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STAT1 gene expression is enhanced by nuclear EGFR and HER2 via cooperation with STAT3

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Abstract

Both EGFR and HER2 are important mediators of tumorigenesis and tumor progression. Despite their best characterized roles as plasma membrane-bound receptors, both receptors undergo nuclear translocation though the impact of this process remains unclear. In this study, we provide evidence showing that EGFR upregulates expression of signal transducer and activator of transcription 1 (STAT1), a transcription factor responding to inflammatory signals and regulates genes involved in inflammatory response. EGFR regulation of STAT1 expression is primarily attributed to the nuclear activity of EGFR. The oncogenic transcription factor STAT3 binds to the STAT1 promoter and synergizes with nuclear EGFR to significantly enhance STAT1 gene expression. Structural characterization of the human STAT1 gene promoter indicates the presence of four functional STAT3-binding sites in the promoter and their importance in STAT1 co-regulation by EGFR and STAT3. The constitutively activated EGFR variant, EGFRvIII, also cooperates with STAT3 to activate the STAT1 gene promoter through the identified STAT3-binding sites within the promoter. Using human breast cancer cell lines, we further found a positive association between levels of STAT1, EGFR and p-STAT3. STAT1 expression is transcriptionally upregulated by HER2 and heregulin stimulation in breast cancer cells, and the level is further augmented by activated STAT3. In summary, we report in this study that STAT1 expression is upregulated by nuclear EGFR, EGFRvIII and HER2 and that STAT3 synergizes with the three receptors to further enhance STAT1 expression. These novel findings establish a novel link between the mitogenic ErbB signaling pathway and the inflammatory pathway mediated by STAT1.

Keywords

EGFR; EGFRvIII; HER2; STAT3; STAT1; gene regulation

INTRODUCTION

The ErbB family of receptor tyrosine kinases consists of four members, namely, ErbB1 (epidermal growth factor receptor; EGFR), ErbB2 (HER2), ErbB3 (HER3) and ErbB4 (HER4). EGFR and HER2 are frequently overexpressed in human cancers and associated with more aggressive malignant tumor behaviors [1–3]. EGFRvIII, the constitutively activated variant of EGFR, is commonly amplified in glioblastomas and is considered a more tumorigenic form of EGFR [4–7]. EGFR is best known for its canonical function as a receptor tyrosine kinase localized on the plasma membrane that becomes activated upon

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ligand binding. Activated EGFR recruits a number of downstream signaling molecules, leading to the activation of several major pathways that are important for tumor growth, progression, and survival [8–10]. Unlike EGFR, HER2 does not have apparent ligands and relies on heterodimerization with HER3 or EGFR to become activated.

Compelling evidence indicates that all ErbB receptors undergo nuclear translocation [11–16]. Nuclear EGFR has been detected in many different types of cancer cells and tumor specimens, including those of breast [14,17], epidermoid [18,19], bladder [18], ovary [20], oral cavity [14,21], lungs [22], and pancreas [23], and also in malignant gliomas [24,25]. Nuclear EGFR is also present in highly proliferative normal tissues, such as regenerating liver and placenta. Currently available evidence indicates nuclear EGFR to be the full-length receptor that originates from the cell-surface [13,14,17,25]. Although presently available information from our laboratory and other groups has shown that EGFRvIII can be detected in prostate cancer [26] and in malignant gliomas [24,25], analysis for nuclear presence of EGFRvIII has not been extensively conducted. Nuclear HER2 has been detected in breast cancer cells [16,27].

Clinical significance of nuclear EGFR has not been well defined. Nevertheless, current evidence suggests that high nuclear EGFR expression in the tumor nuclei is associated with poor patient survival [28]. This link has been reported in breast carcinomas [14], oral squamous carcinomas [14], oropharyngeal carcinomas [21], and ovarian cancer [20]. Nuclear EGFR is linked to resistance to EGFR-targeted therapy [22,29,30]. As for EGFRvIII, high nuclear expression is associated with poor overall survival of patients with hormone-refractory prostate tumors [26]. Although the molecular and cellular mechanisms underlying these observations are still not well understood, it is known that in the nucleus, ErbB receptors regulate gene expression via their C-terminal transactivation domain and their ability to associate with DNA-binding transcription factors [12,13,16,31,32]. Transcriptional targets of nuclear EGFR that have been identified currently include cyclin D1 [13], inducible nitric oxide synthase [17], B-Myb [19], aurora A [33], c-Myc [23], breast cancer resistance protein [34] and cyclooxygenase-2 (COX-2) [25]. In addition to nuclear EGFR, expression of COX-2 pro-inflammation gene can also be upregulated by nuclear HER2 [16] and nuclear EGFRvIII [25], suggesting a potential link between inflammation and nuclear ErbBs such as EGFR, HER2 and EGFRvIII.

