Clarke *et al.*, 2001).

ERα has three distinct pathways of regulating gene expression. The classic model of ERα signaling involves the ligand bound ERα that activates gene expression by direct dimeric binding to DNA response elements in complexes involving co-activators (CoAs) and histone acetyl transferases (HATs). The ER can also influence transcription through protein-protein interactions with other transcription factors, such as activation protein 1 (Ap1) and specific protein 1 (Sp1), which facilitate binding to serum response elements. Lastly, ERs can be activated by downstream signaling events or crosstalk with receptor tyrosine kinases including: epidermal growth factor receptor (EGFR), human epidermal growth factor receptor 2 (HER2, also known as ERBB2), and insulin-like growth factor-1 receptor (IGF-1R) (Kushner *et al.*, 2000; Musgrove & Sutherland, 2009; Ring & Dowsett, 2004; Schiff *et al.*, 2003). Indeed deregulation of estrogen signaling through the estrogen receptors with proliferation through alternative pathways are common mechanisms for tamoxifen resistance (Ali & Coombes, 2002; Musgrove & Sutherland, 2009; Riggins *et al.*, 2007).

1.3 Resistance to tamoxifen

A challenge to nearly all cancer therapies is the development of resistance. Resistance to cancer therapy can be categorized into two major forms, *de novo* and acquired. *De novo* or intrinsic resistance is present in patients that are initially unresponsive to treatment, despite having a phenotypic classification similar to patients that do respond; while acquired resistance is present in patients that respond to treatment initially, but eventually relapse.

Despite tamoxifen's successes, where 70% of ERα⁺ and/or progesterone receptor positive breast cancer patients respond favorably to treatment, 30-50% of patients will have recurrent disease within 15 years (Clarke *et al.*, 2001; Early Breast Cancer Trialists' Collaborative, 2005). Few strategies besides resorting to invasive chemotherapy, radiation therapy, and surgery have found success in circumventing anti-estrogen resistance. The challenge then, is to identify specific biomarkers that can predict therapeutic response to endrocrine therapies and identify new targets to combat endocrine resistant disease (Musgrove & Sutherland, 2009).

1.4 *De novo* resistance

One of the most prevalent factors contributing to *de novo* tamoxifen resistances is lack of ERα expression (Giacinti *et al.*, 2006). The decreased expression of ERα can be the result of hypermethylation in the CpG islands of the ERα promoter. Studies using MDA-MB-231 ERα-negative breast cancer cells have shown that by re-expressing endogenous ERα with a histone deacelylase (HDAC) inhibitor cells can be re-sensitized to tamoxifen (Sharma *et al.*,

2006). Similar studies have shown that by ectopically expressing ERα in the same cell line, cells do not become sensitive to tamoxifen treatment but have a decreased proliferative and metastatic phenotype when exposed to estrogen (Garcia *et al.*, 1992). Thus, the restoration of ERα appears to be a potential therapy for *de novo* resistance or ERα-negative breast cancers. Candidate therapies include the use of demethylating agents or HDAC inhibitors, which could potentially decrease the hypermethylation of CpG island-s within the ERα promoter (Giacinti *et al.*, 2006).

In addition, Hoskins and collogues identified a pharmacokinetic mechanism behind *de novo* tamoxifen resistance (Hoskins *et al.*, 2009). Patients carrying inactive alleles of cytochrome P450 2D6 (CYP2D6), or are co-administered drugs that inhibit CYP2D6, fail to convert tamoxifen into its active metabolites endoxifen and 4-hydroxytamoxifen, as a result, patients seem to derive little therapeutic benefit. Interestingly, the inactive alleles of CYP2D6 vary among different ethnic groups, suggesting an ethnic based difference in response to tamoxifen treatment. Approximately 20% of East Asian women, 16% of women with African ancestry, and 6-10% of Caucasian women have decreased CYP2D6 metabolism. The ethnic differences emphasize the importance of patient based therapies and the potential employment of CYP2D6 genotyping prior to tamoxifen treatment (Hoskins *et al.*, 2009). Other mechanisms of *de novo* resistance and the mechanisms that lead to lack of ERα expression are not as well understood, although they may be similar to some of the mechanisms that lead to acquired tamoxifen resistance.

