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The role of HER2 in early breast cancer metastasis and the origins of resistance to HER2-targeted therapies

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Abstract

The HER2 gene encodes the receptor tyrosine kinase HER2 and is often over-expressed or amplified in breast cancer. Up-regulation of HER2 contributes to tumor progression. Many aspects of tumor growth are favorably affected through activation of HER2 signaling. Indeed, HER2 plays a role in increasing proliferation and survival of the primary tumor and distant lesions which upon completion of full transformation cause metastases. $P185^{HER2/neu}$ receptors and signaling from them and associated molecules lead to increase motility of both intravasating and extravasating cells, decreasing apoptosis, enhancing signaling interactions with the microenvironment, regulating adhesion, as well as a multitude of other functions.

Recent experimental and clinical evidence supports the view that spread of incompletely transformed cells occurs at a very early stage in tumor progression. This review concerns the identification and characterization of HER2, the evolution of the metastasis model, and the more recent cancer stem cell model. In particular, we review the evidence for an emerging mechanism of $HER2^{+}$ breast cancer progression, whereby the untransformed HER2-expressing cell shows characteristics of stem/progenitor cell, metastasizes, and then completes its final transformation at the secondary site.

Keywords

HER2; metastasis; breast cancer; cancer stem cell; untransformed cell

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HER2 identification and characterization

Our initial studies of oncogenes were an extension of our search for a relationship between transforming genes and tumor antigens (Greene et al., 1982). In the early 1980's, we (Schechter et al., 1984) isolated an oncogene from B104 tumors derived from neuroblastoma neoplasms that occurred in the offspring of rats treated during gestation with the carcinogen ethylnitrosourea. We identified the *neu* oncogene, named after its tissue of origin, as responsible for the malignant phenotype of the cells. Immortalized mouse fibroblasts (NIH3T3 cells) were transformed with high molecular weight neuroblastoma DNA containing the oncogene. Transformants were selected for foci formation reflecting neoplastic transformation. This process was repeated several times to enrich the high molecular weight transforming DNA. An NIH3T3 line transfected with the enriched neu oncogene was developed and named B104–1–1. When these cells were injected subcutaneously into mice, rapidly growing tumors ensued. We developed a novel immunization approach to create monoclonal antibodies to the neu encoded transforming protein (Drebin et al., 1984).

This approach was later used by others to generate monoclonals to HER2, the human homolog of neu. Our monoclonal antibodies identified a phosphorylated 185 KDa protein product of the *neu* oncogene in the neuroblastoma cell lysates (Schechter et al., 1984). Using flow cytometry we also showed that the p185 KDa protein existed on the cell surface (Drebin et al., 1984). The Weinberg laboratory (Bargmann et al., 1986) showed that the cDNA of the oncogene was highly homologous to the Epidermal Growth Factor Receptor (EGFR) and that the $p185^{neu}$ protein also possessed a tyrosine kinase subdomain. They also showed that the proto-oncogene found in normal tissues differed from the oncogenic form by a single base mutation that lead to substitution of a valine residue to a glutamic acid residue which introduces a negative charge into the transmembrane region of the receptor.

Our laboratory determined how this negative charge converted this protein into a transforming molecule. Weiner et al. (Weiner et al., 1989b) demonstrated that the oncogenic protein existed as a homodimer whereas the proto-oncogenic form was predominantly a monomer. We then showed that the homodimeric protein, but not the monomeric form, exhibited tyrosine kinase activities (Weiner et al., 1989a). These studies clearly showed that, in this system, the introduction of a negative charge into the transmembrane region promoted dimer formation and that only dimeric receptors possessed kinase activity. Here we designate the rat proto-oncogenic protein as $p185^{c\text{-}neu}$ and the oncogenic protein as $p185^{neu}$. The human homolog is, simply, HER2.

We studied the developmental expression pattern of the *neu* proto-oncogene and found that the protein was expressed at low levels in normal adult and embryonic animal tissues (Kokai et al., 1987). In particular, $p185^{c\text{-}neu}$ was expressed in secretory ciliated epithelial cells of all tissues (notably the lung, small intestine, colon and breast) and diffusely in the brain and central nervous system. The discovery of the expression pattern in normal secretory epithelial cells is especially relevant to the role of HER2 in human tumors of the breast, brain, pancreas and other organs.

HER2 expression patterns in early lesions

Notably, upregulation of HER2 levels can be readily detected in human breast tissues that show the early signs of transformation but have not been completely transformed. Completely transformed cells are able to grow in an anchorage independent fashion *in vitro* and also grow *in vivo*. Incompletely transformed cells display one, but not both, of these traits. Table 1 summarizes HER2 expression within breast lesions at various stages of cancer progression. Generally, HER2 is absent or expressed at low-levels in benign breast lesions (Borg et al.,

1990;Maguire and Greene, 1990;Pechoux et al., 1994;Rohan et al., 1998;van de Vijver et al., 1988;Wells et al., 1995). For instance, HER2 is not detected in terminal duct lobular units (TDLUs) (Allred et al., 1992;De Potter et al., 1989) and has been detected rarely in atypical ductal hyperplasia (ADH) (Allred et al., 1992;De Potter et al., 1989;Gusterson et al., 1988;Lodato et al., 1990).

In contrast, HER2 is amplified and over-expressed in high-grade ductal carcinoma *in situ* (DCIS), particularly of the comedo type, and in high-grade inflammatory breast cancer (IBC) (Allred et al., 1992; Bobrow et al., 1994; Claus et al., 2001; Leal et al., 1995; Liu et al., 1992; Moreno et al., 1997; van de Vijver et al., 1988). The absence of HER2 protein expression in benign breast biopsies suggests that over-expression of HER2 usually occurs at the transition from hyperplasia to DCIS (Allred et al., 1992; Coene et al., 1997; Gusterson et al., 1988; Liu et al., 1992; Lodato et al., 1990; Parkes et al., 1990).