To date, the role of ErbBs in inflammation is still not well characterized despite being suggested by several studies [8,16,35,36]. To explore the potential link between nuclear EGFR and inflammatory pathways, we undertook this current study to investigate the possibility that additional pro-inflammation genes can be regulated by nuclear EGFR. To this end, we focused our efforts on signal transducer and activator of transcription 1 (STAT1), a transcription factor that responds to inflammatory signals and regulates genes involved in inflammatory response [37,38]. This direction was prompted by our recent DNA microarray study [25] that identified STAT1 as one of the candidate genes whose expression was enhanced by wild-type EGFR, but not by mutant EGFR defective in nuclear entry. In this study, we conducted a series of biochemical and genetic validations and the results demonstrated that the human STAT1 gene is a novel transcriptional target of nuclear EGFR. We also provide evidence indicating that EGFRvIII and HER2 can also enhance STAT1 gene expression. Subsequent analyses showed that STAT3, a transcription factor acting downstream of both growth factor- and inflammation-pathways [39], synergizes with EGFR/EGFRvIII/HER2 to significantly upregulate STAT1 expression in human cancer cells. Collectively, our results shed light on the regulatory mechanisms for STAT1 expression and provide evidence linking the ErbB family of receptor tyrosine kinases to STAT1- and STAT3-mediated inflammatory pathway in human cancer and potentially normal cells.

MATERIALS AND METHODS

Cell Lines and Primary Specimens

Human glioblastoma and breast cancer cell lines were obtained from ATCC (Manassas, VA) and were routinely cultured in DMEM supplemented with 10% fetal calf serum and antibiotics. Primary specimens and xenografts of glioblastoma were provided by the Duke University Brain Tumor Center. Stable U87MG human glioblastoma cell lines were established following G418 selection as we previously described [25,40] and were maintained in DMEM supplemented with 700 ug/ml G418 and 10% fetal calf serum (FBS). MDA-MB-231 breast cancer cells were maintained in L-15 medium (Leibovitz) supplemented with 10% FBS. MCF-7 cells were cultured in Eagle's Minimum Essential Medium with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/l sodium bicarbonate, 10% FBS, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, and 10 ug/ml bovine insulin.

Reagents and Chemicals

All chemicals were purchased from Sigma-Aldrich unless otherwise stated. EGF and Heregulin was obtained from EMD. All siRNAs were purchased from Dharmacon Inc. (Lafayette, CO) and the sequences are 5'-GAGAUUGACCAGCAGUAUA-3' (human STAT3 siRNA) and 5'-UGGUUACAUGUCGACUAA-3' (non-targeting control siRNA). Lapatinib was obtained from Selleck Chem (Houston, TX).

Reverse transcription-quantitative PCR (RT-qPCR)

Total RNA isolation was conducted using the SV Total RNA Isolation System (Promega). RT-qPCR was performed in the Mx3005P qPCR System (Stratagene) using the SuperScript III platinum SYBR green one-step qRT-PCR system (Invitrogen), in which GAPDH gene was used as normalization controls. All experiments were in triplicate. The forward and reverse primers used for the PCR were: 5'-CTTACCCAGAATGCCCTGAT-3' and 5'-CGAAGTGTGCTGCAGACTCTC-3' (STAT1), 5'-GGAGGTCAGGTATATGGAACC-3' and 5'-AAAGAGAAGTCTGGGGAAGGT-3' (LMP2), 5'-AAGGAGAAGAGCAGATTCGAG-3' and 5'-AAGGCCCCATCATAACATTAG-3' (vaculin), 5'-TCACAGACGTTCTCGTAAGGA-3' and 5'-TCTTTCCACCCTACCATCTTC-3' (Mcl-1), and 5'-ACTGCCAACGTGTCAGTGGT-3' and 5'-GTGTCGCTGTTGAAGTCAGA-3' (GAPDH).