1.5 Acquired resistance

Tamoxifen is typically prescribed in the adjuvant setting and administered for a 5 year period. Prolonged exposure may drive the development of acquired resistance that can arise from multiple factors including host-specific differences in immunity, endocrinology, and drug pharmacokinetics. Tumor-specific factors such as alterations in ER isoform ratios or ER transactivation and downstream signaling as well as changes in tyrosine kinases or mitogen growth factor signaling may also contribute to acquired tamoxifen resistance.

In cases where patients acquire resistance to tamoxifen, loss of ERα expression occurs in only 15-20% of breast cancers (Gutierrez *et al.*, 2005). Mutations in ERα are also quite rare occurring in <1% of ERα+ tumors (Clarke *et al.*, 2003; Herynk & Fuqua, 2004; Riggins *et al.*, 2007). Indeed, following the development of tamoxifen resistance, many tumors still respond to treatment with aromatase inhibitors or 'pure' anti-estrogens such as fulvestrant, which also block the effects of estrogen but have no estrogen-like effects, unlike tamoxifen. These observations suggest that estrogen still plays a critical role in tamoxifen-resistant tumors and support the idea that endocrine therapies can lead to the activation of novel signaling pathways that circumvent the effects of anti-estrogens (Lewis & Jordan, 2005; Musgrove & Sutherland, 2009; Riggins *et al.*, 2007). In addition, the activation of these pathways may also contribute to *de novo* and acquired resistance to aromatase inhibitors and other anti-estrogens.

In large part, the development of acquired resistance can be attributed to a deregulation of estrogen receptor signaling as well as altered expression and/or modification of several tyrosine kinase growth factor receptors and their downstream signaling targets (Musgrove & Sutherland, 2009; Riggins *et al.*, 2007). Signaling from tyrosine kinase receptors such as EGFR, HER2, as well as the IGF-1 receptor (IGF1R) have been associated with the expression of anti-apoptotic, autophagic, and other pro-survival signaling factors that allow for the

development of endocrine resistance. Others have previously discussed the role of the HER2 and EGFR signaling pathways in relation to tamoxifen resistance (reviewed in: Clarke *et al.*, 2003; Musgrove & Sutherland, 2009; Riggins *et al.*, 2007).

In this chapter, we discuss the significance of IGF signaling and present additional results of a previously published RNA interference (RNAi) tamoxifen resistance screen (Ahn *et al.*, 2010). The screen was used to identify new targets to combat tamoxifen-resistant disease or to identify specific biomarkers that predict response to tamoxifen therapy. One of the identified targets, IGF binding protein 5 (IGFBP5) will be discussed in detail, while the other targets have a role in autophagy which has been shown to be essential for cell survival, differentiation, development, and homeostasis in response to stress and nutrient deprivation (Levine & Kroemer, 2008).

2. Methods

RNAi is a powerful tool to inhibit gene expression at the post-transcriptional level and allows the identification of novel cellular mechanisms that regulate gene expression. As well, its manipulation makes high-throughput genetic screens possible. The silencing of genes within mammalian cells is possible by designing small interfering RNA (siRNA) sequences. The siRNAs can be synthesized chemically, then taken up by the cell. Alternatively siRNAs can be produced within the cell, in the form of short hairpin RNAs (shRNAs) from exogenously introduced vectors.

2.1 siRNAs and shRNAs

Since RNA interference (RNAi) was first discovered in the nematode *Caenorhabditis elegans* it has been used to exploit the function of genes in higher organisms (Fire *et al.*, 1998). In particular, large-scale RNAi based techniques have innovated the existing loss-of-function genetic screens in mammalian cells (Elbashir *et al.*, 2001). The use of chemically-synthesized siRNAs has a couple of key advantages including: the constant quality control of reagents that is important for high-throughput settings; and the diverse modification of siRNA molecules that improves stability and delivery to cells. Vice versa, synthetic siRNAs has disadvantages, including its short-life that results in the transient inhibition of gene expression only, along with difficulty in efficiently delivering siRNAs to non-dividing primary cells and their cost in genome-wide high-throughput screens. To circumvent these limitations, several groups have developed vectors that produce short hairpin RNAs (shRNAs) that are processed within the cell into short duplex RNAs having siRNA-like properties (Brummelkamp *et al.*, 2002). Such vectors provide a renewable source of a gene-silencing reagent that can mediate persistent gene silencing after stable integration of the vector into the host-cell genome. Furthermore, the core silencing 'hairpin' cassette can be readily inserted into retroviral, lentiviral, or adenoviral vectors, facilitating delivery of shRNAs into a broad range of cell types (Brummelkamp *et al.*, 2002; Michiels *et al.*, 2002). Additionally, inducible versions of shRNA vectors have been generated and used successfully in genetic screens (Ngo *et al.*, 2006).

