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Roles of signaling pathways in drug resistance, cancer initiating cells and cancer progression and metastasis

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A B S T R A C T

The EGFR/PI3K/PTEN/Akt/mTORC pathway plays prominent roles in malignant transformation, prevention of apoptosis, drug resistance, cancer initiating cells (CICs) and metastasis. The expression of this pathway is frequently altered in breast and other cancers due to mutations at or aberrant expression of: *HER2*, *EGFR1*, *PIK3CA*, and *PTEN* as well as other oncogenes and tumor suppressor genes. miRs and epigenetic mechanisms of gene regulation are also important events which regulate this pathway. In some breast cancer cases, mutations at certain components of this pathway (e.g., *PIK3CA*) are associated with a better prognosis than breast cancers lacking these mutations. The expression of this pathway has been associated with CICs and in some cases resistance to therapeutics. We will review the effects of activation of the EGFR/PI3K/PTEN/Akt/mTORC pathway primarily in breast cancer and development of drug resistance. The

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targeting of this pathway and other interacting pathways will be discussed as well as clinical trials with novel small molecule inhibitors as well as established drugs that are used to treat other diseases. In this manuscript, we will discuss an inducible EGFR model (v-ERB-B:ER) and its effects on cell growth, cell cycle progression, activation of signal transduction pathways, prevention of apoptosis in hematopoietic, breast and prostate cancer models.

The epidermal growth factor (EGFR) family

The EGFR family consists of four members of membrane spanning growth factor receptors. There are multiple names for some of the family members. In this review, we will refer to them as EGFR1 (a.k.a., EGFR, HER1, c-erbB1), HER2 (a.k.a., EGFR2, c-erbB2), EGFR3 (a.k.a., c-erbB3, HER3) and EGFR4 (a.k.a., c-erbB4, HER4). The expression of the EGFR1, HER2, EGFR3 and EGFR4 has been examined in many types of cancer. We will focus our discussion to primarily breast cancer studies. In a study with 220 breast cancer carcinomas, elevated expression of EGFR1 was detected in 16.4% of the tumors, 22.8% displayed elevated HER expression, 17.5% displayed elevated EGFR3 expression and 11.9 displayed elevated EGFR4 expression (Witton et al., 2003). Thus the expression of the EGFR family is often deregulated in breast cancer. Those patients with elevated EGFR1, HER2 or EGFR3 expression had reduced survival. However, those patients with elevated EGFR4 expression had better survival than the patients that expressed EGFR1, HER2 or EGFR3. The association of estrogen receptor (ER) expression with the different EGFR molecules was also examined in this study. EGFR1, HER2 and EGFR3 expression was associated with ER negativity (ER-). Breast cancer patients that were ER positive (ER+) and also EGFR1+, HER2+ or EGFR3+ were observed to have poorer survival than those breast cancer patients which were either ER+/EGFR1−/HER2−/EGFR3-or EGFR4+.

Decreased disease-free survival and HER2 expression was observed in a study with samples from 100 breast cancer patients (Suo et al., 2002). Expression of both EGFR1 and HER2 was associated with a worse prognosis. While, expression of EGFR4 was associated with a better outcome. Interestingly, EGFR4 expression may antagonize the effects of HER2 on clinical outcome in those breast cancer patients which expressed both.

The association between ERα and EGFR4 expression was examined in 103 breast cancer patient samples (Suo et al., 2001). Approximately 25% of the breast cancer samples did not express ERα and approximately 25% of them did not express EGFR4. Approximately 50% of the ERα-tumors did not express EGFR4. Thus roles for EGFR4 in ERα-mediated signal transduction in breast cancer have been hypothesized but the real roles of EGFR4 expression are not well elucidated.

The patterns of EGFR1, HER2, EGFR3 and EGFR4 expression were examined in 365 unselected primary breast cancers (Pawlowski et al., 2000). EGFR1 and HER2 were associated negatively with ER+ and progesterone receptor (PR)+ breast cancers. While EGFR3 and EGFR4 were associated positively with ER+ and PR+ breast cancers.

The roles of EGFR3 and EGFR4 in breast cancer are not as well defined as HER2 and EGFR1 (Holbro et al., 2003). EGFR3 is kinase-inactive but it interacts with other EGFR family members and serves to transduce signals to the PI3K/PTEN/Akt/mTORC1 pathway. As EGFR3 is kinase-inactive, it is more difficult to isolate specific inhibitors (Hsieh and Moasser, 2007). However, as it can interact with other EGFR family members, it can contribute to drug resistance. EGFR4 encodes a protein containing an extracellular ligand-binding domain, a hydrophobic transmembrane domain, an intracellular tyrosine kinase domain, and carboxyl-terminal tyrosine residues (Mill et al., 2011a). The EGFR4 carboxyl-terminal tyrosine residues are phosphorylated after ligand binding and allows interaction of the receptor with other signaling molecules (Carpenter, 2003). Ligand binding to EGFR4 induces either homodimerization or heterodimerization of EGFR4 with EGFR4 or another EGFR family member. Ligand binding to EGFR4 leads to activation and the cleavage and release of the EGFR4 cytoplasmic

domain from the membrane. This truncated form of EGFR4 may traffic to the nucleus and mitochondria to induce additional biological effects (Jones, 2008; Sundvall et al., 2008; Mill et al., 2011b).

The roles of EGFR4 in cancer are diverse. EGFR4 may function as a tumor suppressor in some cancers (larynx, pancreatic and prostate) (Thybusch-Bernhardt et al., 2001; Saglam et al., 2007; Uberall et al., 2008; Mill et al., 2011b). Expression or the EGFR4 protein in breast, cervical and ovarian cancers is associated with a favorable prognosis (Gilmour et al., 2001; Lee et al., 2005; Sundvall et al., 2008; Mill et al., 2011a). The Q646C *EGFR4* mutant encodes an EGFR4 protein which undergoes ligand-independent homodimerization and tyrosine phosphorylation which suppresses colony formation of breast, pancreatic and prostate cell lines (Williams et al., 2003; Pitfield et al., 2006; Mill et al., 2011a). The constitutively-active *EGFR4* I658Q mutant induced apoptosis upon transfection into breast, ovarian and prostate cell lines (Vidal et al., 2007).

In contrast, EGFR4 has oncogenic activities in certain cell types and biological situations (Mill et al., 2011a). Over-expression of the EGFR4 protein in conjunction with EGFR1 and HER2 overexpression results in breast cancers correlates with poor prognosis (Abd El-Rehim et al., 2004), while over-expression of EGFR4 by itself leads to a more favorable outcome.

Suppressing EGFR4 expression in ER+ MCF-7 and T47D breast cancer cell lines reduced the anchorage-independent proliferation stimulated by the NRG1β EGFR4 ligand (Tang et al., 1999). However it was the HER2 tyrosine kinase activity, rather than the EGFR4 tyrosine kinase activity that was required for NRG1β to promote cell proliferation (Mill et al., 2011a). The sites of EGFR4 tyrosine phosphorylation, but not the sites of HER2 phosphorylation, were required for NFG1β to regulate cell proliferation (Mill et al., 2011a). Thus the roles of EGFR4 in cancer are complex and are often controlled by the expression of other EGFR family members.

Mutations in HER2 which lead to therapeutic resistance

The *T798M* mutation in *HER2* prevents the small molecule kinase inhibitor lapatinib to binding the ATP-binding pocket of HER2. This results in the resistance of the cells carrying this mutation to lapatinib. The *HER2 T798M* mutation is considered a gatekeeper mutation (Kancha et al., 2011).

Aberrant mRNA splicing can alter the sensitivity to herceptin. A splice variant of HER2 (delete16HER2) results from exon 16 skipping. This genetic alteration increases the transformation frequency and results in resistance to herceptin (Jackson et al., 2013). Whereas, retention of intron 8 of the *HER2* gene after mRNA splicing leads to the creation of herstatin which suppresses tumor cell proliferation. Also retention of intron 15 after splicing of HER2 mRNA results in a p100 protein which also inhibits tumor cell proliferation. Thus both point mutation and alterations in mRNA splicing can alter the response to therapeutic treatments.

HER2 normally heterodimerizes with another EGFR family member for functional activity (Holbro et al., 2003). In contrast, when the *HER2* gene is amplified, HER2 activity is induced and this results in abnormal breast tumor growth that is dependent on HER2.

HER2 mutations have been observed in breast cancer patient samples which lack *HER2* gene amplification. Thirteen *HER2* mutations were identified from twenty-five patient samples lacking *HER2* gene amplification. Seven mutations were determined to be activating. These mutations resulted from point mutations and in-frame deletions. The L755S mutation was not an activating mutation but it did lead to lapatinib resistance. The cells containing these *HER2* mutations were sensitive to the irreversible HER2 kinase inhibitor, neratinib (Milanezi et al., 2008).

Loss of HER2 activity can also lead to the loss of phosphorylated EGFR3. HER2 can dimerize with EGFR3 to drive breast cancer proliferation (Holbro et al., 2003). Loss of EGFR3 activity in HER2+ breast cancer cells was determined to inhibit their growth. This growth inhibition could be overcome by introduction of a construct encoding activated *AKT*. A key function of EGFR3 is to couple the response of HER2 to the PI3K/PTEN/Akt/mTORC pathway.

Role of HER2 in breast CICs survival

HER2 has been determined to be express in the CIC population (Korkaya et al., 2008; Ithimakin et al., 2013). HER2 expression is modulated by the tumor microenvironment. HER2 targeting may be an

appropriate therapeutic approach to eliminate breast CICs. Herceptin inhibited tumor growth of the CIC in mouse xenograft models. In contrast, herceptin did not inhibit the growth of established breast tumors in mouse xenograft models (Korkaya and Wicha, 2013a).

Clinical studies have demonstrated that herceptin targets breast cells which do not overexpress HER2 (Paik et al., 2008). HER2 is considered an important molecule expressed on breast CICs. HER2 expression is induced by signals such as cytokines in the microenvironment in breast CICs which lack HER2 gene amplification. The effectiveness of herceptin in CICs which lack deregulation of HER2 is thought to be due to its ability to target the breast CIC population as well as the PI3K/PTEN/Akt/mTORC and other signaling pathways (Korkaya et al., 2008; Korkaya and Wicha, 2009, 2013b).

HER2 is expressed in ER+, HER2-luminal breast cancers and regulates the self-renewal of the CIC subpopulation (Korkaya and Wicha, 2007). HER2 expression was determined to not result from gene amplification but was due to receptor activation of NF-kappaB (RANK)-ligand in the bone microenvironment.

Mutations and amplifications of EGFR gene family members in breast cancer

The *HER2* gene is amplified in 20–25% of breast cancers. However, the roles of mutations/amplifications of other EGFR family members in breast cancer are not so clear. Mutations and amplifications of the *EGFR1* gene have been detected in breast cancer (Generali et al., 2007). Mutations in the *EGFR1* kinase domain were detected in 11% of 70 triple negative breast cancer (TNBC) patient samples (Teng et al., 2011). The region spanning exons 18 to 21 of the *EGFR1* gene were examined in this study. Deletions in exon 19 of *EGFR1*, which encodes part of the kinase domain, were detected. But these mutations appeared to be independent of the expression levels of the EGFR1 protein. Some of the effects of mutations/amplifications in *EGFR1*/*HER2* and other genes in cancer on signal transduction pathways are presented in Fig. 1.

The *EGFR1* gene is amplified in certain breast cancers (Bhargava et al., 2005). Approximately 6% of the breast cancer patients ($n = 175$) examined in this study had *EGFR1* amplified. The patient samples with *EGFR1* amplified displayed increased EGFR1 expression. The patient samples containing amplified *EGFR1* gene were examined for hot-spot mutations in the *EGFR1* gene. However, no mutations were detected in exons 19 and 21.