Western blotting

This was performed as described previously [41,42]. Antibodies used included mouse monoclonal antibodies against α -actin (Sigma), α -tubulin (Sigma) and STAT1 (Santa Cruz; C-136) and rabbit polyclonal antibodies for EGFR (Santa Cruz; C-1005), STAT3 (Santa Cruz; C-20), HER2 (Cell Signaling; 2165), p-EGFR (Y1068/1175; Cell signaling) p-STAT1 (Y701; Cell signaling; 9167) and p-STAT3 (Y705; Cell Signaling; 9145).

Chromatin immunoprecipitation (ChIP) assay to determine binding of nuclear EGFR to the STAT1 gene promoter

This was performed using a ChIP Assay Kit (Upstate, Billerica, MA) as we described previously [41]. Anti-EGFR mouse monoclonal antibody (Neomarkers; Ab-13) and anti-STAT3 rabbit polyclonal antibody (Santa Cruz; C-20) were used in these experiments. Sequences of the primers for amplifying the human STAT1 promoter are 5'-CACGGAGGTCAGTTGCTAAA-3' (forward) and 5'-AGAAGGACGTGCTGTGTTTG-3' (reverse). Normal mouse IgG was used as negative controls for immunoprecipitation. Chromatin input was used as loading control for PCR.

Plasmids, generation of mutant STAT1 promoter reporters via site-directed mutagenesis, and transfections

Construction of the EGFR, EGFRdNLS, EGFRvIII and EGFRvIIIdNLS and STAT3CA expression plasmids was described in our previous studies [25,40]. The STAT1 luciferase reporter construct, pSTAT1-Luc, was purchased from Panomics (Fremont, CA) containing 798 bp human STAT1 promoter (−768 to −29) 5' region of the firefly luciferase reporter gene. Site-directed mutagenesis was performed using QuikChange Mutagenesis Kit (Stratagene), according to the manufacturer's instructions. The pSTAT1-Luc luciferase reporter construct was used as the template. Sequences of the primers used to mutate the STAT1 promoter are 5'-AAGTTTGGGCCGCTGCAAACACAG-3' and 5'-CTGTGTTTGCAGCGGCCAAACTT-3' (pSTAT1-A-Luc), 5'-GTTCCCTGGGTCCAGCAACACG GAG-3' and 5'-CTCCGTGTTGCTGGACCCAGGGAAC-3' (pSTAT1-B-Luc), 5'-AAGAA ACGTGT GGCAAGAACAAGG-3' and 5'-CCTTTGTTCTTGCCACACGTTTCTT (pSTAT1-C-Luc), and 5'-AGCACAGCGTCGAGGAGAAGCCAG-3' and 5'-CTGGGCTTCTCCTCGACGCTGTGCT-3' (pSTAT1-D-Luc). The HER2 WT expression vector [43] was obtained from the Addgene plasmid repository (Cambridge, MA). All transfections were performed with cells in exponential growth using FuGENE HD (Roche) and lipofectamine 2000 (Invitrogen).

Luciferase assay

A Renilla luciferase expression vector, pRL-TK whose expression is controlled by the thymidine kinase promoter was used to control for transfection efficiency. Forty-eight hrs after transfection, the cells were lysed and luciferase activity measured using the Dual Luciferase Assay Kit (Biotium, Hayward, CA) in a TD 20/20 luminometer (Promega). Relative luciferase activity was calculated by normalization of the firefly luciferase activity against that of the Renilla luciferase, as we previously described [44].

Statistical analysis

Student *t*-test and regression analysis was performed using STATISTICA (StatSoft Inc., Tulsa, OK) and Microsoft Excel.

RESULTS

STAT1 gene expression is enhanced by nuclear EGFR

To determine whether STAT1 expression is transcriptionally regulated by EGFR and whether the regulation is via nuclear EGFR, we analyzed the U87MG isogenic cell lines with stable expression of wild-type EGFR and EGFRdNLS mutant for the levels of STAT1 expression. Notably, EGFRdNLS has been characterized in our earlier study to be defective in entering the cell nucleus while EGFR undergoes EGF-induced nuclear translocation [25]. As shown in Fig. 1A, RT-qPCR results indicate that EGFR but not EGFRdNLS enhances STAT1 transcript expression. These observations were confirmed by western blots in Fig. 1B at the protein level. Using intracellular protein:DNA binding ChIP assay (Fig. 1C), we further showed that EGFR, but not EGFRdNLS, associates with the STAT1 gene promoter. In the ChIP assay, IgG was used as the negative controls for immunoprecipitation while the chromatin inputs served as loading controls for PCR. Consistent with the results of the ChIP assay, the luciferase reporter assay (Fig. 1D) indicated that STAT1 gene promoter activity was enhanced by EGF in EGFR-expressing cancer cells but not in those with vector control or EGFRdNLS. Consistent with these results, we further show in Fig. 1E that STAT1 promoter activity is enhanced by ectopic EGFR in two breast cancer cell lines, MDA-MB-231 and MCF-7 cells with low levels of endogenous EGFR. Together, these

results demonstrate that the human STAT1 gene is transcriptionally activated by nuclear EGFR.