2.2 RNAi-screening in breast cancer

The large collections of RNAi libraries have been used in many different ways to identify genes associated with drugs resistance or specific cellular phenotypes such as migration or invasion that act in a cancer-specific genetic context. A list of published screens performed

in breast cancer is presented in Table 1. Using an shRNA library genetic screen, Berns *et al.* (2007) made a significant contribution to breast cancer research when they discovered that PI3K is a determinant in Trastuzumab (Herceptin) resistance for HER2-positive breast cancers. In a siRNA screen with ovarian and breast cancer cells, Swanton *et al.* (2007) revealed that CERT and its client ceramide were integral to paclitaxel-mediated cell death. Furthermore, Swanton and collogues showed that the paclitaxel response metagene is promising as a paclitaxel-specific predictor of pathological complete response in triple-negative breast cancer. Through the genetic screen of tamoxifen resistant breast cancer, Kim and his group identified IGFBP5 as a determinant of its sensitivity (Ahn *et al.,* 2010).

Screening for	Cell line used	shRNA set used	Genes identified	Reference
Herceptin resistance	BT-474	Retroviral shRNAs	PI3K pathway	(Berns *et al.*, 2007)
Paclitaxel resistance	MDA-MB-231, MDA-MB-468	siRNAs	PPM1D, CENP, IGF1..	(Bauer *et al.*, 2010)
Paclitaxel resistance	MDA-MB-231, SKOV-3	siRNAs	Ceramide metabolism regulators	(Juul *et al.*, 2010; Swanton *et al.*, 2007)
Tamoxifen resistance	MCF-7	Retroviral shRNAs	IGFBP5	(Ahn *et al.*, 2010)

Table 1. Application of RNAi library screens in breast cancer.

Overall, genetic RNAi screening holds promise in accelerating the development of drug-specific therapeutics or predictive biomarkers, since they target cancer-specific genetic alterations.