The occurrence of *EGFR1* or *HER2* gene amplification or overexpression has been examined in a study with metaplastic breast carcinoma (MBC) patient samples (Reis-Filho et al., 2005). MBC are basal like tumors that account for less than 1% of all invasive mammary carcinomas. Nineteen of the 25 (76%) MBC patient samples examined overexpressed *EGFR1*. While *EGFR1* gene amplifications were detected in 37% of the MBC tumors which overexpressed EGFR1, only one patient sample displayed *HER2* overexpression. However, *HER2* gene amplification was not detected. Some of the tumors that overexpressed *EGFR1*, did not appear to have *EGFR1* gene amplification. The authors pointed out that the expression of EGFR1 could have resulted from activating mutations in the *EGFR1* gene. The presence of *EGFR1* amplification and activating mutations was examined in an additional study consisting of 47 MBC by the same group (Reis-Filho et al., 2006). Overexpression of *EGFR1* was observed in 32% of the MBC samples. In the MBC subset which had *EGFR1* overexpression, 34% had *EGFR1* gene amplification. In contrast, no activating mutations of *EGFR1* in exons 18, 19, 20 and 21 were detected in these MBC patient samples.

EGFRvIII an activated EGFR1 structurally related to v-ERB-B

The epidermal growth factor receptor variant III (*EGFRvIII*) is a mutant form of *EGFR1* is a genetic truncation of the EGFR1 gene. *EGFRvIII* encodes a constitutively-active truncated EGFR1 protein which has been implication in many types of cancers (e.g., brain, breast, prostate and others) (Tang et al., 2000; Müller et al., 2001; Ge et al., 2002; Rae et al., 2004; Silva et al., 2006; Nieto et al., 2007; Zhang et al., 2009; Thakkar and Mehta, 2011; Yu et al., 2008; Rahimi et al., 2010, 2011; Mukherjee and Zhao, 2013; Del Vecchio et al., 2012). *EGFRvIII* is structurally similar to the activated oncogene contained in the avian erythroblastosis virus *v-ERB-B*.

Transfection of MCF-7 breast cancer cells with a construct encoding *EGFRvIII* resulted in HER2 phosphorylation, which may have occurred through heterodimerization and cross-talk (Tang et al.,

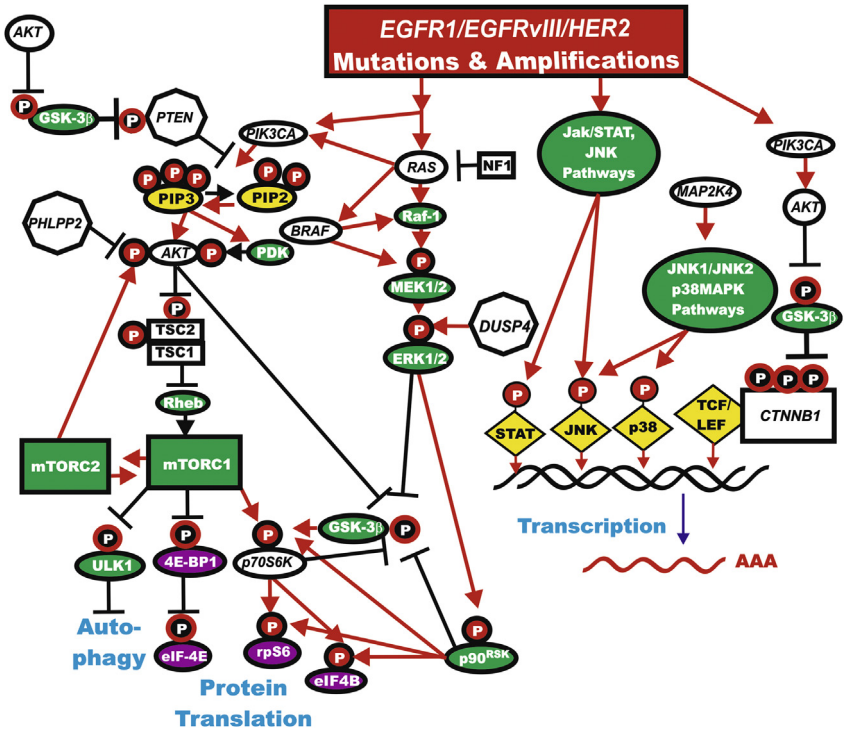

Fig. 1. Effects of mutations at EGFR family members and downstream signaling molecules on signaling, gene expression and autophagy. Aberrant expression of EGFR-family receptors occurs by genetic mutations or amplifications which can lead to activation of the Ras/Raf/MEK/ERK, Ras/PI3K/PTEN/Akt/mTOR and other signaling pathways. Genes with mutations in them which are involved in breast cancer have the gene name in italics. Genes in the Ras/Raf/MEK/ERK and Ras/PI3K/PTEN/Akt/mTOR pathways that have activating mutations or increased expression detected in human cancer and proliferative diseases are indicated in white ovals and squares. Tumor suppressor genes inactivated in certain cancer are indicated in white squares or octagons. Transcript factors are indicated in yellow diamonds. Proteins involved in mRNA translation are indicated in purple ovals. Other key genes are indicated in green ovals and squares. Phosphorylation which results in activation of signaling proteins are indicated by a P in a red circle. Phosphorylation which results in inactivation of signaling proteins are indicated by a P in a black circle. Red arrows indicate activating events in pathways. Blocked black arrows indicating inactivating events in pathways. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2000). The MCF-7/EGFRvIII transfected cells exhibited a 3-fold increase in colony formation and were more tumorigenic than parental MCF-7 cells in athymic nude mice.

The EGFRvIII protein is detected in various human cancers, while it is not or infrequently detected in normal tissues. Laser capture microdissection (LCM)/RT-PCR was used to examine the presence of EGFRvIII mRNA in primary invasive breast cancer. EGFRvIII transcripts were observed in 67.8% of pure breast cancer cells (Ge et al., 2002). 57.1% of the infiltrating breast carcinomas expressed both wild type (WT) EGFR1 WT and EGFRvIII mRNA transcripts in the same tumor specimen. EGFRvIII mRNA transcripts were not detected in samples of normal breast tissue. Immunohistochemical analysis confirmed these results that co-expression of EGFRvIII and EGFR1 WT mRNAs in some human invasive breast cancer tissue but not in normal breast samples. Other studies, by a different group, consisting of 55 breast cancer cell lines and 170 primary breast cancer did not observe similar results and the authors concluded that expression of *EGFRvIII* is extremely rare in breast cancer (Rae et al., 2004). The expression of EGFRvIII mRNA in women with breast cancer was examined by another group by RT-nested PCR (Silva et al., 2006). EGFRvIII mRNA transcripts were observed in the peripheral blood obtained from 30% of 33 low risk, early stage patients, in 56% of 18 patients that were selected for neoadjuvant chemotherapy, in 63.6% of 11 patients with disseminated disease but not in any of 40

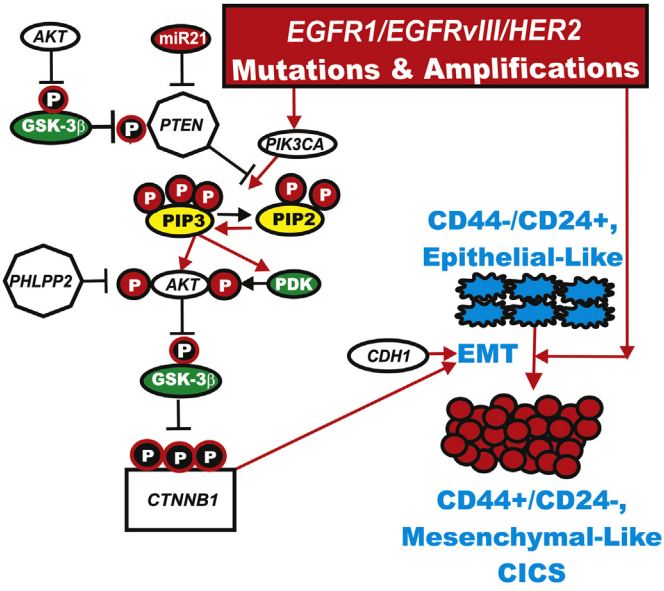

Fig. 2. Effects of mutations at EGFR family members on EMT and CICs. Aberrant expression of EGFR-family receptors and mutations at key genes in the PI3K/PTEN/Akt/mTORC pathway can alter EMT and CIC generation. Genes with mutations in them which are involved in breast cancer have the gene name in italics. Genes in Ras/PI3K/PTEN/Akt/mTOR pathways that have activating mutations or increased expression detected in human cancer and proliferative diseases are indicated in white ovals and squares. Tumor suppressor genes inactivated in certain cancer are indicated in white octagons. Phosphatidylinositol 4,5-bisphosphate (PIP2) and phosphatidylinositol (3,4,5)-trisphosphate (PIP3) are indicated in yellow ovals. Red arrows indicate activating events in pathways. Blocked black arrows indicating inactivating events in pathways. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

"control" women (Silva et al., 2006). EGFRvIII mRNA expression was associated with ER− or HER2+ in the low risk, early stage patients. The expression of EGFR1, phosphorylated EGFR1, and EGFRvIII, was examined by immunohistochemistry and the patient outcomes were also followed in a different study of 225 breast cancer patients (Nieto et al., 2007). 48% of the patients displayed EGFR1 expression, 54% of the patients were positive for phospho-EGFR and 4% of the patients were positive for EGFRvIII expression. EGFR1 expression was shown to correlate with negative hormone receptor status, worse relapse-free and overall survival in comparison to those patients with did not express detectable EGFR1 (Nieto et al., 2007). There did not appear to be any association between the presence of phospho-EGFR or EGFRvIII expression with clinical outcome. The prognostic value of EGFR1 expression was most important in the HER2+ and the ER−/progesterone receptor−(PR−) subgroups.

EGFRvIII expression has effects on PR expression. EGFRvIII downregulates PR expression in certain luminal B tumors (Zhang et al., 2009). These breast tumors were ER+ but were characterized as having an aggressive behavior which was resistant to the estrogen receptor antagonist 4-hydroxyl tamoxifen (4HT). These breast cancers displayed elevated EGFR1, HER2 and downstream PI3K/PTEN/Akt/mTORC1 pathway activation (Zhang et al., 2009).

EGFRvIII can interact with HER2 (Yu et al., 2008) and the chemokine (C-X-C motif) receptor 4 (CXCR4) (Rahimi et al., 2010, 2011). These interactions activate additional signaling pathways which are important in migration, invasion and tumorigenesis. Interactions between EGFRvIII and HER2 or CXCR4 may be prolonged and oncogenic in comparison to interactions between EGFR1 and HER2 or CXCR4. This may result from the mutant EGFRvIII being constitutively active and it is more difficult to down regulate the constitutive nature of EGFRvIII than the ligand-inducible EGFR1. It turns out that CXCR4 is highly expressed in breast cancers and implicated in metastasis (Müller et al., 2001). More recently CXCR4 has been shown to have important roles in cancer initiating cells (CICs) (Mukherjee and Zhao, 2013). A diagram of some of these and other mutations may effect CIC generation is presented in Fig. 2.