Although the mechanism remains unclear, the absence of over-expression in normal TDLUs and ADH, compared with the relatively high incidence of over-expression in DCIS, suggests that the increase in levels of HER2 is an important event in early malignant transformation (Latta et al., 2002; Rohan et al., 1998). In fact, minor perturbations in amplified HER2 expression are sufficient to alter mammary development and induce malignant transformation (Weinstein et al., 2000) (Table 2). Mammary tumorigenesis is influenced by the over-expression and/or amplification of wild-type HER2, somatic activation of wild-type HER2, and the temporal expression pattern of activated HER2 (Table 2).

Pre-malignant and pre-invasive breast lesions are relatively common but only a small proportion progress to IBC. Although these lesions possess some transformed properties such as a loss of growth control, they lack the ability to invade and metastasize. Hence, there must be underlying biological differences causing some to remain stable and others to progress to IBC. Identifying and treating high-risk pre-malignant disease has the potential to prevent lethal invasive breast cancers. HER2, p53, and estrogen receptor (ER) status are important prognostic factors and portend how a lesion responds to different therapeutic strategies. Patients with benign breast lesions with low levels of *HER2* gene amplification (Stark et al., 2000), or slightly elevated levels of p53 protein (Rohan et al., 1998), have a two- to three-fold increased relative risk of developing IBC. Similarly, women with benign breast biopsies demonstrating both *HER2* amplification and a proliferative histopathologic diagnosis may have a substantially increased risk for subsequent IBC (Stark et al., 2000). HER2 may lead to the development and progression of pre-malignant breast disease, and increased proliferation and motility of cells (associated with over-expression) may contribute to this process.

HER2 genes are implicated in epithelial cancers such as colon, ovarian, prostate, pancreatic, and lung (Berchuck et al., 1990; Cohen et al., 1989; Fearon and Vogelstein, 1990; Hynes and Lane, 2005; Kern et al., 1990; Prigent and Lemoine, 1992; Slamon et al., 1989; Zeillinger et al., 1989). In particular, the *HER2* gene is amplified and over-expressed in 20% to 30% of invasive ductal carcinomas (IDCs) and is a well recognized predictor of poor clinical outcome with reduced disease-free and overall survival rates (Ravdin and Chamness, 1995; Slamon et al., 1987).

Activation of *HER2* by over-expression seems to be a dominant transformation mechanism in human tumors, and over-expression alone has the same effect as the oncogenic mutations found in the rat gene, namely promoting activated dimeric receptor complexes (Zhang et al., 2007). There is a correlation between *HER2* amplification/over-expression and aneuploidy in clinical breast cancer samples (Jimenez et al., 2000; Kallioniemi et al., 1991; Lottner et al., 2005; Mrozkowiak et al., 2004; Smith et al., 2000; Wang et al., 2002). *HER2* amplification/over-

expression and aneuploidy are considered downstream effects of p53 dysfunction (Shackney and Silverman, 2003).

In addition, over-expression of ras is often restricted to aneuploid cells that already over-express HER2 (Shackney et al., 1996); and the presence of both *HER2* and *ras* abnormalities defines a subset of aggressive high-grade tumors (Shackney and Silverman, 2003). Patients whose tumors simultaneously demonstrate aneuploidy, HER2 over-expression, and *ras* over-expression in the same cells have worse disease-free survival rate than that of patients whose tumor cells have fewer abnormalities per cell, suggesting co-occurrence in the same cells produces synergistic effects. Although HER2 over-expression is highly correlated with gene amplification (Liu et al., 1992; Press et al., 1993; Robertson et al., 1996; Venter et al., 1987), approximately 20% of malignant tissues, including breast cancers, over-express HER2 while maintaining diploid copies of the gene (Friedrichs et al., 1993; Persons et al., 1997; Robertson et al., 1996; Slamon, 1989).

Thus, in addition to aneuploidy and gene amplification, transcriptional deregulation involving cis-acting enhancer elements near the *HER2* promoter and increased expression of transcription factors that bind to these sequences (Bosher et al., 1996; Bosher et al., 1995; Grooteclaes et al., 1999) may cause HER2 over-expression. Also, differences in the technical sensitivities of PCR and immunohistochemistry may partially account for discordance in *HER2* gene amplification and protein over-expression in breast tumors.

Dimeric forms of erbB receptors

We also examined how the $p185^{c\text{-}neu}$ proto-oncogenic receptors become activated at a biochemical level. David Stern at Yale (Stern et al., 1988) and our laboratory (Kokai et al., 1988) found that $p185^{c\text{-}neu}$ was phosphorylated in cells expressing both EGFR and $p185^{c\text{-}neu}$ proteins. We discovered that transfecting cells with both proto-oncogenic *c-neu* and EGFR enabled the malignant transformation of the cells, while transfecting either of them alone did not. Over-expression of either gene alone, even at a very high level, resulted only in partial transformation of NIH3T3 cells. Moreover, NIH3T3 cells over-expressing $p185^{c\text{-}neu}$ or EGFR alone did not develop progressively growing tumors when implanted into athymic mice (Kokai et al., 1989).

We first determined that $p185^{c\text{-}neu}$ proteins and EGFR proteins formed heterodimers in the absence of EGF ligand but more readily the presence of ligand (Wada et al., 1990). The heterodimer was far more active than EGFR homodimers and could readily transform NIH3T3 cells. These studies suggest that heterodimer formation influences functional properties and may contribute to the diversification of their signaling properties. Two other members of the EGFR receptor family, erbB3 and erbB4, have also been identified. These receptors can also form homomeric and heteromeric associations (Sliwkowski et al., 1994), but the preferred heterodimerization partner is $p185^{c\text{-}neu}$ (Graus-Porta et al., 1997; Wada et al., 1990). Recent molecular modeling (Berezov et al., 2002) and crystallographic studies (Garrett et al., 2003) have elucidated the structural basis of dimer formation. Once these receptors are active, they promote proliferative and phenotype-altering cascades including the MAPK and PI3K/AKT pathways.

HER2 activation induces uncontrolled proliferation, protects against apoptosis, and disrupts normal epithelial organization in epithelial cells (Muthuswamy et al., 2001). Several studies have demonstrated that HER2 affects these functions (Aranda et al., 2006; Guo et al., 2006; Muller et al., 1988). For instance, loss of integrin beta4 signaling delayed the onset of HER2 induced mammary tumors, reduced tumor burden, and suppressed tumor progression and metastasis (Guo et al., 2006). Ex vivo analyses of these tumors identified a beta4– HER2 complex that enhanced activation of the transcription factors c-Jun, which was required for the

hyperproliferative effect of HER2, and STAT3, which contributed to the disruption of epithelial adhesion and polarity. These results demonstrated that distinct signaling mechanisms were responsible for each phenotypic outcome of HER2 activation.

