HER2 Overexpression Elicits a Proinflammatory IL-6 Autocrine Signaling Loop That Is Critical for Tumorigenesis

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Abstract

HER2 overexpression occurs in approximately 25% of breast cancers, where it correlates with poor prognosis. Likewise, systemic inflammation in breast cancer correlates with poor prognosis, although the process is not understood. In this study, we explored the relationship between HER2 and inflammation, comparing the effects of overexpressing wild-type or mutated inactive forms of HER2 in primary human breast cells. Wild-type HER2 elicited a profound transcriptional inflammatory profile, including marked elevation of interleukin-6 (IL-6) expression, which we established to be a critical determinant of HER2 oncogenesis. Mechanistic investigations revealed that IL-6 secretion induced by HER2 overexpression activated Stat3 and altered gene expression, enforcing an autocrine loop of IL-6/Stat3 expression. Both mouse and human in vivo models of HER2-amplified breast carcinoma relied critically on this HER2–IL-6–Stat3 signaling pathway. Our studies offer the first direct evidence linking HER2 to a systemic inflammatory mechanism that orchestrates HER2-mediated tumor growth. We suggest that the HER2–IL-6–STAT3 signaling axis we have defined in breast cancer could prompt new therapeutic or prevention strategies for treatment of HER2-amplified cancers. Cancer Res; 71(13); 4380–91. ©2011 AACR.

Introduction

Breast cancer is a heterogeneous disease classified into subtypes on the basis of gene expression profiles or biomarker expression (1). A subtype overexpressing HER2 accounts for approximately 25% of breast cancers, and therapeutics targeting HER2, such as trastuzumab and lapatinib, have shown clinical efficacy (2). However, because many tumors are resistant either de novo or following therapy, it remains critical to fully understand the molecular and cellular changes elicited by HER2 overexpression during oncogenesis (2–5).

HER2 overexpression has been shown to activate multiple signaling complexes (2–4), which results in a striking dysregulation of the global transcriptome (5). Although these studies have provided a framework for HER2-mediated signaling, the pathways and gene targets critical to HER2 oncogenesis remain incompletely understood. Recent studies have shown that inflammatory pathways and genes [such as interleukin-6 (IL-6) and IL-8] are strongly upregulated by several different oncogenes and are critical to their transformative capacity (6–9). Of note, rat ErbB2 (HER2 homolog) transgenic animals develop tumors with inflammatory patterns by gene expression profiling (10, 11), and these patterns correspond to the proinflammatory pattern of gene expression found in human tumors. Furthermore, clinical studies have shown the activation of inflammatory genes within breast cancer biopsies, whereas several circulating inflammatory cytokines have been found in the serum of breast cancer patients (12–14), with high levels of IL-6 and IL-8 associated with a poor prognosis (12, 13, 15–18).

To investigate whether HER2-mediated signaling could elicit inflammation critical for oncogenesis, we compared gene expression patterns of cells overexpressing wild-type HER2 to a kinase-inactivated HER2 (19). We documented that HER2 overexpression consistently elicited an inflammatory transcriptional signature, including marked elevation of IL-6 expression, which was required for HER2-mediated transformation. HER2-mediated secretion of IL-6 triggered Janus-activated kinase 1 (JAK1)–Stat3 signaling in an autocrine manner, resulting in amplified IL-6 activation of Stat3 in HER2+ cells and, significantly, enhanced HER2-mediated transformation. These findings were confirmed in the MMTV-neu mouse model and a human HER2-amplified breast carcinoma. In sum, we show that HER2 overexpression initiates a HER2–IL-6–Stat3 signaling loop required for HER2-mediated oncogenesis, providing a possible molecular basis for the clinical and pathologic inflammatory markers seen in breast cancer patients. This suggests that IL-6 targeted...
therapies could have significant impact on HER2-overexpressing cancer prevention or therapies.

