**Tumor and Stem Cell Biology**

**HER2 Drives Luminal Breast Cancer Stem Cells in the Absence of HER2 Amplification: Implications for Efficacy of Adjuvant Trastuzumab**


**Abstract**

Although current breast cancer treatment guidelines limit the use of HER2-blocking agents to tumors with HER2 gene amplification, recent retrospective analyses suggest that a wider group of patients may benefit from this therapy. Using breast cancer cell lines, mouse xenograft models and matched human primary and metastatic tissues, we show that HER2 is selectively expressed in and regulates self-renewal of the cancer stem cell (CSC) population in estrogen receptor-positive (ER+). HER2 luminal breast cancers. Although trastuzumab had no effects on the growth of established luminal breast cancer mouse xenografts, administration after tumor inoculation blocked subsequent tumor growth. HER2 expression is increased in luminal tumors grown in mouse bone xenografts, as well as in bone metastases from patients with breast cancer as compared with matched primary tumors. Furthermore, this increase in HER2 protein expression was not due to gene amplification but rather was mediated by receptor activator of NF-kB (RANK)-ligand in the bone microenvironment. These studies suggest that the clinical efficacy of adjuvant trastuzumab may relate to the ability of this agent to target the CSC population in a process that does not require HER2 gene amplification. Furthermore, these studies support a CSC model in which maximal clinical benefit is achieved when CSC targeting agents are administered in the adjuvant setting. *Cancer Res; 73(5); 1635–45. ©2012 AACR.*

**Introduction**

Approximately 20% of breast cancers display amplification of the HER2 gene, a genotype associated with an aggressive course and poor outcome (1). The development of HER2 targeting agents such as trastuzumab and lapatinib represents one of the greatest achievements in clinical oncology showing the effectiveness of molecularly targeted therapeutics (2). In women with advanced metastatic breast cancer, addition of trastuzumab to cytotoxic chemotherapy increases the response rate, time to tumor progression, and survival (2–4). In this setting, the beneficial effect of trastuzumab seems to be limited to breast tumors with HER2 amplification, a finding predicted by preclinical data (1, 5–7).

Based on the demonstrated clinical efficacy of HER2 blockade in women with advanced HER2-amplified tumors, inclusion of patients into adjuvant trials has been largely limited to this patient population. These adjuvant trials showed a remarkable 50% reduction in recurrence rate with the addition of trastuzumab to chemotherapy as compared with chemotherapy alone (8–12). These results have led to establishment of guidelines for HER2 testing (6, 13).

The conventional wisdom that only patients with HER2-amplified breast tumors would benefit from trastuzumab was challenged by a provocative article published in the *New England Journal of Medicine* in 2008, in which, Paik and colleagues reanalyzed HER2 expression in tumors from patients on NSABP-B31, one of the pivotal trials that showed the efficacy of adjuvant trastuzumab (13). They reported that 174 cases originally classified as HER2+ actually lacked HER2 gene amplification when reanalyzed in a central laboratory. Surprisingly, these "HER2-negative" patients benefited as much from adjuvant trastuzumab as did women whose tumors displayed classical HER2 amplification. Although questions have been raised about the reliability of HER2 analyses in this study (14), similar results were recently reported by the Perez and colleagues (15), which makes it less likely that these results were due to chance or laboratory error.
The molecular mechanisms that may account for a clinical benefit of HER2 blockade in the adjuvant setting in patients whose tumors do not display classical HER2 amplification are not known. However, we have recently proposed that the clinical efficacy of HER2 blockade in tumors classified as HER2- "might be explained by the "cancer stem cell hypothesis". According to this model, many human cancers, including breast cancer are driven by a subpopulation of cells that display stem cell properties (16). We have previously shown that HER2 is an important driver of the cancer stem cell (CSC) population in tumors with HER2 amplification (17, 18). Using breast cancer cell lines, xenograft models, as well as primary and metastatic human breast cancer samples, we now show that HER2 is selectively expressed in the CSC population of luminal estrogen receptor-positive (ER+) breast cancers in the absence of HER2 gene amplification and provide evidence that the efficacy of HER2-blocking agents in the adjuvant setting may reflect effects on these cells.

Materials and Methods

Cell culture and treatment and flow cytometry

MCF7, ZR75-1, BT474, SKBR3, and MDA-MB231 cell lines were purchased from American Type Culture Collection and maintained in culture conditions according to supplier’s recommendation. The SUM159 cell line was cultured as previously described (19).