Aranda and colleagues discovered that HER2 activation disrupted membrane polarity in kidney epithelial cells by causing the dissociation of the Par polarity complex components, namely Par6 and aPKC, from Par3, and the association of HER2 receptor dimers with Par6–aPKC (Aranda et al., 2006). Moreover, HER2 activation led to the formation of hyper-proliferative, multi-acinar structures with filled lumens in mammary epithelial cells. Interfering with this interaction blocked the ability of HER2 to disrupt polarized epithelial organization and to protect cells from apoptosis. Together, these studies suggest that HER2 may prevent apoptosis within acinar structures by disrupting the dichotomy between the outer and inner acinar cells.

HER2 promotes early dissemination of incompletely transformed cells

Metastasis, the primary cause of morbidity and mortality of most cancers, is an extremely complex and highly organized process that is organ specific and involves numerous reciprocal interactions between the cancer cells and the host (Fidler and Hart, 1982; Steeg, 2003; Yeatman and Nicolson, 1993). The stage at which individual cells leave the primary tumor was unclear, and new data have caused us to rethink this process. The previous metastasis model suggested that epithelial cells sequentially accumulate multiple genetic and epigenetic changes underlying the disorganization of tissue morphology and uncontrolled growth (Fearon and Vogelstein, 1990). The model predicted that certain additional genomic events initiate invasiveness of the tumor and metastasis in the final stages of tumorigenesis.

However, there has been much data to indicate that metastases occur much earlier during tumor progression than once believed. Indeed, the progenitors of the later-arising metastases must be present among those cells that have disseminated to distant sites before removal of the primary tumor. In fact, based on the correlation of tumor size and time to metastasis, it is estimated that tumor cell dissemination may occur on average five years before diagnosis for breast cancer (Engel et al., 2003). Pantel, et al. showed that epithelial tumor cells were able to disseminate to secondary organs at an early stage of primary tumor development (Pantel et al., 1999). In particular, data suggest that DCIS represents a stage in the development of breast cancer in which most of the molecular changes that characterize invasive breast cancer are already present, though the lesion has not assumed a fully malignant phenotype (Burstein et al., 2004). Most, if not all, clinically relevant features of breast cancer, such as hormone receptor status, the level of HER2 expression, and the histological grade, are probably determined by the time DCIS has evolved (Buerger et al., 1999; Gupta et al., 1997; Lampejo et al., 1994; Warnberg et al., 2001).

The genomic characteristics of a primary tumor may help predict the probability that metastases have already occurred in patients with clinically localized disease. Certain signaling deregulations, for example HER2 activation, provide a selective advantage during tumor initiation and can also foster an invasive and metastatic phenotype (Bernards and Weinberg, 2002; Hanahan and Weinberg, 2000). Likewise, pre-malignant $HER2^{+}$ transgenic glands transplanted into wild-type mice displayed disseminated tumor cells and micrometastases in bone marrow and lungs, and the number of disseminated cancer cells and their karyotypic abnormalities were similar for both small and large tumors (Husemann et al., 2008). Breast cancer progression is dependent on the capacity to invade and to metastasize to distant sites, and loss of tumor cell adhesion is an important factor in this process. Therefore, it is not surprising that in addition to HER2 over-expression, loss of E-cadherin contributes to mammary tumor initiation, progression, dissemination, and metastasis (Cavallaro and Christofori, 2004; Conacci-Sorrell et al., 2002; Derksen et al., 2006).

Data derived from genetic analyses comparing paired primary and metastatic breast tumor samples confirm the hypothesis that disseminated tumor cells evolve independently from the primary tumor (Kuukasjarvi et al., 1997; Pantel and Brakenhoff, 2004; Weigelt et al., 2005). For instance, the patterns of genetic alterations in metastases are often discordant with those of the primary tumor, and differ almost completely in approximately one-third of the cases (Kuukasjarvi et al., 1997). During genetic progression of the primary breast tumor, cancer cells might disseminate continuously, acquiring additional genetic alterations after migration into secondary organs such as the bone marrow. Cytogenetic analyses of breast cancer patients have demonstrated extreme genetic heterogeneity, for instance up to 70% of polyclonality (Kuukasjarvi et al., 1996; Pandis et al., 1995; Teixeira et al., 1996; Teixeira et al., 1995; Trent et al., 1993). Single disseminated cancer cells isolated from bone marrow of breast cancer patients harbor fewer and different genetic aberrations than the primary tumor and do not display signs of telomeric crisis (Schmidt-Kittler et al., 2003). Disseminated tumor cells might, therefore, evolve independently into overt metastases, driven by the specific selective pressures of the bone-marrow environment (Gray, 2003).

In contrast, *in situ* carcinomas already display chromosomal aberrations very similar to invasive carcinomas (Aubele et al., 2000; Hwang et al., 2004; Iakovlev et al., 2008). Likewise, similar genetic alterations were observed in primary tumors and synchronous regional lymph node metastasis (Kuukasjarvi et al., 1997; Torres et al., 2007). In contrast, asynchronous distant metastases often differ extensively from the corresponding matched primary tumors (Torres et al., 2007). Early disseminated cancer cells are genomically very unstable, and selection of clonally expanding cells leading to metastasis seems to occur after dissemination has taken place (Klein et al., 2002). Overall, the data indicates that the molecular heterogeneity that characterizes invasive breast cancers occurs at the time of initial progression (Deng et al., 1996).

Similarly, gene expression alterations conferring the potential for invasive growth are already present in the pre-invasive stages. Gene expression signatures present in the primary tumor cells may predict metastatic potential (Sorlie et al., 2001; van 't Veer et al., 2003; van 't Veer et al., 2002). Most cancer cells in a primary tumor have a “metastatic phenotype”, indicating that metastatic spread is an early event in tumorigenesis, although it manifests only much later after mutation of other genes (Bernards and Weinberg, 2002). There are extensive similarities in the gene expression profiles among the distinct stages of progression suggesting that gene expression alterations conferring the potential for invasive growth are already present in the pre-invasive stages (Ma et al., 2003).