Materials and Methods

Cell lines
Tumor cell lines MCF-10a, MCF-7, 4T1, and 3T3 were obtained from the American Tissue Culture Collection. KPL-4 cells were obtained from the originator, Dr. Kurebayashi (Kawasaki Medical School, Kurashiki, Japan; ref. 20). Human mammary epithelial cells (HMEC) were obtained from Dr. Jeffrey Marks (Duke University, Durham, NC). The 4T1 and 4T1–HER2 cells were obtained from Dr. Michael Kershaw (Cancer Immunology Program, Peter MacCallum Cancer Centre, Victoria, Australia), and all cell lines were validated and tested for contamination by the Duke University Tissue Culture Facility (21).

Adenoviral vector, plasmid, and cell line construction
Adenoviral vectors encoding HER2 and HER2ki were generated as previously described (19). HER2+ cell lines were created through retroviral infection with HER2-expressing vectors. Stat3-Luc reporter cell lines were created by using a lentiviral reporter (SABiosciences). The NF-kB luciferase reporter was purchased from Stratagene, whereas AP-1 and C/EBP reporters were purchased from SABiosciences. Knockdown of JAK1, IL-6, and Stat3 genes was achieved by using retroviral and lentiviral RNA interference constructs purchased from Open Biosystems. A human Stat3 knockdown GFP–expressing lentivirus was kindly provided by Dr. Jageline Bromberg (Memorial Sloan-Kettering, New York, NY).

Microarray and quantitative real-time PCR assessments
RNA was extracted by using TRI-Reagent and RNAzol (Molecular Reagents Center, Madison, WI) and purified with an RNeasy Kit (Qiagen). Microarray analysis was conducted with GeneSpring 7.3 and GX10 (Affymetrix) by using datasets deposited at Gene Omnibus Express (GEO) of the National Center for Biotechnology Information (accession numbers GSE13274 and GSE25282). Datasets were analyzed by using the Database for Annotation Visualization and Integrated Discovery (DAVID) by using standard methods (22). MicroRNA (miRNA) arrays were processed from TRI-Reagent cellular extracts as previously described (23). Quantitative real-time PCR (qRT-PCR) was done on an ABI 7300 system by using standard methods and intron spanning primers. Expression differences were assessed by using the comparative cycle threshold (Ct) method against several control genes (GAPDH, β-actin, HMBS, RN18S, and Rpl13).

In vitro assays and assessments
Proliferation was determined by MTT assay, whereas soft agar assays were done as described (19). Propidium iodide (PI) staining was conducted by fixing cells in 95% EtOH, staining with PI, and assessing DNA content by flow cytometry on a FACScalibur (BD). Luciferase experiments were conducted by transfecting reporters or using stable reporter cell lines and normalizing luminescence with LacZ controls by using a β-Galactosidase Kit (Stratagene) or Renilla-transfected controls using a Dual-Luciferase Assay (Promega). ELISAs for IL-6 were done with IL-6 ELISA kits from Biolegend. Kinase inhibitors were purchased from Enzo Life Sciences and Marligen. Western blotting was done using standard methods with antibodies from Cell Signaling Technology and Abcam.

Mouse experiments
Experiments using BALB/c, NOD CB17-Prkdc SCID/J, and FVB/N-Tg(MMTVneu)202Mul/J mice (obtained from Jackson Labs) were conducted with Duke University Institutional Care and Use Committee–approved protocols. For HER2 measurement of tumors, excised tumors were enzymatically digested as described below and measured by using a HER2-PE labeled antibody (BD Biosciences). For xenograft experiments, cells were injected s.c. into the flank of nonobese diabetic severe combined immunodeficient (NOD/SCID) mice (at indicated concentrations) measured by using calipers with volumes calculated by the formula \[v = \text{width} \times \text{width}^2 / 2\]. For live imaging experiments, mice were anesthetized with the use of isoflurane, injected with 2.85 mg luciferin (in 100 μL of dH2O), and monitored with a Xenogen IVIS 100 in vivo bioluminescence imaging system. Statistical differences were calculated with a mixed effects regression model using autoregressive covariance. Excised tumors were digested into single cell suspensions using a mix of collagenase (1 mg/mL), DNase (20 U/mL), and hyaluronidase (100 μg/mL) at 37°C for 3 to 5 hours, run through a cell strainer (80 μm; BD), and cultured under standard conditions.