Trastuzumab was obtained from the Cancer Center Pharmacy at the University of Michigan (Ann Arbor, MI).

The ALDEFLUOR assay was obtained as previously described (20) according to manufacturer’s guidelines (Stem-Cell Technologies).

Flow-cytometry analyses and immunohistochemical staining were described in detail in Supplementary Data.

Tumorsphere assay was conducted as previously described (17).

Lentivirus infections have been described in the Supplementary Data.

Mice and xenograft models, treatment, and bioluminescence

Details of mouse xenografts and treatment of animals has been given in the Supplementary Data.

Patient selection

After Institutional Review Board (IRB) approval (IRB# HUM0041153), a free-text search of the Department of Pathology, University of Michigan database was conducted using SNOMED. Nineteen patients (between 1986 and 2008) were selected on the basis of the following criteria: matched primary breast cancer and bone metastatic tumor with slides and blocks available for study.

HER2-automated quantitative analysis (AQUA) of matched primary cancer and bone metastasis

The AQUA system (HistoRx) was used for the automated image acquisition and analysis as described in the Supplementary Data.

All statistical analysis has been conducted as explained in the Supplementary Data.

Results

Expression of HER2 correlates with the CSC marker aldehyde dehydrogenase in luminal breast cancer cell lines

We have previously shown that normal and malignant breast CSCs are characterized by aldehyde dehydrogenase (ALDH) expression (20). Furthermore, established breast cancer cell lines show a hierarchical organization in which a subpopulation of ALDH-expressing cells is enriched for tumor-initiating characteristics (21). As assessed by ALDEFLUOR assay, MCF7 and ZR75-1 luminal breast cancer cell lines express the lowest level of ALDH (Fig. 1A), whereas BT474 and SKBR3, HER2-amplified cell lines express the highest levels of ALDH. SUM159 and MDA-MB231, basal/claudin-low cell lines, express an intermediate level of ALDH consistent with previous reports (17, 21). The HER2-amplified BT474 and SKBR3 cell lines expressed the highest levels of HER2, the basal/claudin-low SUM159 and MDA-MB231 cell lines expressed the lowest levels of HER2, and the luminal ER+ cell lines MCF7 and ZR75-1 expressed an intermediate levels of HER2 (Fig. 1B). We used fluorescence-activated cell sorting (FACS) to determine the relationship between HER2 and ALDH expression at the individual cell level. In MCF7 and ZR75-1 luminal cell lines, the level of HER2 expression was considerably lower than in the HER2-amplified cell lines. However, in these cells, HER2 expression was increased 2- to 3-fold in ALDEFLUOR-positive as compared with ALDEFLUOR-negative cells (Fig. 1C; P = 0.012 and 0.047 for MCF7 and ZR75-1, respectively). This association was seen when cells were first gated on ALDEFLUOR-positive and -negative cell populations, or conversely when high and low HER2-expressing cells were separated and assessed for ALDH expression (Fig. 1C and D; Supplementary Fig. S1A and S1B; P = 0.038 and 0.033 for MCF7 and ZR75-1, respectively). In contrast, there was no association between HER2 and ALDH expression in SUM159 and MDA-MB231 basal/claudin-low cell lines (Fig. 1C and D). Immunofluorescence using anti-HER2 antibody in ALDEFLUOR-positive and -negative cells confirmed the concordance between HER2 and ALDH expression in luminal MCF7 cells (Fig. 1E and F).