Podsypanina, et al. reported that untransformed mouse mammary cells that have been engineered to express the inducible oncogenic transgenes and introduced into the systemic circulation of a mouse, can bypass transformation at the primary site and develop into metastatic pulmonary lesions upon immediate or delayed oncogene induction (Podsypanina et al., 2008). Therefore, previously untransformed mammary cells may establish residence in the lung once they have entered the bloodstream and may assume malignant growth upon oncogene activation. Mammary cells lacking oncogenic transgenes displayed a similar capacity for long-term residence in the lungs but did not form ectopic tumors. These studies indicate that incompletely transformed cells are capable of assuming residence in many distal tissues.

Disease-free periods can last from several years up to as long as 20–25 years in breast cancer patients (Karrison et al., 1999). This suggests that a pause in disease progression occurs often and might be explained by different forms of dormancy and could be a common feature of cancer progression. Loss of signals from the microenvironment favors dormancy of disseminated tumor cells (Aguirre-Ghiso, 2007). Loss of a surface receptor, for instance alpha5beta1 integrin, uPAR (urokinase-type plasminogen activator receptor), HER2 or EGFR,

that transduces growth signals from the microenvironment, for instance fibronectin, results in stress signaling (low FAK–Ras–ERK, and high CDC42 (cell division cycle 42)–p38 activity), which in turn might lead to dormancy (Aguirre Ghiso, 2002; Aguirre Ghiso et al., 1999; Aguirre-Ghiso et al., 2003; Aguirre-Ghiso et al., 2001; Aguirre-Ghiso et al., 2004; Liu et al., 2002; White et al., 2004a). Although HER2 positive tumors developed at a high frequency, HER2 positive mammary gland tissue that was null for beta1 integrin resulted in tumor suppression (White et al., 2004b). The cells were in a non-proliferating, dormant state suggesting that a loss of signals from the microenvironment favored dormancy despite the HER2 oncogenic signals. It has also been found that down-regulation of uPAR and HER2 signaling in disseminated tumor cells may contribute to their dormancy (Lacroix, 2006).

Interestingly, the *PLAUR* (encoding uPAR) and *HER2* genes were found to be co-amplified in disseminated breast cancer cells (Meng et al., 2006). Characterization of minimal residual disease revealed that these cells were dormant, and possibly enhanced uPAR and/or HER2 signaling might explain the transition between dormancy and recurrence. Therefore, distant metastases might arise from disseminated tumor cells that acquire additional genetic abnormalities and the re-activation of uPAR and mitogenic signaling (HER2 or EGFR). This suggests that although survival pathways are functional, the mechanisms that propel the growth of the primary lesion are insufficient for metastatic growth and the microenvironment might influence progression. If uPAR and HER2 up-regulation serve as a switch to interrupt dormancy, these might be important targets for combination therapy of residual disease.

HER2 promotes metastases

HER2 is expressed in metastases but also promotes that phenotype. It has been shown that when paired primary tumor and distant metastatic lesions are compared, approximately 94% and 93% of samples have a concordant HER2 status when analyzed by IHC or FISH, respectively (Gancberg et al., 2002). Therefore, routine determination of HER2 on metastatic sites is not needed when FISH results from the primary tumor have been obtained. One of the many functions of HER2 in metastatic cells may be to promote cell motility (De Potter, 1994; De Potter and Quatacker, 1993). Therefore, HER2 over-expressing tumor cells would have the ability to invade through the basement membrane, adhere to endothelial cells, extravasate, and migrate into normal organs.

Although breast cancers metastasize to the bone marrow, lung, liver, and brain, bone marrow is a prognostically relevant indicator organ for the presence of hematogeneously disseminated tumor cells (Leinung et al., 2000; Lindemann et al., 1992; Soeth et al., 1997). The majority of cancer patients have viable tumor cells in the bone marrow at primary tumor diagnosis, and the proliferative potential of these cells determines the clinical outcome (Solakoglu et al., 2002). Breast cancer patients with HER2 -positive tumor cells in the bone marrow have a greater risk for subsequent metastatic relapse than patients with disseminated tumor cells lacking an immunocytochemically detectable expression of HER2 (Braun et al., 2001). Therefore, HER2 over-expression in disseminated tumor cells found in the bone marrow predicts poor clinical outcome (Braun et al., 2001).

The chemokine receptor CXCR4 and its natural ligand, stromal cell-derived factor-1alpha (SDF-1alpha), facilitate the homing of certain metastatic breast cancer cells to both the lung and the bone (Liotta, 2001; Muller et al., 2001). CXCR4-expressing malignant breast cancer cells invade the extracellular matrix and circulate in the blood and lymphatic vessels. SDF-1alpha is released in high amounts by certain organs, such as lung, bone, and liver. The attraction between SDF-1alpha and CXCR4 triggers breast cancer cells to leave the circulation and migrate into these organs where the cancer cells proliferate, induce angiogenesis, and form metastatic tumors.

CXCR4 contributes to invasive processes, such as enhanced migration and adhesion activity, in SDF-1alpha–enriched organs for HER2 over-expressing cancer cells (Muller et al., 2001). In addition, HER2 up-regulates the expression of CXCR4, which is required for HER2-enhanced invasion, migration, adhesion, and metastasis to the lung. HER2 further inhibits ligand-induced CXCR4 degradation. Inhibition of CXCR4 expression suppressed HER2-induced malignancy in *in vitro* and *in vivo* lung metastasis. Finally, there was a significant correlation between HER2 and CXCR4 expression in human breast tumor tissues, and CXCR4 expression correlated with a poor overall survival rate in breast cancer patients. These results provide a plausible mechanism for HER2-mediated breast tumor metastasis and establish a functional link between HER2 and CXCR4 signaling pathways. Subsequent studies have used gene expression analysis to characterize additional molecular species that contribute to breast cancer tissue metastasis to either the lung or the bone (Kang et al., 2003; Lee et al., 2003; Montel et al., 2005).