2.3 Screening strategies

The screening strategy is shown in schematic form in Fig. 1. Several breast cancer cells, including MCF-7, BT-474 and MDA-MB-231 cells were cultured in DMEM supplemented with 10% fetal bovine serum and treated with tamoxifen (5 μM) to find out which cell line is most sensitive. MCF-7 cells were selected for the RNAi screening as they were shown to be the most sensitive to the drug (Fig. 2). Tamoxifen-resistant MCF-7 cells were established by culturing for 4 wks in 10 μM 4-hydroxytamoxifen. Three shRNA oligonucleotides against each of 8,000 genes were designed and cloned into retroviral vector (pRS) containing a shRNA cassette.
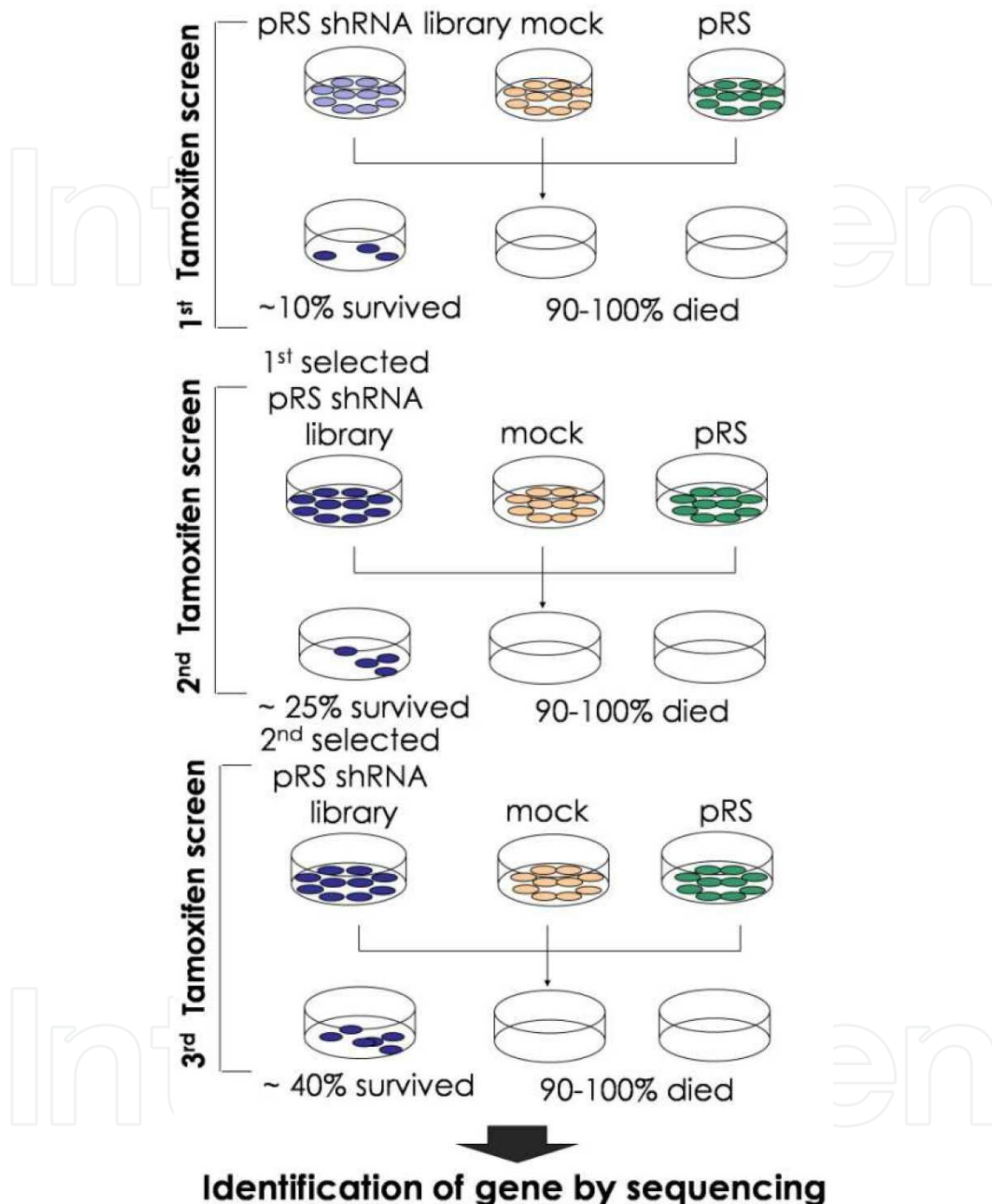
Fig. 1. The screening strategy employed for the identification of tamoxifen resistant genes.

MCF-7 cells were infected with pRS-shRNA library or pRS or mock-infected, selected with puromycin (1 µg/mL) for 2 weeks, and cultured in the presence or absence of tamoxifen (5 µM). After 6 days, genomic DNA was isolated and shRNA inserts were sub-cloned into a pRS vector. Screening was done for two additional rounds to enrich for targets. Targeted genes were identified by sequencing shRNA inserts.
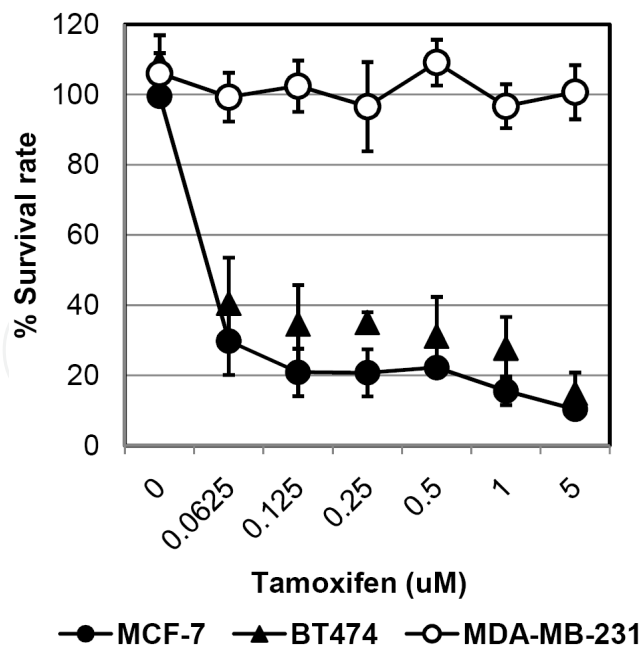
Fig. 2. Various breast cancer cell lines treated with 4-hydroxytamoxifen.