Results

Overexpression of HER2 elicits the activation of a broad inflammatory profile that includes IL-6
Although we have previously reported that global HER2-mediated gene expression changes were dependent on HER2 phosphorylation (19), we now report that a high proportion of these significantly affected genes \((P < 0.05, >3$-fold expression difference) are inflammatory type genes (53 of 424 probesets, ~12.5%; Fig. 1A) that require HER2 overexpression and phosphorylation for their overexpression (Fig. 1A). Concurrent examination of the miRNA profile also revealed significant differences in a cluster of miRNAs (Fig. 1B), which included the expression of different let-7 isoforms, recently shown to affect IL-6 expression (Fig. 1B; ref. 7).

We also investigated HER2-induced inflammatory gene expression in immortalized (MCF-10a) and transformed (MCF-7) human breast cells. We found that HER2 overexpression significantly induced the expression of specific inflammatory genes across different types of human breast cells (Fig. 1C), as well as murine fibroblasts (NIH/3T3) and transformed murine mammary tumor cells (4T1; Fig. 1C). As in human cells, expression of HER2 elicited significant activation of inflammatory gene expression, indicating that HER2 induction of inflammatory gene transcription is independent of species and cell type.

We next examined the impact of HER2 expression on IL-6 protein expression and secretion, showing that supernatants
HER2-mediated upregulation of IL-6 is dependent upon the parallel activation of multiple signaling pathways that activate several IL-6 transcription factors

To identify HER2–IL-6 responsive pathways, we focused on known downstream kinases and transcription factors. We exposed HER2-expressing MCF-10a cells (MCF-10a-HER2) to a variety of specific kinase inhibitors and assessed IL-6 secretion, finding that specific inhibition of mitogen-activated protein kinase (MAPK), c-jun NH kinase (JNK), phosphoinositide 3-kinase (PI3K), Akt, and Src reduce secretion of IL-6 (Fig. 2A). Although PKC inhibition reduced IL-6 secretion, inhibition of the mTOR pathway actually enhanced the HER2-mediated secretion of IL-6. Inhibition of other kinases, such as GSK3β, had no effect on the level of HER2-mediated IL-6 secretion (Fig. 2A, data not shown). To investigate IL-6 transcriptional regulation, we used luciferase reporters for the dominant transcription factors present in the IL-6 promoter complex (NF-κB, AP-1, and C/EBP). In MCF-10a cells, we found that although HER2 strongly induced NF-κB and AP-1 reporters, it had no effect on C/EBP expression (Fig. 2B). However in 3T3 cells, HER2 expression induced the 3 dominant transcription factors (NF-κB, AP-1, and C/EBP), suggesting that HER2 induction of NF-κB and AP-1 is cell type independent but that C/EBP induction may be cell type dependent (Fig. 2C). As NF-κB was strongly induced in both cell types, we directly assessed the importance of NF-κB in HER2-mediated IL-6 secretion through pharmacologic disruption of NF-κB signaling in MCF-10a–HER2 cells and found a dose-dependent inhibition of IL-6 secretion (Fig. 2D). Collectively, these results showed that HER2 overexpression activates multiple pathways which synergistically result in the secretion of IL-6 through the activation of multiple transcription factors (Fig. 2E).

Secretion of IL-6 is required for HER2-mediated transformation and tumor growth in vivo

To determine whether IL-6 secretion was required for HER2-mediated transformation, we inhibited IL-6 expression in 3T3–HER2 transformed cells by stable IL-6KD (Supplementary Fig. S1) and assessed in vivo growth in NOD/SCID mice. IL-6 inhibition significantly attenuated in vivo tumor growth (Fig. 3A and B) and 3T3–HER2–IL-6KD tumors that eventually developed had reacquired baseline IL-6 expression (compared with control 3T3–HER2 cells; Fig. 3C). In addition to the significant role IL-6 has in HER2-mediated transformation, we also investigated its role in the behavior of transformed mammary cells. In transformed 4T1 mammary carcinoma cells, we found that overexpression of HER2 (4T1–HER2) yielded a significant in vivo growth advantage compared with non-HER2-expressing 4T1 cells (Fig. 3D, data not shown), which could be inhibited by blocking IL-6 expression (Supplementary Fig. S1), suggesting that IL-6 also plays a key role in HER2 facilitated growth in transformed cells.