Trastuzumab reduces the CSC population of luminal breast cancer cells

To determine the functional role of HER2, we assessed the effects of the HER2-blocking antibody trastuzumab on cell growth in vitro. As previously reported, under standard tissue culture conditions (22), the effects of trastuzumab on inhibiting cell growth were limited to cells that displayed HER2-amplification (Fig. 2A). While cell growth in tissue culture under attached conditions largely reflects proliferation of bulk cell populations, formation and serial passage of tumourspheres is a validated in vitro surrogate assay for CSCs (23). In contrast to the absence of effects in standard culture conditions, trastuzumab significantly reduced tertiary tumoursphere formation of luminal MCF7 and ZR75-1 mammary carcinoma.
cells (P < 0.05 and P = 0.003, respectively) but had no effect on tumorsphere formation in basal/claudin-low SUM159 cells (Fig. 2B and Supplementary Fig. S2A and S2B). The effects of trastuzumab on the CSC population were further assessed using the ALDEFLUOR assay. Trastuzumab significantly reduced the percentage of ALDEFLUOR-positive cells in luminal MCF7 and ZR75-1 cells (Fig. 2C and Supplementary Fig. S3A) but had no effect on the ALDEFLUOR-positive populations in basal/claudin-low SUM159 or MDA-MB231 cells (Fig. 2D). Breast CSCs also have been characterized as having the phenotype CD44+/CD24–/CD133– as assessed by flow cytometry (24). Trastuzumab significantly reduced the CD44+/CD24– population (P = 0.025) in luminal MCF7 cells (Fig. 2E and Supplementary Fig. S3B). ZR75-1 cells lack a CD44+/CD24– population, whereas SUM159 cells are all CD44+/CD24– precluding the use of those CSC markers in these cell lines. To further investigate the role of HER2 in driving the CSC population, we used flow cytometry to fractionate HER2+ and HER2– cells within the ALDH+ population. ALDH+HER2+ cells showed significantly greater tumorsphere forming capacity than ALDH+HER2– cells, an effect that was inhibited by trastuzumab (Fig. 2F). We also knocked down HER2 expression in MCF7 cells (Supplementary Fig. S4A) and showed that this abrogated tumorsphere formation, furthermore trastuzumab had no effect in HER2 knockdown cells (Supplementary Fig. S4B).

HER2 drives the cancer-initiating population in luminal breast cancer xenografts

To determine the relationship between HER2 expression and tumor-initiating capacity, MCF7 cells were sorted for HER2 expression and injected into the mammary fat pads of nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice. The median time to develop palpable tumors following injection of 1 × 104 cells was significantly less in
HER2-expressing than in HER2-nonexpressing cells (Fig. 2G, P = 0.05). Furthermore, tumor growth rate and size was significantly greater in HER2+ as compared with HER2− cells (Fig. 2G). We have previously reported that ALDH-expressing cells are enriched for tumor-initiating properties (21). To determine whether expression of HER2 in these ALDEFLUOR-positive cells further enhances tumor initiation, we sorted ALDEFLUOR "HER2+" and ALDEFLUOR "HER2−" cell populations by FACS and implanted serial dilutions of these cells into the mammary fat pads of NOD/SCID mice (24). Interestingly, within the ALDEFLUOR-positive population the frequency of tumor initiation was more than 4-fold higher in HER2-expressing than in HER2-nonexpressing cells (Fig. 2G; P < 0.009).

Effect of trastuzumab on the growth of luminal breast cancer xenografts is dependent upon the timing of administration

We used the HER2-blocking antibody trastuzumab to determine the functional role of HER2 in tumor growth in mouse xenografts. CSC models predict that in advanced cancers, stem cell targeting agents would have little effect on tumor shrinkage as these cells constitute only a small fraction of the total cell population. In contrast, growth of tumors from microscopic disease at primary or metastatic sites depends on CSCs, which have unique self-renewal capacity as compared with bulk tumor populations (16). We therefore compared the effects of trastuzumab administered immediately after tumor inoculation (early treatment) with administration after the establishment of measurable tumors (late treatment). Although trastuzumab had little effect on the growth of established luminal MCF7 and ZR75-1 xenografts (Fig. 3A and F), it significantly blocked tumor growth when treatments were initiated immediately after tumor inoculation (Fig. 3B and G). In contrast, early trastuzumab treatment had no effect on basal/claudin-low Sum159 tumor growth showing the specificity of trastuzumab for luminal breast CSCs (Fig. 3C).

To simulate the standard treatment regimen in women in the early (adjuvant) versus advanced disease settings, we