EGFR synergizes with STAT3 to activate the STAT1 gene promoter

EGFR contains a transactivation domain [13]. However, we and others have reported that nuclear EGFR does not directly bind to gene promoters but regulates gene expression by physically interacting with DNA-binding transcription factors such as STAT3 [17,19,25,33]. Thus, we determined whether EGFR synergizes with STAT3 to activate the STAT1 gene promoter. As shown in Fig. 2A, transient co-expression of EGFR with constitutively activated STAT3 (STAT3CA) greatly enhanced STAT1 promoter activity in U87MG cells with very low levels of endogenous EGFR. To determine whether this synergy requires nuclear EGFR, we compared the ability of EGFR with that of EGFRdNLS in activating the STAT1 promoter in the presence of STAT3CA. The results (Fig. 2B) showed that EGFR synergizes with STAT3CA to a greater extent than EGFRdNLS in activating the STAT1 promoter. However, noticeably, EGFRdNLS also appeared to cooperate with STAT3CA, suggesting that the cell-surface EGFR-STAT3 signaling axis contributes to STAT1 promoter activity. Altogether, these results indicate that EGFR synergizes with STAT3 to activate the STAT1 gene promoter and that nuclear EGFR plays an important role in this synergy.

STAT3 binding to the STAT1 gene promoter is important for the indirect association of nuclear EGFR with the promoter

We further determine whether STAT3 binds to the STAT1 gene promoter using the ChIP assay with a STAT3 antibody. As shown in Fig. 2C, STAT3 binds to the STAT1 promoter and the degree of binding increases after EGF stimulation. IgG was used as negative controls which did not produce any non-specific signal. We further asked whether STAT3 is required for the observed indirect association of nuclear EGFR with STAT1 promoter (shown earlier in Fig. 1C). To address this question, we knocked down STAT3 expression using siRNA and examined the ability of nuclear EGFR to associate with STAT1 promoter. As shown in Fig. 2D, the results show that STAT3 expression knockdown significantly reduced the binding of nuclear EGFR to the STAT1 promoter, indicating that the association of nuclear EGFR with the STAT1 promoter is dependent on nuclear STAT3. STAT3 knockdown effectiveness is shown in the lower panel of Fig. 2D. These observations, together, indicate that STAT3 binds to the STAT1 promoter and this binding is essential for nuclear EGFR association with the promoter.

Structural characterization of the human STAT1 gene promoter for activation by the EGFR-STAT3 signaling axis

To determine the structural mechanisms underlying activation of the STAT1 gene promoter by the EGFR-STAT3 signaling axis, we searched the promoter for the consensus STAT3-binding site, 5'-TT-N₄₋₆-AA-3'. The results revealed four putative STAT3-binding sites within the 768 bp STAT1 promoter region (Fig. 3A). We then conducted site-directed mutagenesis to mutate and disrupt each of the four STAT3-binding sites. As summarized in Fig. 3A, four constructs with mutant STAT1 promoters were generated and the mutated nucleotides are shown in bold and underlined. The wild-type and mutant reporter constructs were transfected into U87MG cells, with and without EGFR and/or STAT3CA plasmids for 48 hrs and the luciferase activity of each transfectant was determined. As shown in Fig 3B, all four mutant STAT1 promoters showed lower activities compared to the wild-type promoter. Their reduction in activity was observed in cells with EGFR alone, STAT3CA alone and in combination. Among the four mutant STAT1 promoters, the STAT1-B mutant retained most of the wild-type promoter activity. Together, these results indicate that four

STAT3-binding sites are present in the human STAT1 promoter and essential for EGFR/STAT3-mediated transcriptional activation.