HER2 is expressed in cytokeratin-positive (CK) cells isolated from the bone marrow of breast cancer patients. Single tumor cells can be detected in the bone marrow of 20–60% of carcinoma patients without apparent metastasis using epithelial-specific CK antibodies, but are practically absent in donors without epithelial malignancy (Braun et al., 2000; Pantel et al., 1999). Although CK itself is not a marker of malignancy, many studies demonstrate that the presence of CK-positive cells in bone marrow has a strong prognostic impact on relapse-free as well as overall survival in breast cancer and many other types of cancer (Braun et al., 2000; Pantel et al., 1999). Moreover, in breast cancer, their presence is a strong prognostic indicator specifically for the development of skeleton metastasis (Braun et al., 2000; Gebauer et al., 2001).

About 70% of CK cells isolated from bone marrow of breast cancer patients express HER2 and about 30% express EGFR (Pantel et al., 1993). Whereas expression of proliferation markers such as Ki-67 or p120 on micrometastatic cells was observed only in 15.9% of cancer patients analyzed, HER2/CK18$^+$ cells were found in 67.6% of breast cancer patients. The incidence of HER2/CK18$^+$ cells was positively correlated with the clinical stage of tumor progression. The high incidence of HER2 expression on micrometastatic breast cancer cells in the bone marrow suggests that these cells might have been positively selected during early stages of metastasis. Similarly, genomic analysis of single CK-positive cells from bone marrow reveals early mutational events, including very early *HER2* amplification, in breast cancer (Schardt et al., 2005).

HER2 regulates mammary stem/progenitor cell populations

All tissues in the body are derived from organ-specific stem cells that have the capacity to undergo self-renewal, differentiate into the cell types that comprise each organ, and help maintain tissue integrity. There is some evidence that certain tumors are derived from stem cells or from early descendents of stem cells. Bonnet et al discovered that leukemia is driven by a small population of leukemia cells that have the ability to perpetually self-renew. They termed this population cancer stem cells (CSCs) (Bonnet and Dick, 1997).

In contrast to the traditional “stochastic” model of oncogenesis where transformation results from random mutations and subsequent clonal selection, the CSC model postulates that cancer originates in tissue stem cells (or more likely their recent progenitors cells) through dysregulation of self-renewal pathways (Charafe-Jauffret et al., 2008). As a result, tumors contain and are driven by a subpopulation of cells that retains key stem-cell properties including self-renewal, which drives tumorigenesis, and differentiation, which contributes to cellular heterogeneity. This leads to expansion of this cell population that may undergo subsequent

genetic and epigenetic changes, and the only cells within an organism that live long enough to accumulate the necessary mutations that lead to cancer are stem cells.

Miyamoto et al. discovered that genomic mutations and rearrangements of stem cells that give rise to all the cells of the blood can lead to some forms of leukemia (Miyamoto et al., 2000). They proposed that these changes could underlie the development of cancers in many tissues. Since then, putative CSCs have been isolated from many other tumors including breast, ovarian, brain, colon, pancreas, prostate, lung and head and neck tumors (Al-Hajj et al., 2003; Ceder et al., 2008; Eramo et al., 2008; Li et al., 2007; O'Brien et al., 2007; Prince et al., 2007; Seo et al., 2007; Singh et al., 2003; Zhang et al., 2008).

The breast undergoes most of its development after birth, and adult breast stem cells and their progeny are required for this large expansion in cell number. A wide variety of cancers, including breast cancer, are likely derived from a small subset of tumor-initiating cells or CSCs that display stem cell-like properties. The Wicha laboratory and others have found a sub-population of cells in human breast tumors, with the phenotype $CD44^{+}/CD24^{-}/lin^{-}$, that display CSC characteristics (Al-Hajj et al., 2003). As few as 100 cells with this phenotype were able to form tumors in immunocompromised mice, whereas tens of thousands of cells with alternate phenotypes failed to form tumors. This tumorigenic sub-population, which accounts for 1–5% of primary tumors, can also generate the phenotypic heterogeneity of the initial tumor. In addition, the stem/progenitor cell population, found in both the normal mammary gland and in mammary carcinomas, also has increased expression of the enzyme aldehyde dehydrogenase (ALDH) (Ginestier et al., 2007).

CSCs promote blood vessel formation, support cell motility as well as resistance to a variety of therapies (Phillips et al., 2006), and are implicated in breast metastasis (Balic et al., 2006; Sheridan et al., 2006). Linking CSCs and metastasis is clinically significant since the survival of breast cancer patients with metastatic disease has not changed significantly over the past several decades. Balic and colleagues (Balic et al., 2006) found that most early disseminated cancer cells detected in the bone marrow of breast cancer patients have a breast CSC phenotype.

There is a correlation between the stem cell marker ALDH and *HER2* over-expression in breast cancer patients (Ginestier et al., 2007). In addition, Wicha and colleagues found that PTEN and HER2 regulate self-renewal and invasion of human mammary stem cells (Korkaya and Wicha, 2007). They examined whether HER2 over-expression and downstream signaling may regulate the mammary stem cell population since tumorigenesis, invasion, and metastasis may be mediated by the CSC subpopulation (Korkaya and Wicha, 2007). Indeed, HER2 over-expression increased the stem/progenitor cell population of both normal and malignant mammary cells. The effects of HER2 over-expression on breast CSCs were blocked by trastuzumab in sensitive, but not resistant, cell lines (Korkaya et al., 2008). The ability of trastuzumab to target the CSC population in *HER2*-amplified tumors relates to its clinical efficacy since HER2 over-expression drives mammary carcinogenesis through its effects on normal and pre-malignant mammary stem cells.

The discovery of tumor cells that behave like stem cells offers a possible explanation why breast cancer may be so difficult to eradicate. Conventional cancer therapies effectively kill the bulk of the tumor cells, but may miss cancer stem cells; and, therefore, cancers often recur. In addition, Phillips et al. discovered that CD44+/CD24− mammary stem cells are relatively resistant to radiation and chemotherapy *in vitro* and in mouse models (Phillips et al., 2006). Likewise, there is an increase in the CSC population following neoadjuvant chemotherapy in patients with locally advanced breast cancer (Li et al., 2008). Targeting of this cell population with HER2 targeted therapies may be an efficient treatment option.