HER2-induced secretion of IL-6 can act in an autocrine fashion to elicit Stat3-mediated gene expression and signaling

We next determined whether IL-6 had autocrine effects on HER2-transformed cells in vitro. In vitro assessment of...
cellular proliferation revealed no difference in growth or cell-cycle changes between control and IL-6KD 3T3–HER2 cells (Fig. 4A), nor were any differences detected between these cell types in cell-cycle regulation (Fig. 4B). However, studies of anchorage-independent growth revealed significant growth attenuation by inhibition of IL-6 expression, thus signifying the importance of autocrine IL-6 signaling (Fig. 4C). We thus focused on Stat3, the dominant transcription factor induced by IL-6. Using a lentiviral Stat3 luciferase reporter, we found that HER2 expression significantly induced the activation of Stat3 compared with control 3T3 cells and, furthermore, that inhibition of IL-6 expression ablated Stat3 induction (Fig. 4D). These results were specific for IL-6 induction of Stat3, as tandem investigations using transient transfection revealed that HER2-mediated activation of Stat3, but not Stat1, was dependent upon IL-6 secretion (Supplementary Fig. S2). To further elucidate and confirm that IL-6 activation of Stat3 was mediated by an IL-6–IL-6R–IL6ST signaling complex via JAK kinases, we stably expressed a mutant IL6ST receptor and inhibited JAK1 expression in 3T3–HER2 Stat3–luciferase cells (Supplementary Fig. S3). In the absence of exogenous IL-6 stimulation, inhibition of IL6ST, JAK1, or Stat3 in 3T3–HER2 cells, all significantly inhibited Stat3 activation (Fig. 4E), as previously shown by the inhibition of IL-6 expression itself (Fig. 4D). Notably, in the presence of...
exogenous IL-6 stimulation, we also found that inhibition of these signaling nodes critically inhibited Stat3 induction (Fig. 4E).

We next assessed the role of IL-6 on the expression of other inflammatory genes in 3T3, 3T3–HER2, and 3T3–HER2–IL-6KD cells by qRT-PCR and found that IL-6 inhibition did not affect certain genes such as c-myc and COX2 but that the expression of other genes was significantly attenuated (Fig. 4F). In particular, we had noted that MMP1 was significantly enhanced by IL-6 secretion, so we examined several other MMP genes known to play a role in oncogenesis (ref. 24; Fig. 4G). We found that multiple MMP genes were significantly affected by inhibition of IL-6 secretion, thus showing that HER2-mediated IL-6 secretion elicits autocrine activation of Stat3, perturbing cellular gene expression.

As previous studies have illustrated IL6ST–HER2 interactions in different cell types, we also sought to determine whether HER2 expression could enhance autocrine IL-6-mediated signaling (3, 25). Treatment of 3T3 and 3T3–HER2 cells revealed a nearly identical time course of activation, but at early time points, Stat3 appeared more phosphorylated in HER2-expressing cells in comparison with controls (Fig. 4H). Identical IL-6 treatment of 3T3–HER2–JAK1KD cells confirmed that the enhanced Stat3 activation was being achieved through a JAK1-dependent pathway in 3T3–HER2 cells and not by alternative mechanisms (Fig. 4H). To quantify Stat3 induction, we stably infected cells with Stat3–luciferase reporters, selected 3T3, and 3T3–HER2 cells that had equivalent basal activation of Stat3 (Fig. 4I) and found that IL-6 treatment activated Stat3 signaling to a significantly greater extent in HER2-expressing cells. As 3T3 cells had minimal expression of IL-6Rα (by fluorescence-activated cell sorting, data not shown), we hypothesized that HER2 amplification of IL-6–Stat3 signaling could be potentially abrogated by greater IL-6Rα expression. To test this hypothesis, we overexpressed...
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IL-6Rα in both 3T3 and 3T3–HER2 Stat3-luciferase cells and found that Stat3 activation was again enhanced in HER2-expressing counterparts (Fig. 4I). Furthermore, we found that although IL-6Rα expression increased baseline Stat3 signaling in both cell types, it had a significantly greater effect in cells expressing HER2 upon IL-6 addition. These results suggested that, in addition to stimulating IL-6 secretion, HER2 expression enhances the activation of Stat3 signaling by IL-6. Collectively, these data showed that HER2 expression plays a critical dual role in the activation of an autocrine IL-6–Stat3 signaling complex.