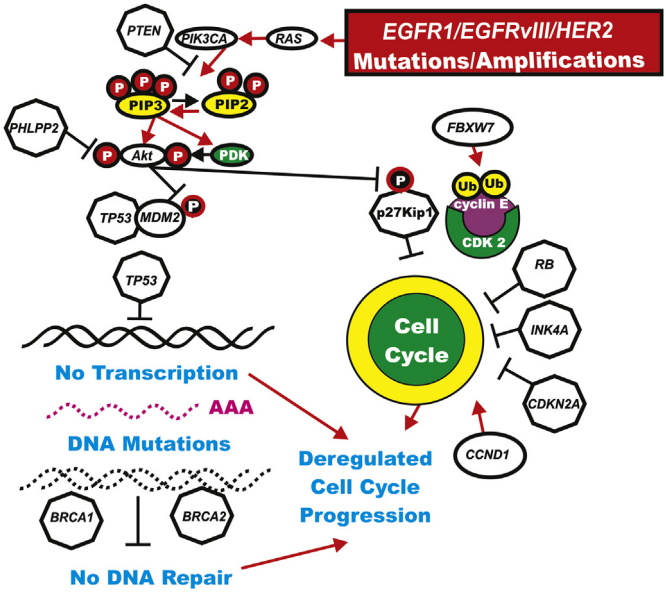

Fig. 3. Effects of mutations at EGFR Family members on cell cycle progression. Aberrant expression of EGFR-family receptors and mutations at key genes in the PI3K/PTEN/Akt/mTORC pathway can alter cell cycle progression. Genes with mutations in them which are involved in breast cancer have the gene name in italics. Genes in the Ras/PI3K/PTEN/Akt/mTOR pathways that have activating mutations or increased expression detected in human cancer and proliferative diseases are indicated in white ovals and squares. Tumor suppressor genes inactivated in certain cancer are indicated in white squares or octagons. Phosphorylation which results in activation of signaling proteins are indicated by a P in a red circle. Phosphorylation which results in inactivation of signaling proteins are indicated by a P in a black circle. Red arrows indicate activating events in pathways. Blocked black arrows indicating inactivating events in pathways. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

EGFRvIII plays important roles in primary breast cancers and breast CICs (Del Vecchio et al., 2012). EGFRvIII expression has been linked with the Wnt/β-catenin pathway and downstream β-catenin target gene expression as well as the expression of genes associated with self-renewal and invasiveness. EGFRvIII expression has been linked with increased *in vitro* mammosphere formation and tumor formation (Del Vecchio et al., 2012).

Mutations/alterations in Ras and PI3K pathway members in breast cancer

The mutational status of the Ras and PI3K pathway genes in 40 breast cancer cell lines, mutations was examined (Hollestelle et al., 2007). Mutations were detected at approximately 25% of Ras pathway members (*KRAS*, *HRAS*, *NRAS*, and *BRAF*) and 54% of PI3K pathway members (*PTEN*, *PIK3CA*). In this study, mutations in both pathway family genes in a given cell line were not frequently detected. In additional studies by the same group with 41 breast cancer cell lines, 146 mutations were detected among twenty-seven cancer causing genes, this corresponded to an average of 3.6 mutations per cell line (Stemke-Hale et al., 2008). Mutations in the tumor suppressor genes *TP53*, and *RB* and PI3K pathway genes were frequently observed in the breast cancer cell lines. These investigators could establish mutational profiles that were associated with luminal-type and basal-type breast cancer cell lines. The mutational profile associated with luminal breast cancer included E-cadherin (*CDH1*) and mitogen-activated protein kinase kinase 4 (*MAP2K4* a.k.a. *MEK4*) genes and amplifications of the cyclin D1, (*CCND1*), *HER2* and mouse double minute 2 homolog (*MDM2*). The mutational profile associated with the basal profile included: *BRCA1*, *RB1*, *RAS* and *BRAF* gene mutations and deletions of tumor suppressors such as p16 (*CDKN2A*) and p14ARF (*CDKN2A* a.k.a. *INK4A*) (Hollestelle et al., 2007). A diagram of how some of these mutations affect cell cycle progression is presented in Fig. 3.

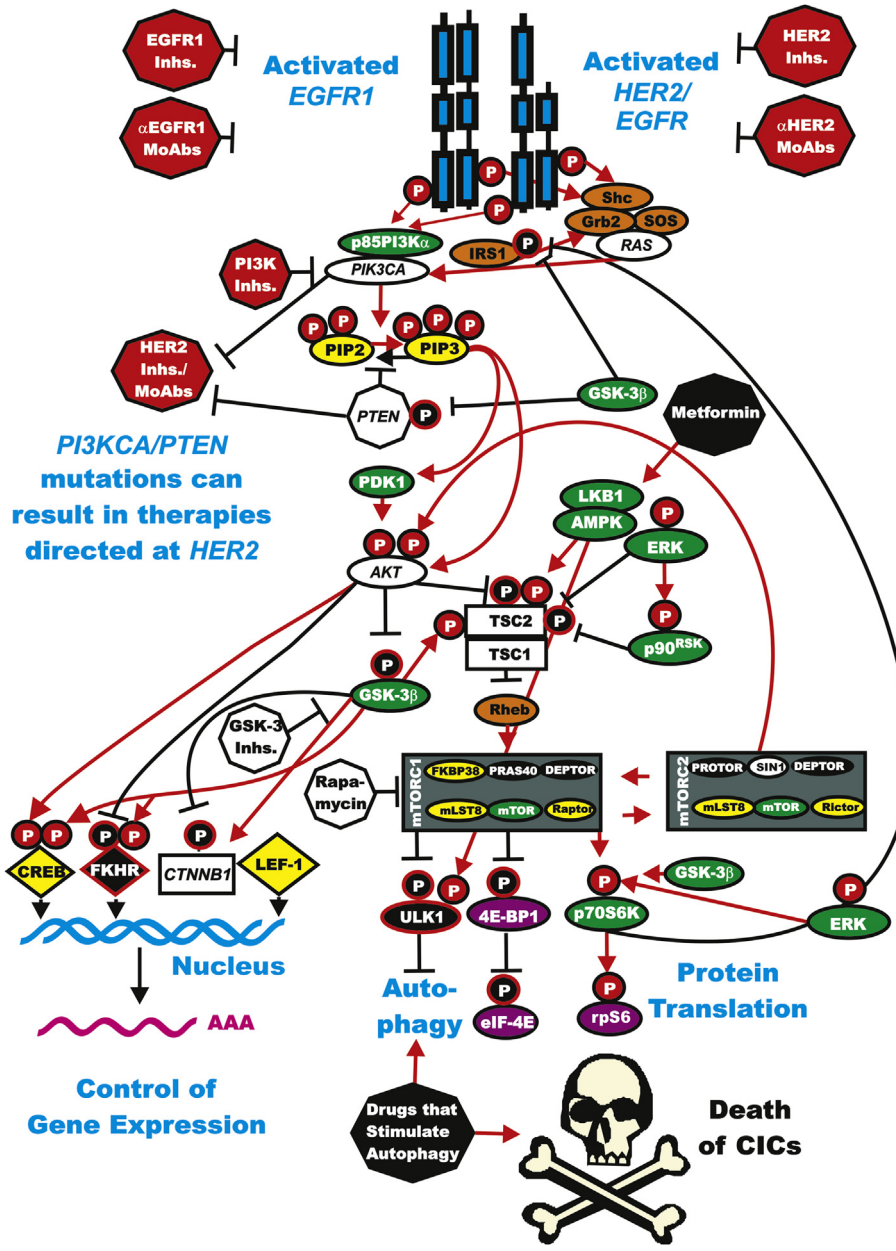

Fig. 4. Overcoming therapeutic resistance by targeting the mutant EGFR/PI3K/PTEN/Akt/mTORC pathway with small molecule membrane-permeable inhibitors and monoclonal antibodies (MoAbs). Genes with mutations in them which are involved in breast cancer have the gene name in italics. The EGFR1 and HER2/EGFR receptors are indicated in blue figures. The downstream PI3K/PTEN/Akt/mTORC1 pathway is regulated by Ras (indicated in clear oval) and PTEN indicated in a clear octagon. Other key receptor associated substrates/coupling molecules such as Shc, Sos, Grb2 and IRS1 are indicated by orange ovals. Phosphorylation which results in activation of signaling proteins are indicated by a P in a red circle. Phosphorylation which results in inactivation of signaling proteins are indicated by a P in a black circle. Sites where various small molecule inhibitors suppress this pathway are indicated by red octagons. Sites which stimulate proteins involved in autophagy are indicated by black octagons and ovals. The serine/threonine-protein kinase ULK1 (ULK1) which is regulated by mTORC1 is indicated in a black oval. The downstream transcription factors regulated by this pathway are indicated in yellow diamond shaped outlines. The Ras/Raf/MEK/ERK pathway also

Mutations of the *RAS* genes are not always necessary for activation of the Ras pathway in breast and other cancers (Eckert et al., 2004; Hongu and Kanaho, 2014; Maertens and Cichowski, 2014). Gene profiling studies indicates that Ras pathway dependence can predict the sensitivity to inhibitors targeting the Raf/MEK/ERK and PI3K/PTEN/Akt/mTORC1 pathways. Activation of the Ras was associated with sensitivity to MEK inhibitors but resistance to Akt inhibitors in breast tumors. A Ras pathway signature was determined to be a better indicator of Ras pathway dependency than mutations at *KRAS*, as there can be many genetic mutations which can lead to Ras pathway dependency (e.g., upstream receptors, *EGFR1* and others as well as *BRAF* mutations and other downstream signaling molecules). Breast cancers with the Ras pathway signature were determined to be sensitive to MEK inhibitors but resistant to Akt inhibitors. A Ras pathway signature was increased in ER-breast cancers.

Significance of PI3K/PTEN/Akt mTOR pathway mutations in breast cancer

PIK3CA mutations were detected in 47.4% of MBC cancers which were aggressive and also chemoresistant (Hennessy et al., 2009). *PI3KCA* mutations were observed in 34.5% of hormone receptor-positive cancers, 22.7% of 75 HER2-positive cancers, 8.3% of basal-like cancers but none of claudin-low tumors examined in this study. Interestingly, MBCs and claudin-low breast cancer subsets display enrichment for markers linked to stem cell function and epithelial to mesenchymal transition (EMT). MBCs and claudin-low tumors are enriched with CICs and are postulated to arise from an earlier, more chemoresistant breast epithelial precursor than either basal-like or luminal cancers. No mutations at *PIK3CA* were detected in the claudin-low cancers, so the functions of *PIK3CA* mutations in these cancers are not clear.

The *PIK3CA*, *AKT* and *PTEN* genes were analyzed for mutations as well as sensitivity to the PI3K inhibitor LY294002 were analyzed in 547 human breast cancer patient samples and 41 established cell lines (Stemke-Hale et al., 2008). ER+ tumors had more *PIK3CA* mutations (34.5%) than in HER2+ tumors (22.7%) or in basal-like tumors (8.3%). *AKT1* (1.4%) and *PTEN* (2.3%) mutations were present ER+ breast tumors. In this study, cells with *PIK3CA* mutations were less sensitive to the PI3K inhibitor LY294002 inhibitor than tumors which lost functional PTEN activity. PI3K pathway aberrations exert roles in the pathogenesis of different breast cancer subtypes. Identification of these aberrations may contribute in the rational design of PI3K-targeted therapies in hormone receptor+ breast cancer.

The genetic structure of the *PIK3CA* gene was examined in 452 breast cancer tumor specimens (Cizkova et al., 2012). *PIK3CA* mutations were detected in 33.4% of the samples. *PIK3CA* mutations were determined to be more frequently detected in ERalpha+ and PR+ breast cancers (41.1%), than in TNBCs (ER−, PR−, HER2−) (12.5%). A longer metastasis-free survival period was observed in the breast cancer patients with *PI3KCA* mutations.