HER2-targeted therapies

We (Drebin et al., 1985; Drebin et al., 1986) discovered that down-regulation of cell surface $p185^{neu}$ blocks downstream signaling and reverses the malignant phenotype of *neu* transformed cells. We purified $p185^{neu}$ reactive monoclonal antibodies (IgG2a) capable of cross-linking the receptor molecules of the *neu*-transfected NIH3T3 cells. This led to rapid $p185^{neu}$ down-regulation and an increased rate of its degradation. The overall effect was reversal of the malignant phenotype both *in vitro* and *in vivo*. The anti- $p185^{neu}$ antibodies had cytostatic effects on anchorage-independent growth of the *neu*-transformed cell lines. The presence of antibody was required for the conversion of phenotype; and when antibody was removed, the malignant properties became evident again.

We were the first to show that disabling a protein complex needed for transformation would reverse the malignant properties of tumor cells *in vitro* and *in vivo*. In a xenograft model, anti-$p185^{neu}$ monoclonal antibodies inhibited tumor growth of *neu* transformed cells, and tumor growth resumed once antibody treatment ceased. These initial studies prompted the development of successful targeted therapeutics, such as trastuzumab (Herceptin®; Genentech Inc., South San Francisco, CA). Herceptin has proven to be an effective treatment for the HER2 over-expressing metastatic breast cancer, with response rates ranging from 17– 35% (Vogel et al., 2002).

Our laboratory, (Drebin et al., 1988) was able to obtain an even greater anti-tumor effect by administering a cocktail of anti-$p185^{neu}$ monoclonal antibodies reactive with distinct $p185^{neu}$ epitopes. Data generated from a Phase II clinical trial presented at the American Society of Clinical Oncology (ASCO) 2008 meeting showed that half of the patients with advanced, Herceptin-resistant, HER2 metastatic breast cancer benefited from a combination of Herceptin and Pertuzumab, another anti-HER2 specific antibody which blocks receptor HER2-erbB3 heterodimerization (Gelmon et al., 2008). These data suggest that two antibodies binding distinct epitopes on HER2 ectodomains can overcome adaptive features of Herceptin resistance in human tumors.

In addition to biologics targeting the ectodomain region of HER2, several tyrosine kinase inhibitors (TKIs) have been developed. For instance, lapatinib is a reversible TKI that inhibits both EGFR and HER2 (Rusnak et al., 2001). By targeting both receptors, lapatinib has, in principle, advantages over other TKIs such as gefitinib and erlotinib that selectively target only EGFR (Zhang et al., 2007). Lapatinib inhibits EGFR and HER2 activation and, consequently, downstream signaling through MAPK and AKT pathways; thus, resulting in the death of human breast and head and neck cancer cells both *in vitro* and *in vivo* (Xia et al., 2002). Since lapatinib blocks AKT phosphorylation by inhibiting both EGFR and HER2, its activity does not depend on phosphatase and tensin homolog (PTEN), the phosphatase that negatively regulates AKT and is required for trastuzumab anti-tumor activity (Xia et al., 2007). Unfortunately, *in vitro* studies indicate that gefitinib-resistant tumors are also resistant to lapatinib (Carter et al., 2005).

Targeted therapies prevent pre-malignant lesions from progressing

Since HER2 plays such an important role in early metastasis, targeted therapies may be critical to prevent pre-malignant lesions from developing into IBC. We have shown that HER2 targeted antibodies not only inhibit growth of already established tumors, but also can prevent tumor development in transgenic mice over-expressing the activated *neu* oncogene in mammary epithelial cells (Katsumata et al., 1995). In this study, treatment of transgenic animals after 20 weeks of age but before tumor emergence led to a dose-dependent reduction in tumor incidence, and the tumor-free mice seemed to be protected for life.

These studies indicated that cells expressing $p185^{neu}$ must still undergo further genetic changes to progress to fully transformed cells. Human cells over-expressing HER2 proteins also require additional allelic and adaptive changes to become fully transformed. Therefore, prevention of tumor emergence can occur by down-regulating HER2 proteins prior to complete transformation. This finding facilitated the use of the antibodies as an adjuvant to prevent tumor emergence (Romond et al., 2005).

Resistance to HER2-targeted therapeutics

A large percentage of HER2-positive cancers demonstrate predisposition to resistance to HER2-targeted therapeutics (Zhang et al., 2007). In addition, many tumors that are initially responsive to HER2-targeted therapies become refractory following treatment (Zhang et al., 2007). An increasing body of evidence indicates that the resistant phenotype can arise from diverse adaptive and genetic changes within transformed cells, which allow the cells to survive in the presence of the HER2-targeting antibodies or TKIs (Wang and Greene, 2008). A better understanding of the mechanisms involved in the resistance of tumor cells to HER2-targeted molecules will provide the framework for developing new therapeutic strategies. Information regarding the genetic hallmarks of drug resistance can be used to predict clinical outcomes. In particular, treatments targeting the early transformed phenotypes may have the best potential to achieve maximal therapeutic effects.

A major factor that determines whether tumors will respond to erbB inhibitors involves changes in the targeted genes. For example, up-regulation of HER2 levels has been associated with resistance to HER2 antagonists. In addition, tumors treated with HER2 TKIs may acquire secondary mutations within the HER2 kinase domain, which leads to TKI resistance (Godin-Heymann et al., 2008). In the case of EGFR, a point mutation in the kinase domain, namely T790M, has been shown to cause resistance to the TKIs by producing steric hindrance for the binding of the small molecules (Engelman et al., 2005; Engelman et al., 2007; Kobayashi et al., 2005; Pao et al., 2005). Although similar mutations have yet to be found in HER2, a number of mutated forms of HER2 have been described and thought to modulate the activities of the receptor ensemble. Many of these mutations are in-frame insertions located in the kinase domain in the analogous structural region of the in-frame EGFR deletions that are associated with some lung cancers (Stephens et al., 2004). In particular, a HER2 mutant containing a G776 (YVMA) insertion in exon 20 exhibits higher activity than the wild-type protein in activating downstream signaling events by forming the EGFR/ HER2 heterodimer, which thereby enhances survival, invasiveness, and tumorigenicity (Wang et al., 2006). Although cancer cells expressing this mutation appear to be sensitive to HER2-targeted therapies, they become more resistant to EGFR TKIs. Knockdown of the mutant HER2 can increase apoptosis and restore sensitivity to EGFR TKIs (Wang et al., 2006).

