Nuclear interaction of EGFR and STAT3 in the activation of the iNOS/NO pathway

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Summary

Epidermal growth factor receptor (EGFR) exists in the nucleus of highly proliferative cells where it functions as a transcription factor. Although EGFR has transactivational activity, it lacks a DNA binding domain and, therefore, may require a DNA binding transcription cofactor for its transcriptional function. Here, we report that EGFR physically interacts with signal transducers and activators of transcription 3 (STAT3) in the nucleus, leading to transcriptional activation of inducible nitric oxide synthase (iNOS). In breast carcinomas, nuclear EGFR positively correlates with iNOS. This study describes a mode of transcriptional control involving cooperated efforts of STAT3 and nuclear EGFR. Our work suggests that the deregulated iNOS/NO pathway may partly contribute to the malignant biology of tumor cells with high levels of nuclear EGFR and STAT3.

Introduction

EGFR/ErbB1 belongs to the ErbB family of receptor tyrosine kinases (RTKs) (Cohen et al., 1982a). Overexpression and/or increased activity of EGFR are key characteristics of human tumors and are frequently linked to more aggressive tumor behaviors, including increased proliferation, metastasis, and therapeutic resistance (Craven et al., 2003). As such, EGFR is considered an outstanding therapeutic target for human cancers (Arteaga, 2003; Mendelsohn and Baselga, 2003).

Our understanding of EGFR-overexpressing tumors remains incomplete, which is evidenced by several recent reports revealing that, in addition to the level of expression, somatic mutations of EGFR played a critical role in the therapeutic response in non-small-cell lung cancer (NSCLC) patients (Lynch et al., 2004; Paez et al., 2004). More recently, we found an inverse correlation between nuclear EGFR and overall survival in patients with breast cancer, suggesting a prognostic value of nuclear EGFR (Lo et al., 2005). Consequently, a more thorough understanding of the EGFR signaling pathway will facilitate the future success of clinically used anti-EGFR agents and the development of novel therapies that target the EGFR pathway.

Emerging evidence indicates a mode of EGF signaling in which growth factor signals can be transmitted, via EGFR nuclear transport, directly from the cytoplasmic membrane to the transcriptional targets in the nucleus (Bourguignon et al., 2002; Lin et al., 2001; Lo et al., 2005; Marti et al., 1991; Marti and Hug, 1995; Wells and Marti, 2002). This direct pathway is distinct from the traditional ErbB pathway that requires activation of signaling cascades, such as those involve phospholipase C-γ, Ras, and phosphatidylinositol-3 kinase (Anderson et al., 1990; Cohen et al., 1982b; Hu et al., 1992). In addition to EGFR, all other receptors in the ErbB family have also been detected in the cell nucleus (Lin et al., 2001; Marti and Hug, 1995; Ni et al., 2001; Offterdinger et al., 2002; Wang et al., 2004; Xie and Hung, 1994). Importantly, nuclear EGFR and HER-2 have been shown to be involved in the transcription of cyclin D1 and cyclooxygenase-2 (COX-2) genes, respectively (Lin et al., 2001; Wang et al., 2004).

Other RTKs also translocate into the nucleus, such as the fibroblast growth factor receptor, TrkA/nerve growth factor receptor, insulin receptor, and type I TGFβ receptor (Chan et al., 2003; Maher, 1996; Podlecki et al., 1987; Raabe et al., 2004; Rakowicz-Szulczynska et al., 1988; Reilly and Maher, 2001; Zhang et al., 2003; Zwaagstra et al., 2000). Inflammatory cytokines, such as interleukin-1 (IL-1), IL-5, and interferon-γ (IFN-γ), and their receptors, also undergo nuclear translocalization; however, their function in the nuclear compartment is largely unknown (Bader and Weitzerbin, 1994; Curtis et al., 1990; Grasl-Kraupp et al., 2002; Jans et al., 1997; Jans and Hassan,

SIGNIFICANCE

Although the nuclear existence of EGFR has been documented for more than a decade, our understanding of the physiological contribution of the nuclear EGFR pathway to the biology of cancer cells remains very limited. Here, we report a transcriptional mechanism by which gene expression can be directly regulated by two oncogenic products, EGFR and STAT3. The transcriptional complex that consists of nuclear EGFR and STAT3 may potentially represent a class of transcription factors that functionally interact at both cytoplasmic and nuclear levels, resulting in a direct transduction of extracellular signals from the cell surface to the nucleus. Thus, the findings reported in this study significantly advance our understanding of the EGFR pathway that occurs in the cell nucleus.