3. Results

Our screen revealed that loss of IGFBP5 contributed to tamoxifen resistance (Ahn *et al.*, 2010). In addition to IGFBP5, we identified several other genes that have a role in regulating autophagy or have a function in the downstream effects of autophagy. Autophagy is the regulated turnover of large components of the cell (e.g., cytosol and organelles) through engulfment in double-membrane sacs, termed autophagosomes, followed by fusion with lysosomes for degradation. These genes include: Vacuolar protein sorting 15 (Vps15), a regulatory subunit of the phosphatidyl inositide 3-kinase (PI3K) complex subunit Vps34 (Lindmo *et al.*, 2008); regulatory-associated protein of mammalian target of rapamycin (mTOR) (Raptor), an inhibitor of mTOR complex kinase activity (Kim *et al.*, 2002); and, interleukin 6 (IL-6), a pro-senescent cytokine (Kuilman *et al.*, 2008). The significance of each of the identified genes will be discussed below.

4. Discussion

4.1 The role of IGFBP5 in tamoxifen resistance

The IGF signaling pathway has important roles in regulating energy metabolism, cellular proliferation, differentiation, and apoptosis (Gluckman *et al.*, 1992; Valentinis & Baserga, 2001). The IGF axis involves a complex regulatory network that operates at a physiological, cellular and sub-cellular level (Pollak *et al.*, 2004). The relationship between IFG signaling and neoplasia has been well documented but the correlation between IGF signaling and endocrine resistance is not as well understood (Pollak, 2008; Pollak *et al.*, 2004). In general, deregulation of IGF signaling may also correlate with the higher incidences of cancer, in particular breast cancer, among affluent nations due to higher incidences of obesity and a preference for a sedentary lifestyle. Both estrogen and IGFs have a synergistic effect on cell proliferation in MCF-7 breast cancer cell lines. Crosstalk between these two pathways has been implicated in the development of resistance to endocrine therapy and cancer recurrence.

The IGF signaling pathway consists of two peptide growth factors, IGF-1 and IGF-2, which can have characteristics of both circulating hormones and tissue growth factors. IGF-1 and IGF-2 can bind their cell surface receptors, IGF-1R and IGF-2R, as well as six different IGF-binding proteins, IGFBP1 to 6 that influence the binding of IGFs to their receptors. The IGF axis is further regulated by IGFBP proteases, and the proteins involved in intracellular signaling distal to IGF-1R which include members of the insulin receptor substrate family, AKT, the target of rapamycin (TOR), and S6 kinase (Pollak *et al.*, 2004).

Compared to HER2 and EGFR, the role of IGF signaling in anti-estrogen resistance is not as well understood and unlike HER2 and EGFR, data is mixed as to whether IGF-1R levels are elevated or decreased in resistant tumors and cell lines (Brockdorff *et al.*, 2003; Gee *et al.*, 2005; Knowlden *et al.*, 2005). Expression of the ER is controlled by IGF-1 in breast cancer cells (Lee *et al.*, 1997). Conversely, genomic and non-genomic interactions of ER can activate the mitogen-inducing signals of the IGF pathway (Fagan & Yee, 2008). Estrogen signaling also causes the expression of insulin receptor substrate 1 (IRS-1), a scaffolding molecule, which activates downstream signaling of IGF-1 and potentiates its proliferative abilities. Salerno *et al.* (1999) suggest that anti-estrogens, like tamoxifen, down regulated IRS-1 and that this down regulation may be one of the possible mechanisms involved in anti-estrogen response. The importance of IGF-1R is further illustrated with studies that show IGF-1R, in ER-negative MDA-MB-231 breast cancer cells, regulates the migration and adhesion abilities of these cells whereas in ER positive MCF-7 breast cancer cells, IGF-1R can modulate mitogenic stimulation (Bartucci *et al.*, 2001). Crosstalk between the IGF signaling pathway and the ER pathway is not only important in breast cancer development but may allow for the development of therapeutics that targets the interactions and communications between the two signaling pathways.