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**Figure 2.** Trastuzumab targets CSCs in luminal breast cancer cells. A, MTT assay in 2-dimensional culture showed inhibition of cell growth by trastuzumab in BT474, HER2-amplified but not in MCF7 or SUM159 cells that do not display HER2 amplification. B, tertiary tumorsphere formation of MCF7, ZR75-1, and SUM159 cells was analyzed after treatment of primary spheres with trastuzumab (at 21 μg/mL) and 2 serial passages were conducted in the absence of trastuzumab. Trastuzumab treatment significantly reduced the number of tertiary tumorspheres of luminal MCF7 and ZR75-1 cells but had no effect on basal/claudin-low Sum159 cells. C, trastuzumab reduced the percentage of ALDEFLUOR-positive cells in luminal cell lines, (D) but had no effect on basal/claudin-low cell lines. E, trastuzumab significantly reduced the proportion of CD44+/CD24− in MCF7 cells. F, trastuzumab significantly reduced the sphere formation in ALDH+HER2+ but had no significant effect on ALDH+HER2−. G, MCF7 HER2+ and HER2− cell populations were FACS sorted and 10,000 cells were then injected into mammary fat pads of NOD/SCID mice. HER2+ cells produced larger tumors compared with HER2− cells as assessed by luciferase imaging. H, HER2 expression in ALDH+ MCF7 cells showed a significantly higher frequency of tumor initiating cells as compared with the ALDH+ MCF7 cells without HER2 expression. (* P < 0.05; ** P < 0.01).
determined the effect of trastuzumab, the cytotoxic chemotherapy docetaxel, or both on the growth of HER2-amplified BT474 or luminal ZR75-1 tumor xenografts. Administration of trastuzumab after tumors were established (late treatment) significantly reduced the growth of HER2-amplified tumors (Fig. 3E) but had no significant effect on the growth of luminal MCF7 and ZR75-1 xenografts (Fig. 3A and F). When administered after tumors were established, the cytotoxic chemotherapeutic agent, docetaxel reduced tumor growth of both xenografts (Fig. 3D and F). In contrast, when administration was begun in the early setting, trastuzumab significantly reduced growth of HER2-amplified BT474 (Fig. 3E) as well as luminal MCF7 and ZR75-1 cells (Fig. 3B and G). Although ZR75-1 luminal tumors recurred following cessation of treatment with docetaxel alone, addition of trastuzumab to docetaxel in the early setting completely prevented tumor growth following cessation of therapy (Fig. 3H). Together, these experiments suggest that in HER2-amplified cells, trastuzumab has a significant effect on tumor growth when administered in either the advanced or early settings. Conversely in HER2-nonamplified luminal tumors, trastuzumab only has a significant beneficial effect when it is administered in the early (adjuvant) setting.

HER2 and ALDH1 are coexpressed in cells at the invasive front of human luminal breast cancers

We used automated quantitative immunofluorescence analysis (AQUA) to correlate HER2 and ALDH expression at the individual cell level in primary human breast cancer tissues (25). Tumor cells were distinguished from stroma by their expression of CK8, an epithelial marker. In luminal tumors without HER2 amplification, HER2+/ALDH1+ tumor cells...
were preferentially found at the tumor invasive front (Fig. 4A and B, yellow cells white arrow). AQUA analysis and a computer algorithm were then used to quantitatively correlate HER2 and ALDH1 expression in individual cells. This algorithm permitted calculation of the percentage of HER2-expressing cells within the ALDH1-expressing cell population or the percentage of ALDH1-expressing cells in the top and bottom 10% of HER2-expressing cells. As was the case in breast cancer cell lines, in primary luminal breast tumors, there was a significant association between expression of HER2 and ALDH1 in these cells (Fig. 4C; \( P < 0.05 \)). Within the ALDH1+ cells more than 90% of cells displayed HER2 expression, whereas only 40% of ALDH1- cells expressed HER2 in primary luminal tumors. In contrast, in HER2-amplified tumors, HER2 expression was more uniformly expressed (Supplementary Fig. S4C) and there was no significant difference in HER2 expression in ALDH1+ versus ALDH1- cells (Fig. 4C).

The bone microenvironment induces HER2 protein expression in luminal tumor cells

Bone represents the most frequent site for metastasis of human tumors and luminal breast cancers are the most frequent subtype that metastasizes to bone (26). Although a number of factors have been postulated to play a role in facilitating the metastasis and growth of breast cancers in the bone microenvironment, the role of HER2 in this setting is poorly understood. To simulate the bone microenvironment, we injected luciferase-labeled MCF7 cells directly into mouse tibias and assessed tumor growth by light emission. While MCF7 cells in the mouse mammary fat pad required estrogen supplementation for tumor growth, MCF7 cells in the tibia grew without estrogen supplementation. As assessed by immunohistochemistry (IHC), HER2 expression was significantly upregulated in MCF7 cells grown within the bone microenvironment compared with cells grown in the mammary fat pad (Fig. 5A and B). This increased expression was not due to HER2 gene amplification as assessed by FISH (data not shown) but was accompanied by an increase in the percentage of ALDH1-expressing cells (Fig. 5A and C).