EGFRvIII cooperates with STAT3 to activate the STAT1 gene promoter

We further investigated whether the constitutively active EGFR variant, EGFRvIII, activates STAT1 promoter and whether the activation requires cooperation with STAT3. As shown in Fig. 4A, we observed that EGFRvIII alone did not significantly enhance STAT1 promoter activity, but that the activity was greatly increased by the combination of EGFRvIII and STAT3CA. In Fig. 4B, we further showed that EGFRvIIIdNLS had a higher propensity than EGFRvIII to induce STAT1 promoter activation which is in contrast to the observation with EGFR and EGFRdNLS in Fig. 2. This is in contrast to the results indicating that nuclear activity is required for EGFR-mediated STAT1 gene upregulation (Fig. 1). Our explanation for this notable observation is that EGFRvIII's potent cell-surface activity may overpower its nuclear activity. Interestingly, EGFRvIIIdNLS and EGFRvIII showed similar ability to synergize with STAT3CA to activate STAT1 promoter, suggesting that nuclear EGFRvIII is not essential for EGFRvIII/STAT3-mediated activation of the STAT1 promoter. We further investigated the role of the four STAT3-binding sites within the STAT1 promoter for EGFRvIII/STAT3-mediated promoter activation and the results (Fig. 4C) indicate that each of the four sites are required for the synergistic activation by EGFRvIII and STAT3. Collectively, these results indicate that EGFRvIII cooperates with STAT3 to activate the STAT1 promoter and that nuclear EGFRvIII does not play a significant role in this cooperation.

Endogenous associations between STAT1, EGFR and p-STAT3 in breast cancer cells

In light of the ability of ectopic expression of EGFR to synergize with STAT3 to transcriptionally activate STAT1 expression and promoter activity, we further determined whether endogenous levels of EGFR and STAT3 are associated with those of STAT1 in cancer cells. We also examined the levels of p-STAT3 because it is the active form that binds to STAT3-targeted gene promoters. To this end, we examined seven breast cancer cell lines (Fig. 5A) for expression levels of all three proteins and p-STAT3 using western blotting. Importantly, the results indicate that breast cancer cell lines with high EGFR and high p-STAT3 levels expressed higher levels of STAT1 than those with low EGFR and low p-STAT3 levels while (Fig. 5A), indicating a positive association between EGFR/p-STAT3 and STAT1 in these cells. Since EGFR can phosphorylate STAT1, we further determined p-STAT1 (Y701) levels in these cells. However, the levels of p-STAT1 were very low in the cells despite the presence of EGFR in 5 of the 7 lines investigated. We reason that this could be due to the competition between STAT3 and STAT1 for EGFR phosphorylation, which should be investigated further in a future follow-up study. Furthermore, we examined the effects of a clinically used small molecule inhibitor of EGFR/HER2 on STAT1 expression in human MDA-MB-468 cells known to express high endogenous levels of EGFR and STAT3. As shown in Fig. 5B, lapatinib effectively inhibits EGFR activation, leading to a reduction in STAT1 expression. Altogether, consistent with the ability of EGFR to phosphorylate and synergize with STAT3 to upregulate STAT1 expression, our results showed that breast cancer cell lines with high EGFR also express high levels of p-STAT3 and STAT1, and that conversely, a pharmacological inhibitor of EGFR leads to reduced expression of STAT1, indicating endogenous associations between STAT1, EGFR and STAT3 in breast cancer cells.

HER2 and STAT3 synergize to upregulate STAT1 gene expression in breast cancer cells

In addition to EGFR, HER2 also interacts with and activates STAT3 [45]. Nuclear HER2 has been shown to associate with STAT3 to upregulate cyclin D1 gene expression [63]. Thus, we examined whether STAT1 gene expression can be regulated by HER2. As shown

in Fig. 6A, we found that STAT1 gene promoter activity was enhanced by ectopic HER2 in HER2-negative MDA-MB-231 cells independent of heregulin exposure. In MCF-7 cells with endogenous low HER2 expression, STAT1 promoter activity was modestly enhanced by ectopic HER2 but was significantly increased in response to heregulin stimulation. It is worth noting that the overall activity of STAT1 gene promoter is higher in MCF-7 cells (with endogenous HER2 expression) compared to MDA-MB-231 cells (without endogenous HER2), indicating a positive association between STAT1 promoter activity and HER2. In Fig. 6B, we further show in MCF-7 cells that the transcripts of STAT1 and its target genes, LMP1, Mcl-1 and vinculin, were enhanced by ectopic HER2 and heregulin. Expression of these three genes can be enhanced by STAT1, independent of STAT1 phosphorylation status. Consistent with the mRNA data, STAT1 protein levels were enhanced by HER2 overexpression (Fig. 6C). Furthermore, we found that HER2-mediated STAT1 promoter activity was significantly increased by STAT3 (Fig. 6D). Collectively, these results indicate that STAT1 gene expression is activated by HER2 and STAT3 in a synergistic fashion.