The structure of the *PIK3CA* gene and clinical outcome after herceptin treatment was examined in eighty HER2+ breast cancer patients (Cizkova et al., 2013). The *PIK3CA* gene was mutated in 21.3% of HER2+ breast cancer patients treated with herceptin for one year. Patients with WT *PIK3CA* had better disease free survival compared with patients having mutant *PIK3CA*.

The expression of the *PIK3CA* gene, the PI3K-p110 was examined in 1394 early stage breast cancer samples. Increased PI3K-p110 protein expression was determined in the basal-like breast cancers, HER2+ breast cancers, and TNBCs. Whereas reduced levels of the PI3K-p110 protein were detected in luminal tumors in comparison to the other classes. Shorter disease free survival was observed in the PI3K-p110+ breast cancer patients. These studies documented that PI3K-p110 (*PIK3CA*) expression is a biomarker associated with poor prognosis in breast cancer (Aleskandarany et al., 2010). It has been estimated that deregulation of the PI3K/PTEN/Akt/mTORC1 pathway by gene mutations has been estimated to occur in >70% of breast cancers (Miller et al., 2011). Activation of the PI3K/PTEN/Akt/mTORC1 pathway can result in both estrogen-dependent and estrogen-independent ER activity in ER+

interacts with key proteins involved in protein translation (indicated in green ovals). Red arrows indicate activating events in pathways. Blocked black arrows indicating inactivating events in pathways. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

breast cancers and contribute to estrogen-independence and potentially loss of sensitivity to hormonal based therapies.

Mutations at *PIK3CA* and *PTEN* which contribute to herceptin resistance

Induction of the PI3K/PTEN/Akt/mTORC1 pathway can also lead to resistance to HER2 inhibitors in HER2+ cells. PI3K/PTEN/Akt/mTORC1 pathway inhibition with small molecule inhibitors can overcome resistant to hormonal and anti-HER2 targeted therapies (Miller et al., 2011). Combinations of various inhibitors have been examined in clinical trials and have yielded encouraging results. Combinations of HER2, PI3K, mTORC2 inhibitors and hormonal based therapeutics may be appropriate for breast cancer patients which are resistant to current therapies. A diagram of potential sites of targeting and the resistance to HER2-based therapeutics is presented in Fig. 4.

Some of the genes involved in resistance to herceptin were identified by an RNA interference screen (Berns et al., 2007). *PIK3CA* mutations or low PTEN expression were determined to be associated with resistance to herceptin by screening of samples from 55 breast cancer patients. Oncogenic *PIK3CA* mutations could confer resistance to herceptin in cell culture models (Berns et al., 2007).

Role of PI3K pathway in breast CICs

The *PIK3CA* gene is mutated in certain breast CICs (Hennessy et al., 2009). The structure of genes in the PI3K/PTEN/Akt/mTOR pathway as examined in DNA isolated from fine needle aspirations of 267 stage I–III breast cancers (Santarpia et al., 2012). Twenty-eight genes were dissected for 163 known cancer-related DNA sequence variations by Sequenom technology. In breast cancers, the PI3K pathway was frequently altered, as at least one mutation in 38 alleles was detected. This corresponded to 15 genes in 108 (40%) of the breast cancer samples. In 16.1% of all samples, the *PIK3CA* gene was mutated. In 8%, the F-box and WD repeat domain containing 7, E3 ubiquitin protein ligase (*FBXW7*) gene was mutated. In 3% the *BRAF* gene was mutated. In 2.6% the *EGFR1* gene was mutated. In 1.9% the *AKT1* and *CTNNB1* genes (β-catenin) were mutated. In 1.5% the *KIT* and *KRAS* genes were mutated. In 1.1% the *PDGFRA* gene was mutated. Polymorphism at the PH domain and leucine rich repeat protein phosphatase 2 (PHLPP2) was detected in 13.5% of the patient samples. PPLPP2 activates Akt. PPLPP2 polymorphism could result in Akt activation. *PIK3CA* mutations were detected more frequently in ER+ cancers in comparison to TNBC (19 vs. 8%). A high frequency of *PIK3CA* mutations (28%) was seen in HER2+ breast tumors. *FBXW7* mutations were significantly more frequent in TNBC compared to ER+ tumors (13 vs. 5%). FBXWZ is a component of ubiquitin ligase (SKP-cullin-F-box) complex. It binds cyclin E and targets it for ubiquitin-mediated degradation.

Akt as to drug therapy sensitivity marker

Activation of Akt predicts the effectiveness of paclitaxel chemotherapy in node-positive breast cancer patients (Yang et al., 2010). The effectiveness of combining paclitaxel with doxorubicin (a.k.a Adriamycin) plus cyclophosphamide (AC) was examined in breast cancer patients (median follow up 9.1 years) in the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-28 trial. Increased effectiveness of adding the combination of the three drugs was observed in those breast cancer patients who expressed elevated, activated P-Akt. However, no enhanced effectiveness of adding paclitaxel to AC was observed in those breast cancer patients who lacked activated P-Akt expression. The PI3K/PTEN/Akt/mTOR clearly an important pathway in the drug resistance of breast and other types of cancer (Elong Edimo et al., 2013, 2014; Shiizaki et al., 2013; Chan et al., 2013; Lattanzio et al., 2013; Boultwood et al., 2013; Pouillon et al., 2013; Toker and Marmiroli, 2014; Laurent et al., 2014; Chan and Katan, 2013).

Involvement of PTEN and cytokines in HER2-resistance and CIC survival

PTEN has been determined to be important in breast CIC survival. Knockdown of PTEN expression lead to increases in normal and malignant human mammary stem/progenitor cells both *in vitro* and

in vivo. The rise in progenitor cells was due to increased Akt activation which resulted in GSK-3β phosphorylation and inhibition of GSK-3β activity which led to activation of the Wnt/β-catenin pathway. The increases in progenitor cells could be suppressed by targeting Akt with the inhibitor perifosine (Korkaya et al., 2009). GSK-3 is an important regulatory kinase implicated in breast and other cancers (Hunt, 2013; Chan et al., 2013; McCubrey et al., 2014a,b; Martelli et al., 2014a,b; McCubrey and Cocco, 2014; Davis et al., 2014; Sokolosky et al., 2014). PTEN is involved in the resistance of breast cancers to herceptin (Rexer et al., 2013) and other therapeutic approaches, such as chemotherapy (Steelman et al., 2008; Barker et al., 2013; Jhanwar-Uniyal et al., 2013; Ribeiro et al., 2013; Fitzgerald and McCubrey, 2014; Bertrand et al., 2014; Fragoso and Barata, 2014).

The cytokine interleukin-6 (IL-6) is an important immune-regulator. It is also important in HER2-resistance as it has the ability to expand the CIC population (Shostak and Chariot, 2011; Korkaya and Wicha, 2013a). As stated previously, decreases in PTEN expression can lead to herceptin-resistance. An IL-6 inflammatory feed back loop resulted in herceptin-resistant HER2+ cell lines which had PTEN knocked-down. This resulted in the expansion of breast CIC which have an EMT phenotype and secrete 100-fold more IL-6 than in the cells which had normal PTEN levels. The generation of the CICs could be suppressed by treatment with an anti-IL-6R Ab which inhibited the IL-6 regulatory loop. Suppression of the IL-6 regulatory loop also reduced tumor growth and metastasis in mouse xenographs.

The tumor microenvironment is critical for CICs. Interactions between the interleukin-8 receptor (IL-8R) and HER2 are necessary for the survival of breast CICs (Korkaya and Wicha, 2013b). NF-κB is also important in breast cancer CIC survival and HER2-dependent tumorigenesis. NF-κB activity is regulated in part by the PI3K/PTEN/Akt/mTORC1 pathway (Shostak and Chariot, 2011).

Inhibiting breast cancer therapeutic resistance by targeting the PI3K/PTEN/Akt/mTORC1 pathway

Treatment of breast cancer patients with the aromatase inhibitor (AI) letrozole leads to suppression of the PI3K/PTEN/Akt/mTORC1 pathway (Generali et al., 2008). The expression of PI3K (p110), P-Akt, and P-mTOR was examined on breast cancer samples from 113 patients by immunohistochemistry. The patients had been treated with letrozole or letrozole and cyclophosphamide. Both letrozole and letrozole plus cyclophosphamide-treated patients exhibited a reduction in PI3K and P-mTOR expression. P-Akt levels in the letrozole-treated patients did not change whereas P-Akt levels were observed to decrease in the letrozole and cyclophosphamide-treated patients. Reduction of P-Akt expression was linked with a better response rate. Also reduction in P-mTOR expression was observed to be associated with a longer disease-free survival. The AI letrozole targets key components of the PI3K/PTEN/Akt/mTORC1 pathway which may be critical in the successful treatment of certain breast cancers.

Roles of PI3K/PTEN/Akt/mTORC1 pathway activation and ER expression in breast cancer

Certain *PIK3CA* mutations present in ER+/HER2-luminal breast cancers actually result in low levels of mTORC1 expression. However these particular breast cancers may have better treatment successes after 4HT therapy (Loi et al., 2010). Certain *PIK3CA* mutations may render breast cancers sensitive to targeted therapy. A *PIK3CA* gene expression pattern was derived from these studies. The pattern was associated with prognosis in those breast cancer patients with *PIK3CA* mutations that remained ER+/HER2−, however, it was not associated with prognosis in breast cancer patients which were either ER− or HER2+.

In ER+ breast cancers, an inverse relationship between ER expression and PI3K pathway activation was documented. A negative correlation was seen, as lower levels of ER were detected when the PI3K pathway was activated. Treatment of breast cancer cells with insulin like growth factor-1 (IGF-1), which activated the PI3K pathway, suppressed ER expression. Treatment of breast cancer specimens with the dual PI3K/mTOR inhibitor BEZ-235 increased ER activity and ER-regulated gene expression (Creighton et al., 2010). ER+ breast cancers displayed elevated PI3K pathway in the more aggressive luminal B breast cancer subtypes than the less aggressive luminal A subtype. The inhibitor increased the effects of 4HT on the more aggressive luminal B breast cancer, likely by increasing ER expression

and restoring sensitivity to hormone based therapies. Thus it may be therapeutically effective to treat certain breast cancer patient with a combination of PI3K inhibitors and 4HT.

The PI3K/PTEN/Akt/mTORC1 pathway is important regulating the abnormal proliferation of breast cancers which have become hormone-independent. Increased phosphorylation of p70S6K, p85S6K and Akt was observed in four hormone-independent breast cancer cell lines recovered after long term estrogen deprivation (Miller et al., 2010). Apoptosis was induced after suppression of the PI3K pathway in these hormone-independent cells. Thus certain hormone-independent breast cancers may be sensitive to the combination of PI3K pathway inhibitors and ER antagonists.

Suppression of PTEN activity is associated with resistance to chemotherapeutic drugs, hormonal based therapies and hypersensitivity to the mTORC1 inhibitor rapamycin (Steelman et al., 2008; Miller et al., 2009). shRNA-mediated inhibition of PTEN expression by shRNA lead to three ERα+ breast cancer cell lines that displayed hormone-independent growth. These cells were also resistant to 4HT and fulvestrant. Fulvestrant is an ER antagonist which downregulates the ER. PTEN inhibition also increased basal and ligand-induced activation of IGF-1R and EGFR3, illustrating the effects that PTEN has on the regulation of these upstream tyrosine kinases. Suppression of IGF-1R or EGFR3 restored hormonal dependency as well as the effects of hormonal therapy on the breast cancer cells with PTEN-knocked down. These results suggest a possible treatment strategy for breast cancers which either lack PTEN or have decreased PTEN expression. These results illustrate the complex interactions between hormonal- and growth factor-receptor mediated signaling.