HER2–IL-6 activation of Stat3 significantly enhances tumor growth in vivo

To investigate the importance of HER2–IL-6–Stat3 signaling in vivo, we first assessed the level of phosphorylated Stat3 in extracted tumor tissue. We found that 3T3–HER2 cells displayed modest phosphorylation of Y705 in vitro, whereas in vivo samples from 3T3–HER2 tumor displayed much higher levels of phosphorylated Stat3 (Fig. 5A). Notably, they also displayed a different pattern of activation in multiple forms of phosphorylated Stat3 as well as different isoforms of unphosphorylated Stat3 (data not shown). Having observed significantly enhanced Stat3 phosphorylation...
in whole tumors \textit{in vivo}, we next wanted to determine whether IL-6 could mediate autocrine activation of Stat3 specifically within 3T3–HER2 tumor cells \textit{in vivo}. We constructed 3T3–HER2 and 3T3–HER2–IL-6KD cell lines with either a Stat3–Luc reporter or LacZ control reporter. When these cells were implanted in mice, striking differences were noted in the level of Stat3 activation 14 days post-treatment (Fig. 5B, Supplementary Fig. S5) and compared \textit{in vitro} conditions. E, the indicated types 3T3–HER2 cells (1 × 10^5) were implanted via s.c. injection into NOD/SCID mice and tumor volume measured over time (n = 5, bars, SE). *, P < 0.05; **, P < 0.01 in comparison to controls.

**Figure 5.** IL-6 activation of Stat3 is critical for HER2-mediated growth \textit{in vivo}. A, total cell extracts from 3T3 and 3T3–HER2 (from \textit{in vitro} cultures or xenografts biopsies) were subjected to Western analysis to determine total and phosphorylated Stat3 expression (pY705). B, 3T3–Stat3–Luc–HER2 and 3T3–Stat3–Luc–HER2–IL-6KD cells (1 × 10^5) were implanted in mice and Stat3 activation assessed by Xenogen luciferase imaging 11 days post-treatment (n = 5; 2–3 representative mice are shown). C, whole cell lysates from 3T3–Stat3–Luc–HER2–LacZ cells which were implanted in mice (sacrificed 24 days post-treatment) or passaged (for corresponding 24 days) were assessed for Stat3 activation, normalized to LacZ expression (samples from individual replicates shown, bars, SD). D, qRT-PCR from 3T3, 3T3–HER2, and 3T3–HER2 xenografts (sacrificed at 25 dpi) to determine MMP gene expression (n = 4; bars, SD). *, P < 0.05; **, P < 0.01 between \textit{in vitro} and \textit{in vivo} conditions. E, the indicated types of Stat3KD and control 3T3–HER2 cells (1 × 10^5) were implanted via s.c. injection into NOD/SCID mice and tumor volume measured over time (n = 5, bars, SE). *, P < 0.05; **, P < 0.01 in comparison to controls.