To simulate the bone microenvironment in vitro, we cocultured DsRed-labeled MCF7 cells with human osteoblasts (27). HER2 expression in MCF7 cells was significantly increased when the cells were cocultured with human osteoblasts compared with culture in the absence of osteoblasts, an effect that was blocked by the receptor activator of NF-κB ligand (RANKL) inhibitor denosumab (Fig. 5D; \( P < 0.01 \); Supplementary Fig. S5A). Recombinant RANKL increased primary and secondary tumor sphere-forming capacity of MCF7 cells (Supplementary Fig. S5B). Using FACS, we also determined that HER2 and RANK receptors are coexpressed. The top 10% of HER2-expressing MCF7 cells contained a significantly higher proportion of RANK receptor expression than did the bottom 10% (HER2-) of MCF7 cells (\( P = 0.0029 \); ref. Fig. 5E and Supplementary Fig. S3C). These results suggest that RANKL produced by osteoblasts in the bone microenvironment (28) may induce the expression of HER2 in RANK-expressing luminal breast cancer cells.

HER2 drives the CSC population and is necessary for maintaining tumor growth in the bone microenvironment

To determine the functional role of HER2 in the growth of breast cancer cells in the bone microenvironment, we knocked down HER2 expression with a short hairpin RNA (shRNA) lentivirus (Supplementary Fig. S4A). This intervention significantly inhibited growth of MCF7 in mouse tibias (Fig. 5F; \( P = \)
Figure 5. HER2 and ALDH1 expression are increased in MCF7 cells growing in the bone microenvironment. A, representative images show an increase in HER2 and ALDH1 expression in MCF7 cells growing in mouse tibia (Fig. 5J). This provides further evidence for the role of HER2 in driving luminal breast cancer stem cells. B and C, quantitation of HER2 expression and ALDH1 expression in MCF7 cells growing in mouse tibia compared with mammary fat pads. D, MCF7 cells cocultured with human osteoblasts expressed increased levels of HER2 and ALDH1. E, HER2 and RANK expression in MCF7 cells was analyzed and the percentage RANK-expressing cells was assessed for the effect of trastuzumab treatment (Fig. 5K and M). We have previously shown that CSCs are regulated by Akt through activation of the Wnt/β-catenin pathway (19). To test whether HER2 upregulation in the mouse tibia (Fig. 5J) was dependent on growth in the bone microenvironment, A, representative images show an increase in HER2 and ALDH1 expression in MCF7 cells growing in mouse tibia (Fig. 5J). This provides further evidence for the role of HER2 in driving luminal breast cancer stem cells. B and C, quantitation of HER2 expression and ALDH1 expression in MCF7 cells growing in mouse tibia compared with mammary fat pads. D, MCF7 cells cocultured with human osteoblasts expressed increased levels of HER2 and ALDH1. E, HER2 and RANK expression in MCF7 cells was analyzed and the percentage RANK-expressing cells was assessed for the effect of trastuzumab treatment (Fig. 5K and M). We have previously shown that CSCs are regulated by Akt through activation of the Wnt/β-catenin pathway (19). To test whether HER2 upregulation in the mouse tibia (Fig. 5J) was dependent on growth in the bone microenvironment, A, representative images show an increase in HER2 and ALDH1 expression in MCF7 cells growing in mouse tibia (Fig. 5J). This provides further evidence for the role of HER2 in driving luminal breast cancer stem cells. B and C, quantitation of HER2 expression and ALDH1 expression in MCF7 cells growing in mouse tibia compared with mammary fat pads. D, MCF7 cells cocultured with human osteoblasts expressed increased levels of HER2 and ALDH1. E, HER2 and RANK expression in MCF7 cells was analyzed and the percentage RANK-expressing cells was assessed for the effect of trastuzumab treatment (Fig. 5K and M).
microenvironment drives Wnt/β-catenin signaling, we assessed nuclear localization of β-catenin in MCF7 xenografts grown in mouse tibia. There was significantly higher nuclear localization of β-catenin in bone tumors in control mice compared with the bone tumors in trastuzumab-treated mice (Supplementary Fig. S5D).