PI3K-p110α and PI3K-p110β subunit expression in breast carcinomas

PI3K-p110α (*PIK3CA*) and PI3K-p110β subunit expression was examined on 315 invasive breast carcinomas. The clinical outcomes were then compared with the expression results (Carvalho et al., 2010). The p110 subunits were detected in 23.8% of invasive breast carcinomas. PI3K-p110α was observed to be expressed in 11.8% while PI3K-p110β was detected in 15.2% of the invasive breast carcinomas. PI3K-p110α expression was associated with hormone receptor expression but was not with overall survival. PI3K-p110β expression was associated with HER2 overexpression and lack of hormone receptor expression. PI3K-p110β+ patients had lower age of onset, lymph node involvement and distant metastasis. A worse prognosis and overall survival in the breast cancer patients that expressed membrane PI3K-p110β. Co-targeting of HER2 and PI3K-p110β may be an appropriate therapeutic approach in certain breast cancer patients.

Regulation of mRNA translation in breast cancer by downstream components of the PI3K/PTEN/Akt/mTORC1 pathway

The translation of certain mRNAs, which are considered difficult to translate due to their structures, is often regulated by the PI3K/PTEN/Akt/mTORC1 pathway (Martelli et al., 2011; McCubrey et al., 2011; Kenney et al., 2014). Key components of the translational apparatus such as: eIF4E, eIF4G, 4E-BP1, rpS6, programmed cell death protein 4 (pdcd4), eEF2 and eEF2K are often regulated by the PI3K/PTEN/Akt/mTORC1 pathway. The expression of some of these molecules was examined in 190 hormone receptor-positive breast cancer patients (Meric-Bernstam et al., 2012). Enhanced eEF2K, rpS6, and p4E-BP and decreased pdcd4 were associated with poor prognosis in hormone receptor+ breast cancer types. Thus certain molecules involved in translation may be prognostic markers and therapeutic targets for certain classes of breast cancer (e.g., hormone-responsive breast cancers).

EGFR1/HER2/PI3K/Akt/mTORC1 pathway and targeting by microRNAs (miRs)

miR21 is linked with tumor progression, metastasis, EMT and CICs. Inhibition of miR21 in MDA-MB-231 cells reversed EMT and the CIC phenotype and resulted in induction of PTEN expression and inactivation of Akt and ERK (Han et al., 2012). Whereas inhibition of PTEN expression reversed the effects of the miR-21 antagomir on EMT and the CIC generation. Antagomirs (a.k.a anti-miRs or blockmirs) are synthetic RNAs complementary to the miR target but have either mispairing at the Ago2 cleavage site or a base modifications which inhibits Argonaute RISC catalytic component 2

(Ago2)-mediated cleavage. Antagomirs are usually chemically modified (2'-methoxy groups and phosphorothioates), which make them more resistant to degradation. Akt and ERK are required for the effects of miR-21 as inhibitors of PI3K/Akt and Raf/MEK/ERK pathways suppressed EMT and CIC phenotype.

Therapy of breast cancer with specific antibodies

Herceptin (trastuzumab) is a genetically engineered antibody. Herceptin is used to treat HER2+ breast cancers. Addition of certain chemotherapeutic drugs and herceptin improves survival and response rates (Nahta and Esteva, 2003).

Pertuzumab (Perjeta®) is a newer antibody developed by Genentech/Roche which also targets HER2. Herceptin and pertuzumab bind to different sites on HER2. It turns out that the co-administration of herceptin and pertuzumab has synergistic inhibitory effects on HER2+ breast cancers (McCormack, 2013).

Pertuzumab blocks the dimerization of HER2 with other EGFR family members (EGFR1, EGFR3, EGFR4) (Kümler et al., 2013). Pertuzumab suppresses the interaction of HER2 with EGFR3 which suppresses activation of the normally pro-proliferative PI3K/PTEN/Akt/mTOR pathway (Metzger-Filho et al., 2013).

Clinical studies with treatment with pertuzumab by itself did not reveal impressive results in suppressing breast cancer growth. The more recent phase III CLEOPATRA trial explored the effects of combined treatment of pertuzumab with herceptin and docetaxel. These results from this clinical trial are more promising. Significantly improved prolonged progression-free survival for a first-line treatment of HER2+ metastatic breast cancer was observed. Furthermore no increase in cardio-toxic effects was observed with these combinations (Baselga et al., 2012). Combined treatment with pertuzumab and herceptin and a taxol was approved by the FDA. It is being evaluated as a first line treatment option for patients with HER2+ metastatic breast cancer who have not been previously treated with anti-HER2 therapy or chemotherapy (Keating, 2012; Blumenthal et al., 2013).

Trastuzumab emtansine is modification of herceptin. Trastuzumab is linked with the cytotoxic agent mertansine (DM1). Trastuzumab-DM1 (T-DM1) is also referred to as Kadcyla, ado-trastuzumab emtansine and PRO132365. The cytotoxic agent mertansine inhibits cell growth upon binding to tubulin (Teicher and Doroshow, 2012). Herceptin (trastuzumab) targets T-DMI to HER + cells (Verma et al., 2012). T-DM1 was evaluated in the EMILIA phase III clinical trial which consisted of 991 cancer patients with unresectable, locally advanced or metastatic HER + breast cancer. T-DM1 enhanced the progression free survival. These patients had been previously treated with herceptin and taxanes or breast cancer patients treated with capecitabine (Xeloda) plus lapatinib (Tykerb, a dual HER2/EGFR1 inhibitor). Capecitabine is a classified as a prodrug. Capecitabine is metabolized to 5-fluorouracil (5-FU) in the body. It is a thymidylate synthase inhibitor.

Lapatinib-resistance in HER2+ cells: activation of PI3K and Src signaling pathways

The HER2/EGFR1 dual kinase inhibitor lapatinib is a small molecule inhibitor which is effective in inhibiting the growth of HER2+ breast cancers. Activation of the PI3K and Src pathways may be a mechanism by which some HER2+ cells grow in the presence of lapatinib. Culturing HER2+ breast cells in the presence of lapatinib for prolonged periods of time resulted in lapatinib-resistant HER2+ cells. These resistant cells did not express activated HER2, but they did express the PI3K/PTEN/Akt/mTORC and Raf/MEK/ERK pathways which were believed to result from activated Src family members. Treatment of the lapatinib-resistant cells with Src inhibitors suppressed the PI3K/PTEN/Akt/mTOR pathway and growth and importantly restored lapatinib sensitivity. Lapatinib treatment of primary HER2+ tumors resulted in the expression of Src-family kinases. Lapatinib and Src inhibitor treatment of HER2+ BT-474 cells was more effective in suppressing the growth of these cells in xenograft models than treatment with lapatinib alone. Thus treatment of certain HER2+ cells with the combination of lapatinib and Src inhibitors might be an effective means to prevent drug resistance of HER2+ cells (Rexer et al., 2011).

Targeting of PI3K/PTEN/Akt/mTORC1 and additional signaling pathways in breast cancer therapy

In order to overcome mTORC1-resistance, the possibility of co-targeting mTORC1 and other signaling pathways such as: IGF-1R, PI3K/PTEN/Akt and Raf/MEK/ERK and other pathways, to treat breast cancer is being examined (Vicier et al., 2013). It has been observed that treatment with rapalogs can induce the IGF-1R or PDGFR pathways which in turn will activate the Raf/MEK/ERK pathway in certain cells. Thus this co-targeting approach may also be appropriate to overcome rapamycin-resistance. There are clinical trials in progress evaluating the effectiveness of co-targeting mTORC1 and other signaling pathways (Davis et al., 2014). Combinations of aromatase inhibitors, rapalogs or mTOR inhibitors with herceptin, pertuzumab, or T-DM1 are new therapeutic approaches for HER2+ breast cancer therapy (Joo et al., 2013). Combining mTOR inhibitors with endocrine therapy may be an appropriate approach for treatment of aromatase inhibitor-resistant metastatic breast cancer patients. The TAMRAD and BOLERO-2 clinical trials have revealed significant effects with these combination approaches (Sendur et al., 2014). Combining a pan PI3K inhibitor (XL 147) with Herceptin may overcome herceptin-resistance in breast cancer by suppressing HER2/PI3K/FOXO/survivin signaling (Chakrabarty et al., 2013).

Clinical trials examining the effects of combining lapatinib, herceptin with paclitaxel in first line HER2+ positive breast cancer patients have been performed. Diarrhea was the dose limiting toxicity observed in the trial (Esteva et al., 2013). The effects of combination of the rapalog everolimus and either 4HT or exemestane (Aromasin) have been discussed (Vinayak and Carlson, 2013). Previous clinical trials combining endocrine therapy with rapalogs to treat metastatic, hormone receptor+ breast cancer patients have yielded variable results. Recent independent clinical trials which selected endocrine therapy-resistant patients, observed that combining the rapalog everolimus (Afinitor) with 4HT or combining everolimus with exemestane (Aromasin) were more effective than either endocrine agent alone. Rapalogs may sensitize the normally endocrine therapy-resistant patients to endocrine therapy. Additional clinical trials with PI3K/mTORC1 and HER2 inhibitors on HER2+ breast cancer patients may improve the therapeutic effectiveness of the HER2 inhibitors.

Herceptin resistance due to induction of EMT

Resistance to herceptin can result from the induction of EMT (Oliveras-Ferraros et al., 2012). HER2 is also associated the aggressiveness of breast CICs (Malik et al., 2012). Herceptin resistance in HER2+ breast cancers is a therapeutic problem may be as 70% (Oliveras-Ferraros et al., 2012).

Herceptin sensitivity was limited to the SLUG/SNAIL2-negative subset of luminal/HER2+ cell lines (Oliveras-Ferraros et al., 2012). While breast cancer lines which expressed SLUG/SNAIL2 were inherently resistance to herceptin. Suppression of SLUG/SNAIL2 inhibited the CIC phenotype by increasing the expression of the luminal epithelial marker CD24 in basal/HER2+ cells. These cells were sensitive to herceptin and underwent the mesenchymal to epithelial transition (MET). A reduction in tumor growth and sensitivity to herceptin was observed when SLUG and SNAIL2 were knocked-down in HER+ cells. Additional mechanisms may be involved in the induction of herceptin-refractory CICs from more differentiated cells via the activation of intrinsic or microenvironmental paths-to-stemness, also involving EMT (Martin-Castillo et al., 2013).

Use of the diabetes-controlling drug metformin in breast cancer therapy

Recent surprising studies have indicated that the anti-diabetes drug metformin may be effective in suppressing certain cancers and even treating herceptin-resistant breast cancers (Cufi et al., 2012; McCubrey et al., 2014b; Martelli et al., 2014b). Adenosine monophosphate-activated protein kinase (AMPK) is a target of metformin. AMPK is involved in regulation of components of the PI3K/Akt/mTORC1 as well as other pathways. Stat3 is an additional target of metformin. Metformin can inhibit the growth of TNBC (Deng et al., 2012). Additional Stats may be important in the progression of breast and other cancers (Raven et al., 2011; Dorritie et al., 2014a,b).