DISCUSSION

In this study, we report for the first time that STAT1 gene expression can be enhanced by STAT3 and that the regulation is further augmented in the presence of the ErbB family of receptor tyrosine kinases, namely, EGFR, EGFRvIII and HER2. Our findings characterize STAT1 as a novel nuclear EGFR target gene and a new transcriptional target of STAT3. The results also shed light on the relationship between inflammation and tumorigenesis by linking ErbB-associated oncogenic pathway to STAT1-regulated inflammatory pathway.

The STAT transcription factors are important regulators of many normal and pathological processes, including proliferation, apoptosis, inflammation, tumorigenesis, and tumor progression [46–48]. Both STAT1 and STAT3 have been shown to play an important role in inflammation [49]. In particular, STAT3 is implicated in inflammation-induced tumor initiation and malignant progression [49,50]. Constitutive activation of STAT3 is also a common event in many types of human cancers, including, those of the breast [17,51,52], brain [40,53], prostate [54], ovary [55], colon [56] and others. In agreement with these notions, our results showed that STAT3 is highly expressed in human breast cancer cells and glioblastoma tumors. Interestingly, we further demonstrated that STAT3 upregulates STAT1 expression in cancer cells, suggesting its ability to amplify tumor inflammatory response. This possibility is also supported by the results of our previous studies showing that STAT3 activates the expression of other pro-inflammatory factors, iNOS [17] and COX-2 [25].

Our data indicate a positive association between levels of STAT1 and STAT3 as a result of transcriptional activation of the STAT1 gene by STAT3. This is supported by the observations reported in this study and consistent with the results of previous studies by other groups [57] that both proteins can be overexpressed in human cancer cells. Both STATs can be activated by pro-inflammatory cytokines and form homodimers and heterodimers to regulate gene expression [48,49]. However, it has been shown that the two STAT proteins can elicit opposing effects on tumor initiation, cell growth and death and other cellular processes [48]. In this context, STAT1 has been shown to suppress tumor growth [58] whereas STAT3 is considered an oncogene [47,59]. STAT1 can promote apoptosis [58] whereas STAT3 promotes survival [17,40]. In contrast, STAT1 has also been shown to contribute to gastric tumorigenesis by promoting inflammation [38]. In light of these mixed results, it is likely that in cells expressing both STAT proteins, the STAT1:STAT3 balance is tightly controlled by the growth factors and inflammatory signals and that the state of the balance determines the fate of the cells.

Unlike STAT3, the role of ErbBs in inflammation is still not well characterized. Nonetheless, the results of this current study provide evidence directly linking three ErbB receptors to STAT1, and accordingly, to inflammatory response. In agreement with our observations, EGFR is involved in airway inflammation and asthma [35,60] as well as liver cancer [61]. Conversely, an EGFR kinase inhibitor suppresses airway inflammation [57]. In the case of HER2, it has been shown that UV-induced HER2 activation leads to skin inflammation [62] and that HER2 overexpression activates a pro-inflammatory signaling loop leading to mammary tumorigenesis [36]. Importantly, the results of this study showed STAT1 to be a novel effector of EGFR- and HER2-associated inflammation.

Our results indicate that HER2 induces STAT1 expression and that the induction is enhanced by STAT3. This is in agreement with the fact that HER2 interacts with and phosphorylates STAT3 [45]. However, whether HER2/STAT3-induced STAT1 gene activation is the consequence of the binding of nuclear HER2-STAT3 complex to the STAT1 gene promoter remains to be investigated. This possibility is supported by the observation that HER2 undergoes nuclear translocation after heregulin stimulation [16,27] and that nuclear HER2 has been shown to associate with STAT3 to upregulate cyclin D1 gene expression [63]. Interestingly, HER2 can interact with STAT1, suggesting that they may associate in the nucleus and potentially co-regulate gene expression, like the nuclear HER2-STAT3 complex.