\textbf{ErbB2 induction of IL-6 plays a critical role in an endogenous model of ErbB2-mediated oncogenesis}

The MMTV-neu mouse model spontaneously develops mammary carcinomas dependent upon expression of activated ErbB2 (the rat homolog of HER2). Using published microarray datasets of developing MMTV-neu tumors (26), we found that a significant portion of genes were dysregulated in ErbB2+ tumors in comparison with control mammary gland tissue (Fig. 6A, ∼5% 309 probes with P < 0.05, >3 fold), of which approximately 10% (31 of 309 probesets) had
mice and tumor volume measured over time (n = infected or uninfected MMTV-neu of MMTV-neu fluid contained significant amounts of IL-6 (Fig. 6D). Exposure to ErbB2-expressing murine breast cancer (4T1; Fig. 6D). MMTV-neu tumors and compared these with a transformed non-ErbB2 expressing murine mammary gland tissue revealed tumor Stat3 activation, further confirming these findings (Fig. 6B), revealing strong induction of immune-related functions. Quantitative RT-PCR analysis confirmed these findings (Fig. 6B), revealing strong induction of several relevant inflammatory mediators including IL-6, Stat3, and SOCS2. Western blots of control and transformed MMTV-neu mammary tissue revealed tumor Stat3 activation, further confirming this IL-6 inflammatory phenotype (Fig. 6C).

Although IFN and inflammatory signatures have been reported in MMTV-neu tumors (10, 11), we focused on IL-6 expression in tumor cells and biofluid from multiple MMTV-neu tumors and compared these with a transformed non-ErbB2-expressing murine breast cancer (4T1; Fig. 6D). MMTV-neu tumor cells secreted high levels of IL-6, and peritumoral fluid contained significant amounts of IL-6 (Fig. 6E). Exposure of MMTV-neu tumor cells to ErbB2 inhibitors ablated IL-6 secretion (Fig. 6E), and IL-6KD MMTV-neu tumor cells were significantly growth attenuated compared with control infected or uninfected MMTV-neu cells (Fig. 6F). Our findings thus showed that endogenous ErbB2 expression supports an inflammatory phenotype, typified by IL-6 secretion, which plays an important role in MMTV-neu mammary tumor growth in vivo.

ErbB2-mediated IL-6 expression in human tumor cells causes Stat3 activation and facilitates oncogenic growth

To ascertain the relationship between spontaneously amplified ErbB2 and IL-6 secretion in human cells, we used the human KPL-4 breast cancer line, which overexpresses HER2 and secretes IL-6. When HER2 was stably knocked down, we found a significant, but not complete reduction of IL-6 secretion (Fig. 7A and Supplementary Fig. S7). As the high endogenous HER2 expression in KPL-4 cells was not completely knocked down by short hairpin RNA (Supplementary Fig. S7), we next used pharmacologic inhibition of HER2 (Fig. 7B), which resulted in a near complete ablation of IL-6 expression, showing the importance of HER2 signaling in promoting IL-6 secretion in HER2-expressing tumor cells.

KPL-4 cells were then stably infected with Stat3-luciferase reporters and then treated with IL-6 in tandem with HER2 kinase inhibitors to assess Stat3 activation (Fig. 7C). These studies revealed that HER2-inhibited cells had lower basal levels of Stat3 activation, correlating with their lower levels of IL-6 secretion (Fig. 7C). More significantly, we found that high
concentrations of IL-6 were not able to activate Stat3 in HER2-inhibited cells, suggesting that HER2 plays a prominent role in the IL-6-mediated activation of Stat3. These studies used levels of IL-6 (10 ng/mL) that approximated levels that we found in pleural effusions from breast cancer patients (Supplementary Fig. S8). When KPL-4–Stat3–Luc cells were directly exposed to malignant pleural effusions, we again observed significant activation of Stat3 (Fig. 7D), which