Metformin may prevent the onset of cancer in diabetic patients potentially by targeting the CICs (Sanchez-Alvarez et al., 2013) Metformin may function as a weak mitochondrial poison. Metformin

may induce mitochondrial dysfunction in the breast cell compartment which contains the breast CIC cells. This may be responsible for the beneficial anticancer effects of metformin. Metformin may function as a complex I inhibitor in mitochondria and prevent oxidative mitochondrial metabolism which is required for tumor growth. Mitochondrial uncoupling proteins (UCP) were used to induce mitochondrial dysfunction in TNBC MDA-MB-231 cells, which usually contain a high proportion of cells with the CIC phenotype. Three UCP family members induced autophagy and mitochondrial dysfunction in the MDA-MBA-231 cells. Similar studies were performed with cancer-associated fibroblasts (hTERT-BJ1 cells). Overexpression of UCP-1 in hTERT-BJ1 cells resulted in increased β-oxidation, ketone body production and the release of ATP-rich vesicles. These high-energy nutrients released by hTERT-BJ1 cells increased tumor MDA-MDA-231 growth in a paracrine fashion. The beneficial effects of metformin on breast CICs were associated with the induction of mitochondrial dysfunction in the MDA-MBA-231 cells.

Metformin treatment also decreased the size and number of MCF-7 mammospheres (Jung et al., 2011). Metformin inhibited Oct-4 expression. The transcription factor Oct-4 is a marker for breast CICs. Bisphenol A, estrogen and 2,3,7,8-tetrachlorodibenzodioxin (TCDD), increased Oct-4 expression as well as the sizes of the MCF-7/CICs. Bisphenol A is normally used to produce certain plastics and epoxy resins, for example water bottles. Upon metformin treatment, Oct-4 expression decreased in estrogen and TCDD-treated cells but not in bisphenol A-treated cells. Thus metformin may have beneficial effects in the treatment of breast and other cancers. It will be informative to further explore the effects of metformin on the incidence of cancer in various diabetic patients that have taken metformin for years as part of the treatment.

Roles of oncogenes and growth factors on sensitivity to chemotherapeutic drugs

Previously we have observed that activated Raf-1 and Akt-1 altered the drug sensitivity of MCF-7 breast cancer cells (Weinstein-Oppenheimer et al., 2001; McCubrey et al., 2014b, 2008; Sokolosky et al., 2011; Steelman et al., 2011). Furthermore mutations in PTEN and GSK-3β can serve as dominant negative mutations to suppress WT PTEN or GSK-3β activity and alter the drug sensitivity of breast cancer cells (Steelman et al., 2008; Sokolosky et al., 2014; McCubrey et al., 2014c,d).

Cytokines such as EGF elicit their effects through the EGFR. EGF and other cytokines may play critical roles in the tumor microenvironment by supporting proliferation and preventing apoptosis. We also have observed that cytokines such as EGF can increase the resistance of cells to chemotherapeutic drugs. We determined that EGF treatment could make breast, prostate and pancreatic cancer cells more resistant to common chemotherapeutic drugs normally used to treat cancer patients. For example, EGF treatment made certain cancer cells approximately 10-fold more resistant to chemotherapeutic drugs. Furthermore, treatment of drug resistant cells with antibodies which targeted EGF-R or IGF-1R reduced the resistance of the cells to chemotherapeutic drugs. These results indicate the critical roles that activation of the EGFR and other growth factor receptors can have on chemotherapeutic drug sensitivity.

Materials & methods

Cell lines and growth factors

Cells were maintained in a humidified 5% CO_2 incubator with RPMI-1640 [(RPMI) Invitrogen, Carlsbad, CA, USA] supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Atlanta, GA, USA).

Analysis of cell sensitivity to anticancer agents

The sensitivities of the cells to doxorubicin, paclitaxel, daunorubicin, 5-fluorouracil or cisplatin (all purchased from Sigma, Saint Louis, MO) were investigated by characterizing the effects of these agents on proliferation as described (Chappell et al., 2013) Inhibitory concentration 50% (IC_{50}) is defined in this context as the drug dose that causes the cells to proliferate at a rate that is half as rapid as cells incubated in the absence of drugs as determined by MTT assays as described (Chappell et al., 2013).

Annexin V/PI binding assay to determine induction of apoptosis

In order to determine the importance of certain signaling pathways, cells were treated with: the MEK inhibitor U0126, the PI3K inhibitor LY294002, the JAK inhibitor AG490, the EGFR inhibitor AG1478, the $p38^{MAPK}$ inhibitor SB203580, the JNK inhibitor SP600125 and the negative control for this JNK inhibitor. Signal transduction inhibitors were purchased from EMD Millipore (Calbiochem, San Diego, CA) with the exception of UO126 which was purchased from Promega (Madison, WI). These inhibitors were dissolved in dimethyl-sulfoxide (DMSO). Annexin V/PI binding experiments were preformed as described (Shelton et al., 2003).

Western blot analysis

Western blots were performed with antibodies specific for phospho or total MEK, ERK, Akt, GSK-3β, JNK, STAT5, $p38^{MAPK}$, PTEN as we have previously described (Chappell et al., 2013). Antibodies were purchased from Cell Signaling Technologies (EMD Millipore, Danvers, MA).

Infection of cells with retroviral vector encoding v-ERB-B:ER

Cytokine-dependent FDC-P1 hematopoietic cells, MCF-7 breast cancer and PC3 prostate cancer cells were infected with v-ERB-B:ER viral stocks as described (McCubrey et al., 2004). The v-ERB-B:ER retrovirus contains the Neo^r gene which allows the selection of cells resistant to neomycin (G418, Life Technologies, Bethesda, MD). Neo^r FDC-P1, MCF-7 or PC3 were isolated by selection in medium containing 2 mg/ml G418 as described (McCubrey et al., 2004). The nomenclatures of the v-ERB-B:ER-infected cells are FD/v-ERB-B:ER, MCF-7/v-ERB:ER and PC3/v-ERB-B:ER. The FD/v-ERB-B:ER were determined to be v-ERB-B:ER responsive by limiting dilution analysis by comparing their plating efficiency in the presence of β-estradiol or IL-3 (McCubrey et al., 2004; Shelton et al., 2005).

Cell cycle analysis by flow cytometry

Cells were grown in 6-well plates in the presence of appropriate growth factors or inhibitors. Approximately $1–2 \times 10^6$ cells were collected into 12 × 75 mm polystyrene tubes (Fisher Scientific, Pittsburgh, PA). The cells were washed once with cold PBS. The cells were then resuspended by adding 70% ethanol to the tube dropwise while vortexing. This was to prevent clumping of the cells while being fixed. The tubes containing the cells were then placed at −20 °C overnight. The next day the cells were pelleted, the alcohol aspirated and the cells were resuspended in 200 μl of the PI master mix (100 μg/ml RNase A, 40 μg/ml propidium iodide, in PBS). The cells were then incubated in a 37 °C water bath for 30 min and analyzed on a FACScan flow cytometer as previously described (Shelton et al., 2005).

Assays of [^{3}H]-thymidine incorporation

Cells were incubated for 24–72 h in the presence or absence of β-estradiol, 4HT (both obtained from Sigma–Aldrich, Saint Louis, MO) or supernatant from the WEHI-3B cell line which produces murine IL-3. [^{3}H]-thymidine (6.7 Ci/mmol, Perkins Elmer, Boston, MA) was added for the last 4 h and then the levels of [^{3}H]-thymidine incorporation were determined as described (40–44).

Clonogenic assays MCF-7 and MCF-7/v-ERB-B:ER were collected and seeded in 6-well cell culture plates at densities of 1000 cells/well as described. The cells were allowed to adhere to the plates for 24 h and then treated with nothing, 100 nM doxorubicin or 1000 nM 4HT. Plates were incubated for 3–4 weeks and then stained with giemsa dye (Sigma) colonies determined.

Results and discussion

Conditional growth of hematopoietic cells which proliferate in response to activated EGFR

We have developed a model by which we could follow the effects of an activated EGFR molecule (v-ERB-B:ER) on the cytokine-dependency of hematopoietic cells (McCubrey et al., 2004; Shelton et al.,

Table 1
Effects of activated v-ERB-B:ER on cell cycle progression.[a]

Cell cycle phase↓	Treatment condition				
	None	IL-3	β-Estradiol	IL-3 + 500 nM AG1478	β-Estradiol + 500 nM AG1478
% in G_1	95	37	17	58	99
% in S	5	41	78	30	1
% in G_2/M	0	22	5	12	0

[a] Cell cycle progression was determined as described in Shelton et al. (2005).

2005). IL-3-dependent FDC-P1 were infected with a construct encoding v-ERB-B:ER. Pools of cells (FD/v-ERB-B:ER) were selected which grew in response to v-ERB-B:ER activation by addition of β-estradiol to the tissue culture medium. The addition of β-estradiol or the estrogen receptor antagonist 4HT induced the v-ERB-B activity in the v-ERB-B:ER chimeric protein.

FD/v-ERB-B:ER cells entered G_1 after being cultured in medium containing 10% FBS but lacking either IL-3 or β-estradiol for 24 h (Table 1). In contrast, when FD/v-ERB-B:ER cells were cultured with either IL-3 or β-estradiol, the cells were present in all three phases of the cell cycle. Activation of the v-ERB-B activity by β-estradiol induced 78% of the FD/v-ERB-B:ER cells to be in S phase, while IL-3 treatment induced 41% of the cells to be in S phase. The requirement for the activation of v-ERB-B:ER activity for progression to S phase was examined by treatment with the EGFR inhibitor AG1478. When the FD/v-ERB-B:ER cells were treated with β-estradiol and AG1478, entry into S phase of the cell cycle was prevented. In contrast when the FD/v-ERB-B:ER cells were treated with IL-3 and AG1478, the cells entered S phase. These results indicate the conditional transformation of the FD/v-ERB-B:ER cells, they will grow in response to v-ERB-B activation or cytokine-mediated (IL-3) signal transduction, however, they will not grow in absence v-ERB-B activation or IL-3. This is in contrast to FDC-P1 cells transformed by an activated oncogene such as BCR-ABL or v-Ha-Ras, where the cells grow in presence of just medium and 10% FBS (McCubrey et al., 1995).

The effects of activation of v-ERB-B and the requirements of the PI3K/Akt/mTOR and Raf/MEK/ERK pathways on DNA synthesis in FD/v-ERB-B:ER cells were examined in Table 2. When FD/v-ERB-B:ER cells were cultured in the absence of IL-3 and β-estradiol for 24 h, 5.1- and 9.7- fold less [^{3}H]-thymidine was observed when the cells were cultured in the presence of either IL-3 or β-estradiol respectively. When FD/v-ERB-B:ER cells were cultured in the presence of IL-3 with EGFR (AG1478), PI3K (LY294002), or MEK (UO126) inhibitors did not significantly suppress [^{3}H]-thymidine incorporation. In contrast, when FD/v-ERB-B:ER cells were cultured with β-estradiol and the EGFR, PI3K and MEK inhibitors, DNA synthesis was decreased 55.4-, 43.8- and 28.2-fold respectively, documenting the importance of the PI3K/Akt/mTORC1 and Raf/MEK/ERK pathways on v-ERB-B mediated DNA synthesis.

The effect of inhibition of the EGFR kinase activity in the FD/v-ERB-B:ER cells on the induction of apoptosis was examined by Annexin V/PI binding (Table 3). FD/v-ERB-B:ER cells were washed with PBS and then cultured with either IL-3 or β-estradiol and increasing concentrations of the EGFR inhibitor

Table 2
Effects of signal transduction inhibitors on DNA synthesis.

Treatment↓	Mean ± SD[a]	Fold inhibition[b]
No cytokine or β-estradiol	1855 ± 161	5.1× (IL-3), 9.7× (β-Estradiol)
IL-3	9439 ± 115	–
IL-3 + 400 nM AG1478	9350 ± 51	1×
IL-3 + 2 μM LY294002	9550 ± 150	1×
IL-3 + 2 μM UO126	9362 ± 154	1×
β-Estradiol	17,934 ± 2382	–
β-Estradiol + 400 nM AG1478	324 ± 37	55.4×
β-Estradiol + 2 μM LY294002	409 ± 14	43.8×
β-Estradiol + 2 μM UO126	636 ± 18	28.2×

[a] [^{3}H]-thymidine incorporation after 24 h, performed in triplicate.
[b] Calculated by dividing either IL-3 or β-estradiol-treated cells by IL-3 or β-estradiol and inhibitor treated cells.