Also important is that our data defined STAT1 as a novel transcriptional target of the nuclear EGFR-STAT3 transcriptional complex. Notably, iNOS [17], COX-2 [25] and Myc [23] can be upregulated by the nuclear EGFR-STAT3 signaling axis. COX-2 expression can also be activated by both the nuclear EGFRvIII-STAT3 signaling complex [25] and by nuclear HER2 [16]. Interestingly, the results in this study suggest that the cell-surface mode of the EGFRvIII pathway may play a more essential role in activating STAT1 expression compared to the nuclear counterpart (Fig. 4B). This is in contrast to the results indicating that nuclear activity is required for EGFR-mediated STAT1 gene upregulation (Fig. 1). Our explanation for this notable observation is that EGFRvIII's potent cell-surface activity may overpower its nuclear activity. This is possible as EGFRvIII phosphorylates and activates STAT1 while they interact near the plasma membranes. In support of this speculation, our previous study showed that loss of nuclear activity reduced the ability of EGFR, but not EGFRvIII, to mediate colony forming ability of glioblastoma cells [25]. In summary, the findings reported in this study shed light on the molecular mechanisms by which STAT1 gene expression is regulated and provide evidence linking ErbB oncogenic receptor tyrosine kinases to inflammatory response through the STAT3-STAT1 signaling axis.

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Figure 1. STAT1 gene expression is enhanced by nuclear EGFR

U87MG isogenic cell lines with stable expression of control vector, wild-type EGFR and EGFRdNLS mutant were used in panels A-D. Notably, EGFRdNLS has been characterized in our earlier study to be defective in entering the cell nucleus while EGFR undergoes EGF-induced nuclear translocalization [25]. EGF treatments were administered at 100 ng/ml for 4 hrs after serum starvation for 16 hrs.

A: EGFR, but not EGFRdNLS, enhances STAT1 transcript expression as shown by RT-qPCR. The results were derived from three independent experiments. The student *t*-test was performed to compute *p*-values.

B: STAT1 protein levels are higher in cancer cells with EGFR following EGF treatment, as shown by western blotting.

C: EGFR, but not EGFRdNLS, associates with the STAT1 gene promoter. In the intracellular protein:DNA binding ChIP assay, a mouse monoclonal EGFR antibody was used to immunoprecipitate EGFR-bound chromatin while mouse IgG was used as the negative controls for immunoprecipitation. Chromatin inputs served as loading controls for PCR.

D: STAT1 gene promoter activity was enhanced by EGF in EGFR-expressing cancer cells but not in those with vector control or EGFRdNLS. Cancer cells were transfected with the pSTAT1-Luc reporter, serum-starved, treated with EGF and then subjected to luciferase assay. The pRL-TK reporter was co-transfected as normalization controls. The results were derived from three independent experiments and analyzed by the student *t*-test to compute *p*-values.

E: STAT1 promoter activity is enhanced by EGFR in breast cancer cells. MDA-MB-231 and MCF-7 cells with low levels of endogenous EGFR were co-transfected with pSTAT1-Luc reporter, with and without an EGFR expression vector for 48 hrs, harvested and subjected to luciferase assay. The student *t*-test was performed to compute *p*-values.

Figure 2. EGFR synergizes with STAT3 to activate the STAT1 gene promoter

A: Co-expression of EGFR with STAT3CA synergistically enhanced STAT1 promoter activity. U87MG cells with very low levels of endogenous EGFR were used in these studies. STAT3CA is a constitutively activated variant of STAT3. Forty-eight following transfections, cancer cells were subjected to luciferase assay. The pRL-TK Renilla luciferase reporter was co-transfected to normalize for transfection efficiency. The data represent those of three independent experiments and were analyzed by the student *t*-test to derive p-values. $P < 0.05$ was considered statistically significant and marked by asterisks.

B: EGFR synergizes with STAT3CA, to a greater extent than EGFRdNLS, in activating STAT1 promoter. U87MG cells were used in these experiments.

C: STAT3 binds to the STAT1 promoter and the degree of binding increases after EGF stimulation. MDA-MB-468 breast cancer cells with endogenous EGFR and STAT3 were serum-starved for 16 hrs and treated with EGF (100 ng/ml) and without for 20 mins and then subjected to the ChIP assay. A STAT3 antibody was used to IP STAT3-associated chromatin whereas rabbit IgG was used as negative IP control. Chromatins were used in PCR as loading controls.