Figure 7. HER2-mediated secretion of IL-6 in human mammary carcinoma cells is critical for Stat3 activation and in vivo tumor growth. A, KPL-4 cells (endogenously HER2+) were knocked down for HER2 expression and IL-6 secretion assessed (n = 6; bars, SD). B, KPL-4 were treated with HER2 inhibitors (10 μmol/L) or DMSO and IL-6 secretion assessed at 24 hours (n = 4; bars, SD). C, KPL-4–Stat3–Luc stable cells that were exposed to varying concentrations of IL-6 in the presence or absence of 10 μmol/L lapatinib for 24 hours and Stat3 activation assessed (n = 6; bars, SD; *, P < 0.05; **, P < 0.01 from DMSO control–treated KPL-4 and KPL-4–IL-6 counterparts). D, KPL-4–Stat3–Luc stable cells were exposed to varying concentration of IL-6 (as indicated) and pleural effusion fluid (consisting of increasing concentrations of 0, 0.25%, 2.5%, and 25% of total media volume indicated by increasing bars) for 24 hours, after which Stat3 activity was quantified by luciferase assay (n = 6; bars, SD). E, KPL-4–Stat3–Luc cells were treated with 10 ng/mL of IL-6 and PEF (at a 10 ng/mL IL-6 concentration, which represented 25%, 58%, and 100% of respective PEF concentrations), along with either mock treatment with 1 ng/mL of an anti-IL-6 neutralizing antibody or 1 μg/mL of a control immunoglobulin G (IgG) antibody. After a 24-hour incubation, Stat3 activity was quantified by luciferase assay (n = 6; bars, SD; *, P < 0.05; **, P < 0.01 from IL-6–IgG–treated counterparts). F, the indicated types of modified KPL-4 cells (5 × 10⁵) were implanted via s.c. injection into NOD/SCID mice and tumor volume measured over time (n = 5; bars, SE). *, P < 0.05; **, P < 0.01 in comparison with untreated controls.
was inhibited by addition of neutralizing IL-6 antibody (Fig. 7E).

Finally, to determine whether HER2-mediated expression of IL-6 was critical for the growth of human HER2⁺ breast carcinomas in vivo, both IL-6 and Stat3 were stably knocked down in KPL-4 cells, which were then implanted in mice (Fig. 7E, Supplementary Fig. S9) and assessed for tumor growth. The growth of Stat3KD cells was significantly inhibited, whereas IL-6KD cells displayed the most dramatically inhibited tumor growth, again suggesting that both autocrine and paracrine modes of IL-6 signaling likely play important roles in human tumor growth.

Discussion

Although oncogenes such as Ras, src, myc, and EGFR are known to trigger inflammatory pathways critical for oncogenesis (6–9, 27), the relationship between HER2/neu and inflammation had previously been speculative (28). In this study, we documented that HER2 overexpression activates multiple inflammatory pathways, including the secretion of IL-6, which we identify as critical for HER2-mediated transformation. We found that several pathways downstream of HER2 synergistically affected IL-6 expression and showed that secreted IL-6 elicited autocrine Stat3 activation. We also found that Stat3 activation was enhanced in HER2-expressing cells and associated with cellular transcriptional changes, as well as anchorage-independent growth. Studies with endogenously arising ErbB2 tumors also revealed that ErbB2–IL-6–Stat3 activation enhances tumor growth, signifying that these phenomena were not limited to a cellular model of HER2-mediated transformation. Likewise, investigation of a human breast carcinoma line with amplified HER2 also showed that HER2-mediated IL-6 expression was critical for autocrine Stat3 activation, signaling amplification, and human tumor growth in vivo. In sum, these experiments reveal that HER2 activation and amplification of autocrine IL-6–Stat3 signaling are critical to its oncogenic capacity.

We found that inflammatory related genes encompass approximately 10% of the most significant transcriptional changes induced by the overexpression of HER2 and that this inflammatory transcriptional response occurs in various cell types at different stages of transformation. The inflammatory effect on cellular properties is likely dependent upon cellular context as oncogene-induced inflammatory pathways (such as IL-6) can lead to autocrine-induced cellular senescence in nonimmortalized cells (29), whereas inflammatory genes can enhance cellular oncogenicity in tumor cells (9, 30–33). In addition, inflammatory responses can influence other cells (such as fibroblasts, adipocytes, or immune cells) and modulate tumor-mediated immunity.