In our own studies, when screening for genes that confer resistance to tamoxifen, we identified IGFBP5 (Ahn *et al.*, 2010). IGFBP5 is a secreted protein and known to inhibit growth factor binding and signaling through IGF-1R (Beattie *et al.*, 2006). In addition, IGFBP5 has recently been shown to play a critical role in breast cancer progression and metastasis, although the exact mechanism remains obscure (Akkiprik *et al.*, 2008). We showed that knock-down of IGFBP5 resulted in resistance to tamoxifen treatment in MCF-7 cells and in mice tumor xenografts. The IGFBP5 knockdown-induced resistance to tamoxifen occurred potentially via altered IGF signaling and loss of ER expression (Bunone *et al.*, 1996; Kato *et al.*, 1995; Lee *et al.*, 1997). We found that treatment with recombinant IGFBP5 reversed *in vitro* and *in vivo* tamoxifen resistance. We also demonstrated, with a cohort of 153 breast cancer patients, that low IGFBP5 expression is associated with shorter overall survival after tamoxifen therapy. *In vitro*, others have shown that with tamoxifen or fulvestrant treated MCF-7 cells, growth inhibition is associated with increased expression of IGFBP5 and increased IGFBP5 secretion into the cell culture media (Huynh *et al.*, 1996; Parisot *et al.*, 1999; Pratt & Pollak, 1993). Preliminary evidence, in addition to some microarray data, supports the idea that IGFBP5 expression level determines tamoxifen responsiveness (Becker *et al.*, 2005).

4.2 Interleukin-6

Results from our screen indicate that reduced expression of IL-6 can confer tamoxifen resistance *in vitro*. IL-6 is a pleiotropic cytokine that has been found to have both tumor-promoting and tumor-inhibiting functions in breast cancer development (Knupfer & Preiss, 2007). Cytokines can be defined as small protein signaling molecules that tend to be

glycosylated and exert their biological function at low concentrations (pg to ng/mL). Cytokines are secreted predominantly by lymphocytes and macrophages and exert their effect by binding to receptors on the target cell surface, altering the function of the target cell by either paracrine or autocrine signaling. The effects of different cytokines can be additive, synergistic, or antagonistic, and the integration of these effects determines the overall outcome and function of a particular cytokine.

Recently, Gilbert *et al*. (Gilbert & Hemann, 2010) have identified IL-6 as a mediator in the development of chemotherapeutic-resistant niches, a novel physiological mechanism behind drug-resistant lymphoma and a likely contributor to the development of cancer recurrence. Specifically, IL-6 along with metallopeptidase inhibitor 1 (Timp-1) were released from the thymus in response to chemotherapy-induced DNA damage, creating a "chemo-resistant niche". The niche helps promote the survival of undetectable residual disease, serving as a reservoir for eventual tumor relapse. Currently it is not known if chemo-resistant niches are present in other cancers or if tamoxifen can elicit the development of such niches.

Notwithstanding, in breast cancer cells, tumor necrosis factor alpha (TNFα) can induce the expression of IL-6 along with IL-11, promoting osteoclast formation and mediating osteolysis at the site of breast cancer bone metastases (Suarez-Cuervo *et al*., 2003). IL-6 has also been implicated in mediating oncogene-induced cellular senescence (Kuilman *et al*., 2008). Cellular senescence, where cells continue to be metabolically active but generally lose their ability to divide may act as a safeguard against a variety of cellular insults, including anti-estrogen therapy. Interestingly, Young *et al*. (2009) identified autophagy as a new effector mechanism of senescence by allowing for rapid protein turnover and cellular re-modeling that occurs when a cell transitions form a proliferative to a senescent state. How knockdown of IL-6 confers resistance to tamoxifen therapy is unclear. The pleiotropic actions of IL-6 complicate our understanding of its role in anti-estrogen resistance and highlight a limitation of RNAi based screens, in that one gene may confer multiple phenotypes.