D: STAT3 binding to the STAT1 promoter is essential for the indirect association of nuclear EGFR with the promoter. MDA-MB-468 cells transfected with control (C) siRNA or STAT3 (S) siRNA for 30 hrs were serum-starved for 16 hrs and treated with EGF (100 ng/ml) for 20 mins. The ChIP assay was performed using an EGFR antibody or mouse IgG. WB in the lower panel indicates STAT3 knockdown effectiveness.

Figure 3. Structural characterization of the human STAT1 gene promoter for activation by the EGFR-STAT3 signaling axis

A: Simplified structures of five luciferase reporter constructs under transcriptional control of the wild-type and mutant STAT1 gene promoters. Top, four putative STAT3-binding sites are present within the wild-type STAT1 gene promoter. Following site-directed mutagenesis, four constructs with mutant STAT1 promoters were generated to disrupt each of the four STAT3-binding sites. Mutated nucleotides are shown in bold and underlined.

B: STAT3-binding sites are important for upregulation of the STAT1 promoter by the EGFR-STAT3 signaling axis. The reporter constructs were transfected into U87MG cells, with and without EGFR and/or STAT3CA plasmids for 48 hrs and the luciferase activity of each transfect was determined. All four mutant STAT1 promoters showed lower activities than the wild-type promoter. The STAT1-B mutant retained the most of the wild-type promoter activity. The means and standard deviations were derived from three independent experiments and analyzed by the student *t*-test to derive *p*-values. *P*<0.05 was considered statistically significant and marked by asterisks.

Figure 4. EGFRvIII cooperates with STAT3 to activate the STAT1 gene promoter

U87MG cells were used in these studies as they do not express EGFRvIII. The reporter constructs were transfected into U87MG cells, with and without EGFRvIII and/or STAT3CA plasmids for 48 hrs and the luciferase activity of each transfect was determined. The pRL-TK reporter was co-transfected as normalization controls. Data were derived from three independent experiments and analyzed by the student *t*-test to compute *p*-values.

*denotes *p* values < 0.05.

A: Combination of EGFRvIII and STAT3CA greatly increased STAT1 promoter activity.

B: EGFRvIII Δ NLS had a higher propensity than EGFRvIII to induce STAT1 promoter activation.

C: STAT3-binding sites within the STAT1 promoter are required for its synergistic activation by EGFRvIII and STAT3.

Figure 5. Endogenous associations between STAT1, EGFR and p-STAT3 in breast cancer cells

A: Positive association between EGFR, p-STAT3 and STAT1 in human breast cancer cells. Seven breast cancer cell lines were subjected to western blotting. Cell lines with high EGFR expression contained higher levels of STAT1 and p-STAT3 than those with low EGFR and p-STAT3 levels.

B: Lapatinib effectively inhibits EGFR activation, leading to a reduction in STAT1 expression. MDA-MB-468 cells with endogenous expression of EGFR and STAT3 were treated with 25 uM lapatinib for 24 hrs, harvested and subjected to WB for EGFR, p-EGFR and STAT1 expression.

Figure 6. HER2 and STAT3 synergize to upregulate STAT1 gene expression in breast cancer cells

Two human breast cancer cell lines were used in these studies, namely, MDA-MB-231 cells (HER2-negative) and MCF-7 cells (with endogenous low HER2).

A: HER2 enhances STAT1 gene promoter activity. Cancer cells were transfected with control vector or HER2 plasmid for 48 hrs, treated with and without 100 ng/ml heregulin for 2 hrs, and then subjected to luciferase assay. Data were calculated from three independent experiments and analyzed by the student *t*-test to compute p-values. $P < 0.05$ was considered statistically significant and marked by asterisks. Overall activity of STAT1 gene promoter is

higher in MCF-7 cells compared to MDA-MB-231 cells, indicating a positive association between STAT1 promoter activity and HER2.

B: Transcript levels of STAT1 and its downstream target genes were enhanced by ectopic HER2 and heregulin in MCF-7 cells. RT-PCR was performed.

C: STAT1 protein expression was upregulated by ectopic HER2 and heregulin in MCF-7 cells. Western blotting was conducted.

D: HER2-mediated STAT1 promoter activity was significantly increased by STAT3. MCF-7 cells were co-transfected with the pSTAT1-Luc reporter and HER2/STAT3CA plasmids for 48 hrs and treated with and without heregulin, followed by luciferase activity determination. Mean and standard deviations were calculated from the results of three independent experiments and analyzed by the student *t*-test to compute p-values. $P < 0.05$ was considered statistically significant and marked by asterisks.