Heteromer formation and resistance

The HER2/EGFR heterodimer may undergo antibody-induced internalization, ubitiquination, and proteolysis (Gilboa et al., 1995; Maier et al., 1991; Qian et al., 1997; Srinivas et al., 1993). The internalization of HER2/EGFR represents a mechanism by which the HER2-specific antibodies disable the transforming activity of the receptor. HER2 internalization and degradation involve clathrin-mediated endocytosis and trafficking of the cell surface proteins from the endosomal compartment to the lysosome (Maier et al., 1991). Thus, resistance to HER2-specific immunotherapy may involve genetic or epigenetic changes that compromise receptor endocytosis and degradation.

Activation of the PI3K pathway, which has been shown to be dominant in transformation-related signaling events caused by erbB kinase complexes, also contributes to resistance to erbB-targeted therapeutics (Yakes et al., 2002). Both loss of PTEN and activation of the PI3-

kinase are implicated in the resistance of HER2-positive breast tumors to trastuzumab (Nagata et al., 2004).

HER2 signaling activity, as well as resistance to inhibition, is mediated in part by its dimerization with other erbB family members. In particular, the dimerization of HER2 with EGFR or erbB3 may be a major source for resistance (Chen et al., 2000), and thus may be explored as the targets for novel therapeutic approaches. For example, a subset of $HER2^+$ and $EGFR^+$ cancers are refractory to inhibitors of a single co-receptor. Gefitinib-resistant cells exhibit increased EGFR expression, which leads to constitutive activation of the MAPK pathway and thereby compensates for down-regulation of the PI3K pathway following inhibition of HER2 (Yokoyama et al., 2006). A separate study showed that Herceptin-resistant cells exhibit higher levels of phosphorylated EGFR and EGFR/HER2 heterodimers as well as over-expression of the erbB ligands such as transforming growth factor alpha, heparin-binding EGF, and heregulin (Ritter et al., 2007). These findings suggest that amplification of ligand-induced activation of erbB receptors is a plausible mechanism of acquired resistance to trastuzumab in breast cancers. Small-molecule inhibitors of both EGFR and HER2 can induce apoptosis in the resistant cells (Ritter et al., 2007). Thus, simultaneous inhibition of EGFR and HER2 may produce superior therapeutic effects. Indeed, lapatinib, a selective TKI that disables both EGFR and HER2 kinase activities, has shown promise in early clinical trials (Cameron et al., 2008; Di Leo et al., 2008; Gomez et al., 2008; Storniolo et al., 2008).

HER2 heterodimerization with erbB3 may initiate signaling events that provide cellular resistance to HER2-targeted therapeutics (Agus et al., 2002). ErbB3 lacks a functional kinase domain, but its dimerization with HER2 preferentially promotes activation of the PI3K pathway, which compensates for the disabled HER2-dependent signaling (Holbro et al., 2003). In addition, erbB3 also mediates the activation of Src and, thereby, may provide additional survival and proliferative signaling events (Contessa et al., 2006).

More recently, Wang et al showed that the transforming growth factor beta (TGF-beta) signaling pathway can increase resistance of the HER2 over-expressing cells to trastuzumab (Wang et al., 2008). Activation of the TGF-beta type I receptor can induce phosphorylation of TACE/ADAM17 and its translocation to the cell surface, leading to increased secretion of the erbB ligands including TGF-alpha, amphiregulin, and heregulin. In turn, these ligands stimulate erbB3 and activate the PI3K pathway. Conversely, inhibition of Alk5, PI3-kinase, TACE, or erbB3 restored sensitivity to trastuzumab (Wang et al., 2008). These findings describe a mechanism by which TGF-beta activates PI3K/Akt and enhances resistance to trastuzumab.

Furthermore, resistance to HER2-targeted therapy may arise from activation of other autocrine signaling pathways. For example, insulin-like growth factor-I (IGF-I), which activates the cell survival signaling network such as the PI3K pathway, renders cells resistant to trastuzumab (Lu et al., 2001; Nahta et al., 2005). Breast cancer cells with over-expression of IGF-IR are not subjected to trastuzumab-mediated inhibition of proliferation (Lu et al., 2001). Consistent with these observations, the addition of IGF-binding protein-3, which down-regulates IGF-IR signaling, can restore trastuzumab-induced growth inhibition (Lu et al., 2001).

A recent study showed that Notch-1 may contribute to trastuzumab resistance (Osipo et al., 2008). The Notch receptors are potent cell-fate regulatory proteins and can act as survival factors that promote breast cancer (Dievart et al., 1999; Politi et al., 2004). In particular, activation of Notch-1 (Stylianou et al., 2006) or Notch-4 (Imatani and Callahan, 2000) cause mammary tumors in animal models. In humans, high levels of Notch-1 and its ligand, Jagged-1, are associated with poor overall survival of breast cancers, including cancers over-expressing HER2 (Dickson et al., 2007; Reedijk et al., 2005). Notch-1 may play a role in self-renewal of

mammary epithelial stem cells (Dontu et al., 2004) and DCIS (Farnie et al., 2007). Of note, cells treated with trastuzumab or a dual EGFR/HER2 TKI exhibit increased Notch activities (Osipo et al., 2008). Inhibition of the Notch signaling pathway increases the inhibitory effect of HER2-targeted therapeutics (Osipo et al., 2008).

Processes of cancer metastasis appear to provide selection pressure for the early incompletely transformed tumor cells to accumulate genetic and epigenetic changes in order to survive at the secondary sites. These changes may result in activation of diverse signaling pathways that are capable of compensating for the signaling events disabled by HER2-targeted therapeutics. In this regard, therapeutic approaches that target HER2-mediated early tumor dissemination may be particularly useful to prevent or counteract the development of resistance.

Conclusion

Metastasis can occur very early during breast cancer progression and exist in different locations with an incompletely transformed phenotype. Despite complete removal of their primary tumor, patients with localized, lymph node-negative tumors also relapse, demonstrating that disseminated tumor cells must have spread before surgery or even diagnosis.