Table 3
Effects of the EGFR inhibitor AG1478 on induction of apoptosis.[a]

Treatment ↓	% Apoptotic cells (fold increase)	Treatment	% Apoptotic cells (fold increase)
IL-3	38% (1×)	β-Estradiol	34% (1×)
IL-3 + 0.04 nM AG1478	40% (1.1×)	β-Estradiol + 0.04 nM AG1478	25% (0.7×)
IL-3 + 0.4 nM AG1478	42% (1.1×)	β-Estradiol + 0.4 nM AG1478	59% (1.7×)
IL-3 + 4 nM AG1478	53% (1.4×)	β-Estradiol + 4 nM AG1478	58% (1.7×)
IL-3 + 40 nM AG1478	48% (1.3×)	β-Estradiol + 40 nM AG1478	96% (2.8×)
IL-3 + 400 nM AG1478	54% (1.4×)	β-Estradiol + 400 nM AG1478	90% (2.6×)
IL-3 + 4000 nM AG1478	59% (1.6×)	β-Estradiol + 4000 nM AG1478	90% (2.6×)

[a] % Apoptotic cells determined by Annexin V/PI binding as described (Shelton et al., 2005).

AG1478 for 1 day. As can be seen in Table 3, treatment with the EGFR inhibitor induce more apoptosis when the FD/v-ERB-B:ER cells were grown in β-estradiol. When FD/v-ERB-B:ER cells were cultured in β-estradiol at concentration 40 nM and greater, essentially all the cells registered as apoptotic by the annexin V/PI flow cytometric assay.

The effects of the Raf/MEK/ERK and PI3K/Akt/mTORC1 pathway on the induction of apoptosis was determined by cell cycle analysis by examining the percentage of subG$_0$/G$_1$ cells after 48 h in the indicated culture conditions in both the parental cytokine-dependent FDC-P1 and FD/v-ERB-B:ER responsive cells (Table 4). Treatment of IL-3-deprived FDC-P1 or β-estradiol-deprived FD/v-ERB-B:ER cells resulted in 3- and 4.5-fold more apoptotic cells than when the cells were cultured in the presence of IL-3 and β-estradiol respectively. Also the IL-3-deprived FDC-P1 or β-estradiol-deprived FD/v-ERB-B:ER were very sensitive to the induction of apoptosis mediated by addition of the MEK inhibitor as 10.2- and 15.7- fold increases in the induction of apoptosis were observed respectively. The IL-3-deprived FDC-P1 or β-estradiol-deprived FD/v-ERB-B:ER were not more sensitive to the induction of apoptosis mediated by addition of the PI3K inhibitor as 2.8- and 4.5-fold increases in the induction of apoptosis were observed respectively. Similar values were observed with the IL-3-deprived FDC-P1 or β-estradiol-deprived FD/v-ERB-B:ER were cultured in the absence of the PI3K inhibitor. When cytokine-dependent FDC-P1 cells were cultured in the presence of β-estradiol, in the absence of IL-3, they underwent apoptosis as expected. When the FD/v-ERB-B:ER cells were cultured in the presence of β-estradiol and the MEK inhibitor, a 6.8-fold increase in the induction of apoptosis was observed as compared to when they were cultured in the presence of β-estradiol by itself. When the FD/v-ERB-B:ER cells were cultured in the presence of β-estradiol and the PI3K inhibitor, a 1.7-fold increase in the induction of apoptosis was observed as compared to when they were cultured in the presence of β-estradiol by itself. Addition of the MEK and PI3K inhibitors together did not appear to increase the presence of SubG$_0$/G$_1$ FD/v-ERB-B:ER cells detected when the cells were cultured in β-estradiol in these experiments as 4.7-fold more apoptotic cells were detected when both inhibitors were added while 6.8-fold more apoptotic cells were detected when the MEK inhibitor was added.

Signal transduction pathways activated by v-ERB-B:ER

We examined some of the signal pathways induced by activated v-ERB-B:ER in FD/v-ERB-B:ER cells by western blot analysis with antibodies recognizing activated and total form of key signaling proteins. Cells were deprived of estrogen for 24 h and then treated with either DMSO or 500 nM of the EGFR inhibitor AG1478 for 1 h. Then the cells were treated with β-estradiol for the indicated time periods and aliquots were removed and protein lysates prepared. We observed activation of the PI3K/Akt/mTORC1, Raf/MEK/ERK, Jak/Stat and JNK pathways upon v-ERB-B:ER activation (Fig. 5). When the cells were treated with the EGFR inhibitor, less P-ERK, P-AKT, P-JNK and P-STAT-5 were detected. Thus the activated v-ERB-B:ER can induce many signaling pathways. In contrast, more P-p38MAPK was detected after treatment with the EGFR inhibitor. In addition, we detected phosphorylation of PTEN which is associated with its inactivation after v-ERB-B:ER activation (Fig. 6).

Table 4
Effects of signal transduction pathway inhibitors on induction of apoptosis in FDC-P1 and FD/v-ERB-B:ER cells as determined by SubG0/G1 population by cell cycle analysis.[a]

Treatment↓	FDC-P1 cells↓	Fold induction of apoptosis↓[b]	FD/v-ERB-B:ER cells↓	Fold induction of apoptosis↓[c]
Neither IL-3 or β-estradiol	15	3×	27	4.5×
IL-3	5	1×	6	1×
Neither IL-3 or β-estradiol + 2000 UO126	51	10.2×	47	15.7×
Neither IL-3 or β-estradiol + 2000 LY294002	14	2.8×	27	4.5×
β-Estradiol	21	4.2×	6	1×
β-Estradiol + 2000 nM UO126	61	12.2×	41	6.8×
β-Estradiol + 2000 nM LY294002	24	4.8×	10	1.7×
β-Estradiol + 2000 nM UO126 + 2000 nM LY294002	32	6.4×	28	4.7×

[a] % Apoptotic cells determined after culturing the cells in the indicated conditions for 2 days by determining the % of cells in $SubG_0/G_1$ by FACS cell cycle analysis as described in Steelman et al. (2008).
[b] Fold inductions of apoptosis in FDC-P1 cells were determined by dividing the % apoptotic cells in the treated FDC-P1 by the % apoptotic cells in the IL-3 treated FDC-P1 cells.
[c] Fold inductions of apoptosis in FD/v-ERB-B:ER cells were determined by dividing % apoptotic cells in the treated FD/v-ERB-B:ER by the % apoptotic cells in the β-estradiol-treated FD/v-ERB-B:ER cells.

Effects of inhibition of JAK, JNK and p38^{MAPK} signaling pathways on the induction of apoptosis in FD/v-ERB-B:ER cells

We next investigated the significance of activation of Jak/STAT and JNK pathways by examining the effects of specific pathway inhibitors. We examined the induction of apoptosis after treatment with these inhibitors. Treatment of FD/v-ERB-B:ER cells with the 5 μM of the AG490 JAK resulted in the induction of apoptosis (Table 5). Treatment of FD/v-ERB-B:ER cells with 5 μM of the JNK II inhibitor SP600125 induced apoptosis in FD/v-ERB-B:ER cells. In contrast, the structurally related negative control for the JNK II inhibitor (CAS 54642-23-8), induced less apoptosis in FD/v-ERB-B:ER cells than the active JNK inhibitor. In contrast treatment of FD/v-ERB-B:ER cells with 5 μM of the p38^{MAPK} inhibitor SB203580 did not induce much apoptosis in FD/v-ERB-B:ER cells. Thus the FD/v-ERB-B:ER cells were sensitive to JNK and JAK inhibitors, but did not appear to be significantly sensitive to the p38^{MAPK} inhibitor at least at these drug concentrations. Interesting, activation of the v-ERB-B:ER induced, JNK,

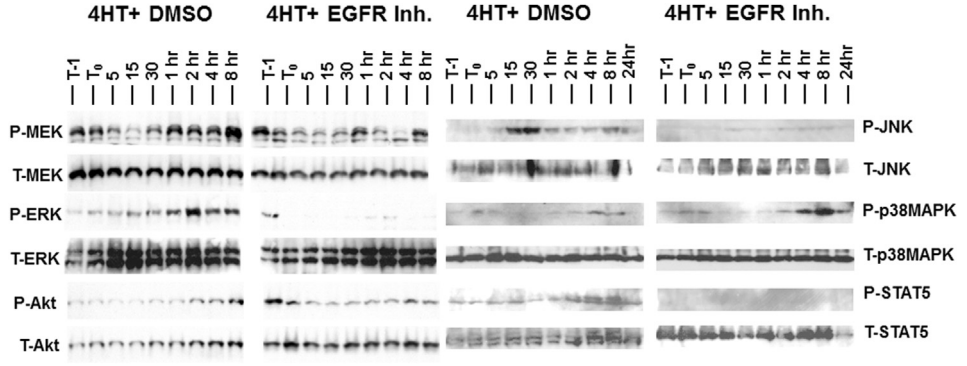

Fig. 5. v-ERB-B:ER activates phosphorylation of downstream signaling pathways. FD/v-ERB-B:ER cells were cultured in the absence of β-estradiol for 24 h in phenol red free RPMI 1640 containing 5% charcoal stripped FBS to avoid estrogenic effects of phenol red and endogenous estrogens present in FBS. The cells were then treated with either DMSO or 4 μM AG1478 for 1 h and a portion of the cells were collected. Then the cells were then treated with 500 nM 4HT for the indicated time points and the remaining protein lysates made. 4HT serves to activate the v-ERB-B:ER (McCubrey et al., 2004). Western blots were performed with antibodies which recognized activation specific and total proteins.

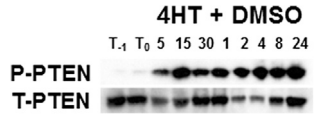

Fig. 6. v-ERB-B:ER activates PTEN phosphorylation. FD/v-ERB-B:ER cells were cultured in the absence of 4HT for 24 h in phenol red free RPMI 1640 containing 5% charcoal stripped FBS to avoid estrogenic effects of phenol red and endogenous estrogens present in FBS. The cells were then stimulated with 500 nM 4HT for the indicated time periods and then protein lysates made. Western blots were performed with PTEN antibodies which recognized phosphorylated (inactive) and total PTEN.

STAT5 but did not appear to significantly induce $p38^{MAPK}$, in fact, higher levels of $p38^{MAPK}$ were detected when the cells were treated with the EGFR inhibitor.

The effects of various combination treatments were also examined. When we combined low doses of the JAK and EGFR inhibitors, more apoptosis was observed than after treatment with identical concentrations of JAK and EGFR inhibitors. More apoptotic cells were observed when the FD/v-ERB-B:ER cells were treated with 0.5 μM JAK + 0.5 μM PI3K inhibitors (27.7%) than when the cells were cultured with 0.5 μM JAK or 0.5 μM PI3K inhibitors (15.8% and 18.3% respectively). Co-addition of the JNK and PI3K inhibitors increased the induction of apoptosis as compared to addition of either inhibitor by itself as 86.5% and 33.2% apoptotic cells were observed when 5 μM JNK + 5 μM PI3K and 0.5 μM JNK + 0.5 μM PI3K were observed respectively while 64.1% and 26.6% apoptotic cells were observed when 5 μM JNK and 0.5 μM JNK were added by themselves and 54.9% and 18.3% apoptotic cells were observed when 5 μM PI3K and 0.5 μM PI3K were added by themselves. Co-addition of 0.5 μM JNK + 0.5 μM JAK inhibitors increased the % of apoptotic cells to 49.2% as compared to when 0.5 μM JNK and 0.5 μM JAK were added by themselves as 26.6% and 15.8% apoptotic cells were observed when the two inhibitors were added respectively. These experiments document that co-targeting of relevant signal transduction pathways may be an approach to inhibit the growth of cells which grow in response to aberrant activation of an oncogene.