**HER2 expression is increased in bone metastasis of luminal breast cancers compared with primary tumors in matched patient samples**

We assessed HER2 levels in matched primary and bone metastasis in 19 patients with breast cancer using immunohistochemical and AQUA analysis. In 12 of 14 (87%) of luminal tumors classified as ‘HER2-negative’ by classical criteria, HER2 protein expression was significantly higher in bone metastases as compared with matched primary tumors from the same patients (Fig. 6A and B and Supplementary Fig. S6). Interestingly, HER2 levels as determined by AQUA were above the clinical positive threshold in 6 of 14 bone metastases of luminal tumors even though primary tumors from these patients were HER2-negative (Fig. 6B). Furthermore, none of these HER2-expressing bone metastases displayed HER2 gene amplification as determined by FISH (Fig. 6C and Supplementary Fig. S6A). In contrast, there was no significant increase in HER2 expression between primary tumor and bone metastasis in 5 patients with HER2-amplified breast tumors. In fact, 3 of these patients who received trastuzumab treatment had a significant decrease in HER2 protein expression in bone metastases as compared with their primary tumors (Fig. 6B and Supplementary Fig. S7). The fact that we did not detect increased HER2 expression in bone metastases of HER2-amplified breast tumors compared with their matched primary tumors suggest that the findings in luminal tumors are not an artifact of bone fixation as has been previously suggested (29). Furthermore, the absence of HER2 gene amplification in these metastases suggests that the bone microenvironment induces the expression of HER2 in luminal breast cancer cells metastatic to that site (Fig. 7A).

![Image of Figure 6](image-url)
The CSC hypothesis posits that many tumors, including human breast cancer, are hierarchically organized and driven by a cellular subcomponent that displays stem cell properties (30). These cells drive tumor growth and metastasis and by virtue of their relative resistance to traditional therapies such as cytotoxic chemotherapy and radiation may contribute to tumor recurrence (19, 31–34). We have previously shown that HER2 is an important regulator of breast CSCs in HER2-amplified breast tumors where it regulates CSC self-renewal through activation of the Akt–Wnt signaling pathway (17, 19).

Our studies suggest that contrary to the dichotomous model used clinically, HER2 expression in breast tumors follows a distribution related to molecular subtype with luminal tumors expressing an intermediate level of HER2 compared with basal/claudin-low (low) and HER2-amplified (high) tumors.

The development of technologies such as AQUA quantitative immunofluorescence assay, have enabled assessment of HER2 expression as a continuous variable. In addition to intertumor heterogeneity, we observed intratumor heterogeneity of HER2 expression in both HER2-amplified and non-amplified luminal breast cancer cell lines as well as in human primary and metastatic breast tissues. In luminal cell lines defined as "HER2-negative" by classical criteria, HER2 is preferentially expressed in the CSC population. These observations extend previous studies (35, 36) linking HER2 expression and CSC phenotypes in cell lines.

We determined the effect of trastuzumab on tumor growth in HER2-amplified and nonamplified cell lines under standard culture conditions in vitro, as well as by CSC assays. Although it had no discernable effect on bulk tumor populations, trastuzumab was able to target the CSC populations in MCF7 and ZR75-1 luminal cell lines, as assessed by tumorsphere formation and by reduction in cells expressing the CSC markers ALDH and CD44+/CD24−. This effect was not seen in basal/claudin-low cell lines. Knockdown of HER2 recapitulated the effect of trastuzumab in tumorsphere formation confirming the specificity of trastuzumab in targeting HER2.

The important role of HER2 in the regulation of CSCs in luminal tumors was confirmed using mouse tumor xenografts. HER2-expressing MCF7 cells had significantly greater tumor-initiating capacity than HER2-nonexpressing cells. We found that the effects of trastuzumab were highly dependent on the timing of administration. When trastuzumab treatments were begun after palpable tumors had been established (late treatments) trastuzumab beneficial effects were limited to HER2-amplified tumors. However, when treatment was started immediately (early treatment) after inoculation, trastuzumab significantly reduced the growth of both luminal ZR75-1 and MCF7 cells, as well as the HER2-amplified BT474 tumors. In contrast, early trastuzumab treatment had no effect on the basal/claudin-low SUM159 tumor growth in fat pads or in the tibia model suggesting a specificity of this treatment for luminal tumors. Furthermore, the combination of adjuvant trastuzumab and cytotoxic chemotherapy but not cytotoxic chemotherapy alone completely prevented tumor growth in these luminal tumors even after cessation of treatment. These results emphasize the pivotal role of CSCs in mediating tumor recurrence following adjuvant therapy, an important prediction of the CSC hypothesis (16).