Recent studies have found breast tumor cells have cancer stem cell properties and are regulated by HER2. HER2 is responsible for an increase in proliferation and survival of the primary tumor and also plays a role in the distant but not completely transformed lesions. An increase in motility of intravasating and extravasating cells, a decrease in apoptosis, an enhancement of communication with the microenvironment, and regulation of adhesion alters these distantly migrated cells. HER2 over-expression enhances the transformation potential. Current understanding of how untransformed, stem/progenitor cell-like, HER2-expressing cells metastasize and how they could be therapeutically targeted to prevent $HER2^{+}$ breast cancer progression should lead to new treatment schedules of targeted antibodies which are directed at incompletely transformed cells.

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Table 1

HER2 expression level at various stages of tumor progression

Invasiveness	Stage	HER2 Expression	HER2 Gene Amplification	Other characteristics	References
Pre-malignant	TDLU	0–9%	Rare		(Krishnamurthy and Sneige, 2002)
Pre-malignant	ADH	0–9%	Rare		(Allred et al., 1992; De Potter et al., 1989; Gusterson et al., 1988;Lodato et al., 1990)
Pre-invasive	DCIS (low grade)	10%	Rare	p53 abnormalities rare, >90% ER positive	(Bobrow et al., 1994; Hoff et al., 2002; Kallioniemi et al., 1991;Krishnamurthy and Sneige, 2002; Leal et al., 1995; McCann et al., 1991; Ridolfi et al., 2000;Soslow et al., 2000)
Pre-invasive	DCIS (high grade)	70%	Common	p53 abnormalities common (2/3), 2 5 % E R positive	(Allred et al., 1992; Collins and Schnitt, 2005; Hoff et al., 2002;Kallioniemi et al., 1991;Krishnamurthy and Sneige, 2002; Leal et al., 1995; Poller et al., 1993; Quenel et al., 1995;Ridolfi et al., 2000; Tsuda et al., 1993; Warnberg et al., 2001;Wilbur and Barrows, 1993)
Pre-invasive	LCIS	2%			(Krishnamurthy and Sneige, 2002)
Invasive	IDC	High	Common	p53 abnormalities common	(Bloom et al., 2001; Kobayashi et al., 2002; Mrhalova et al., 2003; Slamon et al., 1989)
Invasive	ILC	Low	Rare	Frequent E-cadherin mutations, absent E-cadherin expression	(Gusterson et al., 1992; Hoff et al., 2002; Janocko et al., 2001;Kallioniemi et al., 1991; Marks et al., 1994; McCann et al., 1991;Ridolfi et al., 2000; Seshadri et al., 1993; Somerville et al., 1992;Soslow et al., 2000; Toikkanen et al., 1992)

Table 2

$p185^{NEU}$ transgenic mice

$p185^{HER2/NEU}$	**Promoter**	**Expression pattern**	**Phenotypic effect**
Homozygous null (knockout)	Endogenous	Congenital	Embryonic lethal (before E11) due to myocardial malformation and peripheral nervous system abnormalities
Homozygous null (knockout) + wild type (rat)	Endogenous + Nkx2.5	Congenital + heart	Heart development is rescued, though the heart is smaller by ~30%, Schwan cells and motoneuron subpopulations are severely affected, perinatal death
Homozygous (knock in) wild-type (rat)	Endogenous	Congenital	Viable
Homozygous (knock in) kinase-dead (rat)	Endogenous	Congenital	Similar phenotype with Lee et al., 1995
Homozygous (knock in) activated (rat)	Endogenous	Congenital	Similar phenotype with Lee et al., but prolonged survival (up to E12.5) with lower-than-expected message levels
Heterozygous (knock in) activated (rat)	Endogenous	Congenital	Viable, failed to develop tumors
Wild-type(human)	MMTV*	Mammary epithelial	Adenocarcinomas and a variety of tumors including B lymphomas were induced at relatively late onset
Wild-type (rat)	MMTV*	Mammary epithelial	Mammary tumors after a long latency period, tumor progression was associated with somatic activating mutations in 70% of the mammary tumors analyzed
Activated (rat)	MMTV*	Mammary epithelial	Rapid induction of multifocal mammary tumors, mammary adenocarcinomas that involve the entire epithelium in each gland, tumors arise synchronously and are polyclonal in origin, sufficient to induce malignant transformation
Activated (rat)	MMTV*	Mammary epithelial	Independent but multiple mammary tumors arose asynchronously, between 5 and 10 months of age, as stochastic events
Heterozygous (knock in) activated (rat)	Endogenous +MMTV*-Cre recombinase	Cre-inducible, mammary epithelial	Mammary adenocarcinomas, tumor progression was associated with a dramatic elevation of both P185HER2/NEU protein and transcript and correlated with genomic amplification, accelerated lobuloalveolar development and formation of focal mammary tumors after a long latency period
Wild-type (rat)	Endogenous (multiple copies)	Congenital with slight elevation	Abnormal lobuloalveolar development in virginal glands and incomplete regression in multiparous glands, malignant foci form following multiple rounds of pregnancy and regression
Activated (rat)	Endogenous (multiple copies	Congenital with slight elevation	Stronger but similar phenotype as wild-type rat $p185^{HER2/NEU}$
Activated (rat)	Keratin 5 or Keratin 14	Epidermal basal cells	Severe epithelial hyperplasia in multiple organs, including the skin, particularly striking in hair follicles and perinatally lethal
Activated (rat)	Keratin 14 + rtTA/TetRE	Dox-inducible epidermal basal cells	Similar to the above but controllable by exposure to and withdrawal of doxycycline
Wild-type (rat)	Keratin 5	Epidermal basal cells	Developmental abnormalities in both skin and hair follicles, skin hyperplasia, and squamous cell carcinomas; tumorigenesis resistance
Wild-type (human)	Ig/Tp**	B cell	Late onset of clonal pre-B cell lymphomas
Activated V695E (human)	Ig/Tp**	B cell	Clonal pre-B cell lymphomas were induced neonatally in all mice

*MMTV – mouse mammary tumor virus

**Ig/Tp - immunoglobulin enhancer--SV40 early gene promoter