4.3 Raptor, Vps15, and autophagy

Several gene targets identified in our RNA interference screen for tamoxifen resistance were found to be involved directly in macroautophagy, including Raptor and Vps15. Macroautophagy, hereafter referred to as autophagy, involves the delivery of cytoplasmic cargo, such as long-lived proteins and organelles, sequestered inside double-membrane vesicles, termed autophagosomes, to the lysosomes for degradation. Digested components are recycled back to the cell; therefore, autophagy is a cellular process essential for tissue development and homeostasis, and as an adaptive response to stress and nutrient deprivation (Levine & Kroemer, 2008).

The mTOR, a central regulator of cell growth, is activated through signaling by receptor tyrosine kinase-mediated PI3K activation (Efeyan & Sabatini, 2009). Two distinct protein complexes of mTOR mediate its effects on controlling the rate of cell growth and timing of cell cycle progression including, mTORC1 and mTORC2, respectively (Efeyan & Sabatini, 2009). The mTORC1 complex consists of mTOR, Raptor, and mLST8 and activation leads to increased protein translation, ribosome biogenesis and inhibition of autophagy, the combined effects resulting in increased cell growth (Efeyan & Sabatini, 2009). Raptor participates in mTORC1 complex as a key scaffolding protein to bind mTOR substrates such as eukaryotic initiation factor 4E binding protein 1 (4EBP1) and p70 S6 kinase α (p70α), facilitating phosphorylation and activation by mTOR (Hara *et al*., 2002; Kim *et al*., 2002).

Under nutrient-replete conditions mTOR-Raptor association is weak but is stabilized by nutrient deprivation resulting in inhibition of mTOR kinase activity (Hara *et al.*, 2002; Kim *et al.*, 2002). Raptor-mediated inhibition of mTORC1 is particularly relevant for its role in autophagy as Raptor inhibition of mTOR phosphorylation of ULK1/2 kinase complex leads to ULK1/2 dephosphorylation and activation to initiate autophagosome formation by Atg family of proteins (Kroemer *et al.*, 2010).

Recent attention to development and application of mTOR inhibitors in cancer therapy, including breast cancer, highlights the significance of our finding that loss of Raptor contributes to tamoxifen resistance (Dancey, 2010). In the context of autophagy, loss of Raptor may lead to mTOR phosphorylation of ULK1/2 and autophagy inactivation. In breast cancer, inhibition of mTOR activity has been found to restore tamoxifen sensitivity in breast cancer (deGraffenried *et al.*, 2004).

Vps15 is a putative serine/threonine protein kinase (Stack & Emr, 1994), required for Vps34 activity, a Class III PI3K that produces phosphatidylinositol-3-phophate (Backer, 2008; Schu *et al.*, 1993; Volinia *et al.*, 1995). Vps34 lipid kinase activity has recognized critical functions in early endosome fusion (Christoforidis *et al.*, 1999), maturation (Futter *et al.*, 2001), vesicular trafficking (Stack & Emr, 1994) and starvation-induced autophagy (Takahashi *et al.*, 2007). *In vitro*, Vps15 enhances Vps34 lipid kinase activity (Panaretou *et al.*, 1997); however, Vps34 does not appear to be a substrate of Vps15. Further, Vps15 deletion leads to loss of phosphatidylinositol-3-phosphate production and vesicular trafficking (Stack & Emr, 1994). Lastly, regulation of Vps34 activity by autophagy-related proteins or nutrients requires Vps15 (Yan *et al.*, 2009). The Beclin-1 complex containing Beclin 1, Vps34, Vps15, UVRAG, Atg14L and Rubicon orchestrates demarcation of sites for formation of double membrane organelles associated with Atg5 and Atg12, termed autophagosomes (Zhong *et al.*, 2009). Vps15 regulation of Vp34 activity is necessary for marking sites of autophagosome formation and eventual autophagic removal of protein aggregates (Lindmo *et al.*, 2008).