Taken together, these results provide a potential biologic explanation (illustrated in ref. Fig. 7B) for the unexpected findings from 2 separate prospective randomized trials that suggest that the benefit of adjuvant trastuzumab in women...
with HER2− cancers as assessed retrospectively is roughly the same as those with HER2+ cancers (13, 15). Our data provide a biologic rationale supporting the ongoing prospective randomized clinical trial currently being conducted by the National Surgical Adjuvant Breast and Bowel Project (NSABP, Protocol B47), which is designed to evaluate the clinical benefit for adjuvant trastuzumab in women with breast cancers that are HER2−1− or −2+ by IHC and without HER2 gene amplification. Our findings are further supported by a recent neoadjuvant trastuzumab study, in which 89% of women with breast tumors that were classified as HER2− by classical criteria had circulating tumor cells (CTC) that expressed HER2. Furthermore, in these patients neoadjuvant trastuzumab treatment significantly reduced the HER2-expressing CTC population, an effect associated with a significant increase in disease-free survival (29).

Our studies also show that HER2 expression is regulated by the tumor microenvironment. Previous studies examining discordance between HER2 expression in primary and metastatic tumors have suggested a discordance rate of 8% to 50% (14) including loss of HER2-amplification following trastuzumab-based neoadjuvant therapy (37, 38). We show that MCF7 cells growing in mouse tibias express higher levels of HER2 compared with those growing in the mammary fat pad. Increased HER2 expression in bone metastases was not due to gene amplification as assessed by FISH, suggesting that HER2 expression is regulated by the bone microenvironment. We also provide evidence that osteoblast generated RANKL may play a role in this regulation. RANK mediates NF-κB signaling has been previously shown to be able to directly regulate HER2 expression (39) driving CSCs in MMTV-HER2 mouse breast cancer (40). In addition, progesterone-induced RANKL drives mammary stem cell self-renewal through NF-κB activation (41, 42). Interestingly HER2 also activates NF-κB signaling through Akt-mediated IκB phosphorylation (43) generating a positive feedback loop. These results suggest that the RANKL inhibitor denosumab, already approved for the treatment of bone metastasis, may also target CSCs by blocking RANKL-induced HER2 expression. It has been reported that approximately 30% of women with early-stage breast cancer harbor occult micrometastasis in their bone marrow at the time of diagnosis, a state associated with a worse prognosis (44). These micrometases have been found to be highly enriched in cells expressing CSC markers (45). The ability of trastuzumab to target occult bone marrow metastasis in both HER2-amplified and -nonamplified breast cancer may also play a role in reducing tumor recurrence in the adjuvant setting (illustrated in ref. Fig. 7B).

These studies have important implications for the development of clinical trials using HER2-targeting agents by suggesting that a much larger group of women with breast cancer may benefit from HER2 blockade in the adjuvant setting than currently receive these treatments. Our data also support a CSC model in which a subpopulation of cells with stem cell properties mediates tumor recurrence. If this is the case then effective adjuvant therapies will need to successfully target this CSC population.

Disclosure of Potential Conflicts of Interest
H. Korkaya has commercial research grant from MedImmune. M.S. Wicha has commercial research grant from Dompe and ownership interest (including patents) in Oncomed Pharmaceuticals and is a consultant/advisory board member of Verastem and Paganini. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K.C. Day, F. Malik, Q. Zen, S.J. Dawsey, A.A. Quraishi, K.W. Ignatowski, A. Davis, C.L. Hall, N. Palaisamy, A.N. Heath, N. Tawakkoli, T.K. Luther, S.G. Clouthier, D.G. Kleer, D.G. Thomas, D.F. Hayes, H. Korkaya

Acknowledgments
The authors thank Dr. Stephen Ethier for supplying SUM159 cells, Nancy McAnish, Alan Burgess, and Anosike Nwokeoye for their technical help in pathologic examinations, and Denise Pourier for her tireless effort and UMMCC Core facilities. The authors also thank the Indo-US fellowship (F. Malik).

Grant Support
This work was supported by NIH grants, CA129765 and CA101860, the Breast Cancer Research Foundation, Komen for the Cure, the Taubman Institute, and Fashion Footwear Charitable Foundation of New York/QVC Presents Shoes-On-Loan. M.S. Wicha is supported by a Stand Up To Cancer Dream Team Translational Research Grant, a Program of the Entertainment Industry Foundation (SU2C-AACR DT0409).

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Received August 22, 2012; revised November 28, 2012; accepted December 14, 2012; published OnlineFirst February 26, 2013.

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