Our study is the first to show that overexpression of kinase active, but not inactive, HER2 induces IL-6 secretion and is thus dependent upon HER2 phosphorylation and preservation of multiple signaling pathways downstream of HER2. HER2 activation correlated with NF-κB and AP-1 activation, and NF-κB was critical to IL-6 expression. These findings are similar to those observed in the RAS-mediated activation of IL-8 (9), which we also found to be induced by HER2, suggesting that oncogene-mediated cytokine gene expression is dependent on multiple coordinated signaling pathways. Although this does not exclude the influence of other factors in the activation of IL-6 (such as let-7 involvement; ref. 7), it shows that interference with many signaling nodes downstream of HER2 can perturb IL-6 expression and thus implies the possibility of therapeutic intervention against HER2-mediated IL-6 secretion at multiple levels.

Our investigation also revealed that IL-6 secreted in response to HER2 expression was critical for HER2-mediated transformation and activation of Stat3 in vitro and in vivo, a finding corroborated by other studies that show IL-6 mediation of transformative properties in mammary epithelial and tumor cells (7, 33). Collectively, these findings suggest that HER2–IL-6–Stat3 activation is a critical component of HER2-mediated oncogenesis, although a full evaluation of Stat3-mediated effects may vary on the basis of cell type. Notably, we found that HER2 plays an additional role in the IL-6–Stat3 signaling axis, through the amplification of Stat3 signaling after IL-6 treatment. Although the exact nature of this role is unknown, previous studies have documented the involvement of HER2 with the IL6ST receptor (3, 25), suggesting that HER2 expression on the cell surface could be an important part of the IL-6–IL6ST–IL-6Rα complex. As such, HER2 could play a critical dual role in this pathway acting as an initiator and amplifier of cellular IL-6 signaling. However, it should also be noted that in multiple contexts, our knockdown of Stat3 did not fully recapitulate the suppression of tumor growth achieved with IL-6 knockdown.

We found that Stat3 was more highly activated in tumor cells in vivo in comparison with identical cells in vitro, consistent with the high levels of activated Stat3 reported in different types of tumor biopsies (34, 35). Although we found that tumor cell Stat3 activation was directly associated with tumor cell IL-6 expression in vitro and in vivo, stronger Stat3 activation in vivo could be a product of infiltrating cells as well as environmental stimuli that would provide additional sources or stimulations to permit Stat3 activation. For instance, the presence of high levels of soluble IL-6Ra in vivo (36) could permit IL-6 trans signaling in tumor cells, as IL-6Ra could be a limiting activating factor in certain cell types. Although it is unclear whether pharmacologic HER2 inhibition could alleviate Stat3 activation in vivo, our data suggest that such an approach may provide Stat3 suppression through inactivation of HER2-mediated IL-6 secretion, as well as abrogation of HER2-mediated enhancement of IL-6–Stat3 signaling. In sum, our finding of enhanced Stat3 signaling in HER2⁺ tumor cells in vivo supports the importance of Stat3 activation in tumor cell populations in clinical settings.

Finally, although multiple studies have shown IL-6 expression in breast cancer patients and linked expression with certain subsets and grades of malignancy (12, 14, 37, 38), the source and mechanisms generating IL-6 in cancer patients has been undetermined. Likewise, other studies have determined that many breast cancers have activated Stat3, although the activators and significance of Stat3 in these...
tumors remains unknown (34, 39). Our study shows that HER2 overexpression activates a transcriptional inflammatory profile, which includes the significant secretion of IL-6 in multiple cell types, as well as in a mouse model of ErbB2 overexpression and in a human HER2+ breast carcinoma line. We further found that secreted IL-6 was critical for HER2-mediated oncogenesis and was mediated by autocrine activation of Stat3 in tumor cell populations, which was enhanced by cellular HER2 expression and in in vivo contexts. Thus, our findings show a potential origin and mechanism for IL-6 expression and its relevance to breast cancer progression. Although further study of HER2-mediated inflammation is needed, these findings suggest that therapeutic targeting of IL-6–Stat3 activation could augment existing prevention strategies and treatments of HER2+ cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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