While little is known about Vps15 in breast cancer, Vps34 function appears to have a pro-survival function mediated through promotion of autophagy. First, increased Vps34 expression levels and tyrosine phosphorylation by pp60c-Scr contributes to enhanced tumorigenic activity in breast cancer cells (Hirsch *et al.*, 2010). Second, in MCF-7 cells, knockdown of Vps34 by RNA interference reduced cytoprotective autophagy mediated by Bcl-2 homology domain 3 (BH3) mimetic gossypol and potentiated apoptosis induction (Gao *et al.*, 2010). Lastly, Vps34 has tumor suppressor function in MCF-7 mouse xenograft tumors mediated through distinct Beclin-1 binding and enhancement of starvation-induced autophagy (Furuya *et al.*, 2005). These studies corroborate lines of evidence demonstrating that PI3K/AKT/mTOR pathway, implicated in cell survival, contributes to anti-estrogen resistance (Musgrove & Sutherland, 2009).

In contrast, our studies have identified loss of Vps15 and Raptor as candidate gene target for mediating breast cancer tamoxifen resistance *in vitro*, suggesting that Vps15- or Raptor-mediated autophagy promotes cell death mediated by tamoxifen. In support of this, inhibitors of each of PI3K, Akt, and mTOR are in clinical trials (Dancey, 2010) and inhibition of mTOR activity is thought to restore tamoxifen sensitivity in breast cancer (deGraffenried *et al.*, 2004). Also, others have also demonstrated that tamoxifen and anti-estrogen-binding site ligands induce autophagic cell death in human breast cancer cells (de Medina *et al.*, 2009; Payre *et al.*, 2008). Tamoxifen-induced cell death through autophagy was associated with cholesterol accumulation. Interestingly, our laboratory's RNA interference screen also identified several genes involved in cholesterol metabolism including Niemann-Pick C 2

(NPC2) protein, reportedly involved NPC disease, in which cells accumulate sterols in multilamellar bodies resulting in onset of autophagy (Pacheco *et al.*, 2007). Furthermore, an ectopic over-expression screen identified a small heat-shock protein family member, which limited autophagic cell death mediated by tamoxifen (Gonzalez-Malerva *et al.*, 2011).

Controversy remains as to whether autophagy limits or promotes tumor malignancy, including drug resistance. Genetic inactivation of autophagy is known to promote tumorigenesis and constitutes a new category of tumor suppressors including Beclin 1. And while oncogenes including PI3K/AKT/mTOR and Bcl-2 inhibit autophagy and hence the ability of tumor cells to proliferate, other oncogenes including Ras and myc stimulate autophagy (Kondo *et al.*, 2005). These variegated effects could represent the significance of autophagy at different stages of tumor progression. Further investigation into the impact of autophagy inactivation on clinical tamoxifen resistance is warranted, specifically on the candidate targets Vps15 and Raptor.

5. Final remarks

Over the last several decades the therapeutic use of tamoxifen has undoubtedly saved countless lives. However, predicting responsiveness and understanding resistance is critical to advances in treatment. Although this chapter has focused on tamoxifen, future studies investigating the mechanisms of resistance to aromatase inhibitors and other anti-estrogens will likely reveal similar mechanisms as the clinical data and experience with these drugs matures. Progress towards better treatment and understanding resistance may be made by further examining the role of IGF signaling and crosstalk with the ER and the role of autophagy.

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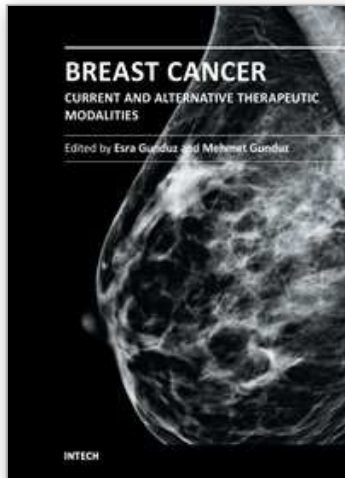
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Cancer is the leading cause of death in most countries and its consequences result in huge economic, social and psychological burden. Breast cancer is the most frequently diagnosed cancer type and the leading cause of cancer death among females. In this book, we discussed various therapeutic modalities from signaling pathways through various anti-tumor compounds as well as herbal medicine for this deadly cancer. We hope that this book will contribute to the development of novel diagnostic as well as therapeutic approaches.

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