Therefore in this model, we have investigated the effects of a mutant EGFR gene on proliferation of a normally cytokine-dependent hematopoietic cell line. Furthermore, the activation of certain key downstream signaling pathways and their importance in the prevention of apoptosis has been determined.

Table 5
Effects of targeting JAK, p38MAPK and JNK Pathways on induction of apoptosis in FD/v-ERB-B:ER cells.[a]

Treatment	% Apoptotic cells	Combined treatment	% Apoptotic cells
JAK Inh		0.05 μM JAK + 0.4 nM EGFR	74.3%
5 μM AG490	53.3%	0.005 μM JAK + 0.04 nM EGFR	22.6%
0.5 μM AG490	15.8%		
0.05 μM AG490	14.5%	0.5 μM JAK + 0.5 μM PI3K	27.7%
0.005 μM AG490	16.6%		
		5 μM JNK + 5 μM PI3K	86.5%
JNK Inh		0.5 μM JNK + 0.5 μM PI3K	33.2%
5 μM JNK inhibitor II	64.1%		
0.5 μM JNK inhibitor II	26.6%	0.5 μM JNK + 0.5 μM JAK	49.2%
0.05 μM JNK inhibitor II	12.3%		
0.005 μM JNK inhibitor II	11.6%	Controls:	
		Neither IL-3 or β-estradiol	94.5%
p38MAPK Inh		IL-3	54.3%
5 μM SB203580	16.1%	β-Estradiol	15.2%
0.5 μM SB203580	10.4%	0.4 nM EGFR Inh AG1478	54.2%
0.05 μM SB203580	13.5%	0.04 nM EGFR Inh AG1478	14.7%
0.005 μM SB203580	12.8%	5 μM PI3K Inh LY29402	54.9%
		0.5 μM PI3K Inh LY29402	18.3%
		5 μM JNK negative control	43.4%
		0.5 μM JNK negative control	13.2%

[a] Cells were cultured with β-estradiol, with the exception of the neither IL-3 or β-estradiol or IL-3 cultured cells. % Apoptotic cells were determined by Annexin V/PI binding and FACS analysis as described previously (Shelton et al., 2003; McCubrey et al., 2004; Shelton et al., 2005).

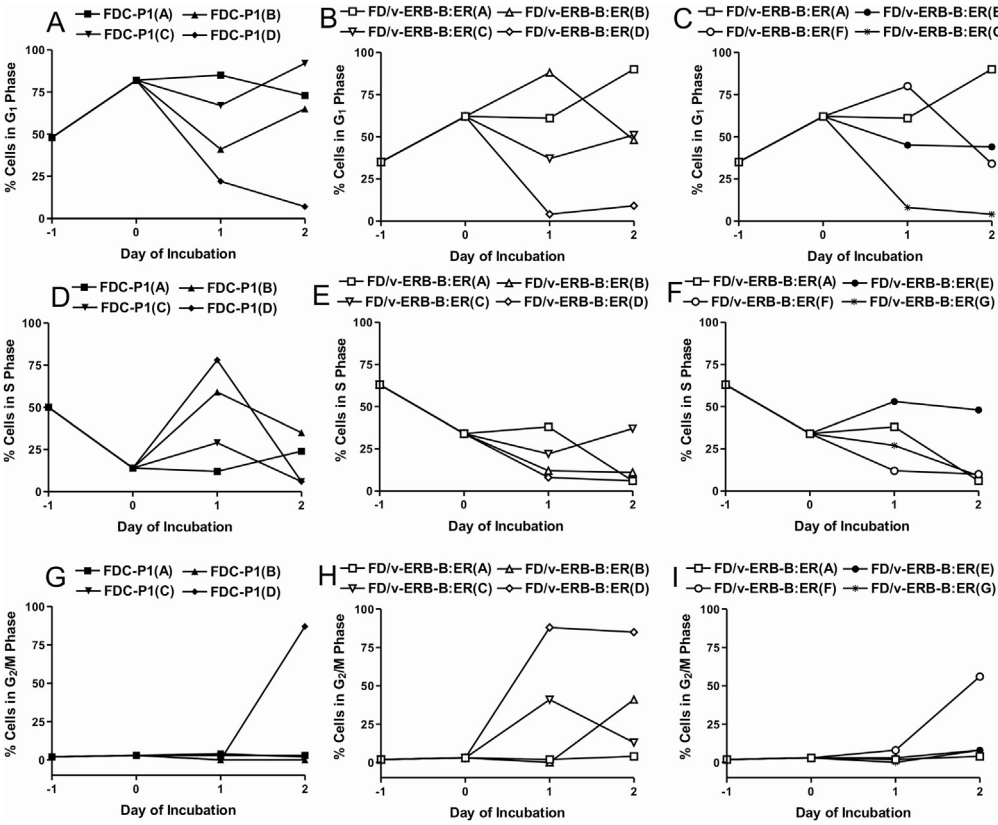

Fig. 7. Effects of v-ERB-B activation on cell cycle progression of FD/v-ERB-B:ER cells in the presence and absence of IL-3, β-estradiol and chemotherapeutic drugs. Cell cycle progression in FDC-P1 and FD/v-ERB-B:ER cells was examined over a period of 4 days. Code panels A, D & G, FDC-P1 cultured in the absence of IL-3 indicated in solid squares (■ = A), FDC-P1 cultured in the presence of IL-3 after day 0 indicated in solid triangles (▲ = B), FDC-P1 cultured in the presence of IL-3 and 25 nM doxorubicin after day 0 indicated in solid downward triangles (▼ = C) and FDC-P1 in the presence of IL-3 and 10 nM paclitaxel after day 0 indicated in solid diamonds (◆ = D). Code panels B, E & H, FD/v-ERB-B:ER cultured in the absence of β-estradiol indicated in open squares (▫ = A) FD/v-ERB-B:ER cultured in the presence of 500 nM β-estradiol after day 0 indicated in open triangles (△ = B), FD/v-ERB-B:ER cultured in the presence of 500 nM β-estradiol and 25 nM doxorubicin after day 0 indicated in open downward triangles (▽ = C) and FD/v-ERB-B:ER cultured in the presence of 500 nM β-estradiol and 10 nM paclitaxel after day 0 indicated in open diamonds (◇ = D). Code panels C, F & I, FD/v-ERB-B:ER cultured in the absence of β-estradiol indicated in open squares (▫ = A), FD/v-ERB-B:ER cultured in the presence of IL-3 after day 0 indicated in closed circles (● = E), FD/v-ERB-B:ER cultured in the presence of IL-3 and 25 nM doxorubicin after day 0 indicated in open circles (○ = F) and FD/v-ERB-B:ER cultured in the presence of IL-3 and 10 nM paclitaxel after day 0 indicated in asterisks (* = G). Panels A, B & C = % cells in G_1 phase of cell cycle. Panels D, E & F = % cells in S phase of cell cycle. Panels G, H & I = % cells in G_2/M phase of cell cycle.

Activation of the mutant EGFR increased the resistance of the FD/v-ERB-B:ER cells to chemotherapeutic drugs such as doxorubicin (Fig. 7). This was determined by comparing cell cycle progression in the presence and absence of doxorubicin in cytokine dependent FDC-P1 and FD/v-ERB-B:ER cells cultured in IL-3 and β-estradiol respectively. When FDC-P1 cells were cultured in IL-3 and doxorubicin they entered the G_1 phase after two days of culture (Panel A). In contrast, a similar percentage of FD/v-ERB-B:ER cells was present in the G_1 phase after culture with β-estradiol of β-estradiol and doxorubicin for 2 days (Panel B). When FDC-P1 cells were cultured in IL-3 and doxorubicin a very low percentage of cells were present in the S phase of the cell cycle after two days of culture (Panel D). In contrast, the percentage of FD/v-ERB-B:ER cells present in the S phase

Table 6
Effects of activated v-ERB-B:ER on chemotherapeutic drug resistance.[a]

Chemotherapeutic drug↓	MCF-7/v-ERB-B:ER/MCF-7	PC3/v-ERB-B:ER/PC3	DU145/v-ERB-B:ER/DU145
	Fold increase	Fold increase	Fold increase
Doxorubicin	2.4×↑	10.5×↑	20↑
Daunorubicin	2×↑	13.3×↑	7.5×↑
Paclitaxel	10×↑	33.3×↑	65×↑
5-Fluorouracil	1×	25×↑	3.7×↑
Cisplatin	6.2×↑	ND	ND

[a] Determined by analysis of IC_{50}s by MTT analysis as described (Chappell et al., 2013). The fold increase is calculated by dividing the IC_{50} of the v-ERB-B:ER cell line by the IC_{50} of the parental cell line. ND = not done.

Table 7
Effects of activated v-ERB-B:ER on colony formation.[a]

Treatments↓	Fold increase MCF-7/v-ERB-B:ER compared to MCF-7
None	1×
4HT (1000 nM)	2.1×↑
Doxorubicin (100 nM)	15.7×↑

[a] Colony formation performed as described (Steelman et al., 2011). Fold increases were determined by dividing the average number of normalized colonies observed in MCF-7/v-ERB-ER by normalized colonies observed in MCF-7 in the indicated conditions.

after culture with β-estradiol and doxorubicin was increasing after two days of culture, demonstrating the drug resistance of these cells when the mutant EGFR was activated (Panel E). In addition, the proportion of cells in the G_2/M phase was altered when the mutant EGFR was activated in FD/v-ERB-B:ER (Panel H). Drug resistant cells have been previously shown to have altered G_2/M phase distribution (O'Loughlin et al., 2000; Wang et al., 2008). Activation of the mutant EGFR did not appear to increase the resistance of the cells to paclitaxel. When the FD/v-ERB-B:ER cells were cultured in IL-3 and the various drugs, the v-ERB-B:ER was not activated their cell cycle distributions resembled the parental FDC-P1 cells and they were not resistant to doxorubicin (Panels C, F and I). Thus the v-ERB-B:ER makes the cells conditionally-resistant to certain chemotherapeutic drugs such as doxorubicin.

To further determine the effects of activation of the EGFR on the sensitivity of cells to chemotherapeutic drugs, we transfected breast and prostate cell lines with an activated EGFR (v-ERB-B:ER) which is similar in structure to the naturally occurring EGFvIII truncated EGFR1 mutant observed in breast and other cancers including prostate cancer (Edwards et al., 2006). We observed that the activated v-ERB-B:ER increased the resistance of breast and cancer cells to chemotherapeutic drugs as determined by MTT analysis (Table 6).

Furthermore the activated v-ERB-B:ER increased the colony formation of MCF-7/v-ERB-B:ER cells compared to MCF-7 when they were plated in the presence of either doxorubicin or 4HT (Table 7).

In conclusion, we have observed that the activated v-ERB-B:ER, which is similar in structure to the truncated *EGFR1* gene called EGFvIII, was able to alter the growth properties of hematopoietic, breast and prostate cells. The *EGFR* gene family is frequently deregulated in various cancers, including breast, brain, prostate and others. Further knowledge of how *EGFR* gene family mutations can result in therapeutic resistance could lead to the identification of alternative approaches to treat patients which have become resistant to *EGFR* family member inhibitors.

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