1998; Larkin et al., 2000; Subramaniam and Johnson, 2002; Zwaagstra et al., 2000). While the physiological and pathological consequences of the nuclear presence of cell-surface receptors remain unclear, a role of nuclear EGFR in tumor aggressiveness and poor clinical outcome is suggested (Lo et al., 2005).

Several questions remained unanswered regarding the nuclear function of ErbB RTKs. For example, additional unidentified transcriptional targets may exist. It is also unknown how these receptors activate gene transcription, as they lack a putative DNA binding domain. Nevertheless, the EGFR complex specifically recognizes AT-rich sequences, designated ATRSs (Lin et al., 2001). The nuclear EGFR complex associates with the ATRSs within the cyclin D1 promoter and activates its gene promoter (Lin et al., 2001). Nuclear HER-2 binds to the HER-2-associated sequence (HAS) in the COX-2 promoter, resulting in its transcriptional activation (Wang et al., 2004). The carboxyl terminus of EGFR, HER-2, rat p185/neu, and ErbB4 contains transactivational activity (Lin et al., 2001; Ni et al., 2001; Wang et al., 2004; Xie and Hung, 1994). Together, these observations suggest the possible existence of transcription cofactors with DNA binding ability that cooperate with nuclear EGFR to regulate gene expression.

STAT3 is a DNA binding transcription factor that is activated by cytokines and growth factors, and, upon activation, elicits its function by regulating gene expression (Darnell et al., 1994; Fu, 1999). STAT3 becomes phosphorylated at Y705 and activated (Park et al., 1996) by cell-surface EGFR following its association with the C terminus of EGFR (Coffer and Kruijer, 1995; Shao et al., 2003). Whether such association occurs in the nuclear compartment, however, has not been investigated. Interestingly, a recent report demonstrated that both EGF and activated STAT3 translocate to the perinuclear region via endosomes (Bild et al., 2002), implying that EGFR and STAT3 may interact in cellular locations other than the plasma membrane. STAT3 activates the transcription of genes involved in cell-cycle progression and antiapoptosis (Barre et al., 2003; Bowman et al., 2001; Karni et al., 1999; Shirogane et al., 1999; Sinibaldi et al., 2000; Yang et al., 2003). More recently, constitutively active STAT3 was shown to activate vascular endothelial growth factor (VEGF), leading to increased angiogenesis and tumor metastasis (Niu et al., 2002; Wei et al., 2003). Consequently, STAT3 has a major role in oncogenesis (Bromberg et al., 1999), angiogenesis (Niu et al., 2002), and tumor metastasis (Wei et al., 2003) and is thus a favorable target for cancer drug development (Turkson and Jove, 2000).

EGF has been shown to elevate nitric oxide (NO) production (Cianchi et al., 2004; Fantappie et al., 2002). The gaseous second messenger, NO, is frequently elevated in cancerous cells, and such elevation has been linked to tumor growth and metastasis (Jenkins et al., 1995; Lala and Orucevic, 1998). Increased expression of iNOS, a potent NO-producing enzyme, is another key characteristic of many human malignancies, including breast cancer (Ekmekcioglu et al., 2000; Vakkala et al., 2000). Overexpression of iNOS is significantly associated with tumor growth and angiogenesis (Cianchi et al., 2003; Vakkala et al., 2000). For these reasons, iNOS is considered an attractive target for both chemoprevention and chemotherapy (Jadeski and Lala, 1999; Rao et al., 2002). Although overexpression of both EGFR and iNOS is commonly observed in human cancers, a direct and consistent link between the EGFR and iNOS/NO pathways has not been established. For example, EGF induced expression of iNOS in normal astrocytes (Liu and Neufeld, 2003) and in head and neck squamous cell carcinomas (Gallo et al., 2002). However, such regulation was not found in other cellular systems (Salzman et al., 1996; Wang et al., 1999).

In an attempt to search for a nuclear protein that binds to EGFR, we found that nuclear EGFR physically and functionally interacts with STAT3, leading to activation of the iNOS gene whose promoter contains regulatory elements for both molecules. Furthermore, in a cohort of 111 breast carcinomas, we found an inverse correlation between iNOS expression and overall patient survival. Together, these findings describe a new mode of transcription machinery that involves physical and functional interaction between STAT3 and its associating receptor, EGFR. Our work also implies that deregulated iNOS expression may, in part, contribute to the malignant biology in tumors with increased nuclear EGFR and STAT3.

Results

Nuclear interaction of EGFR and STAT3

Since it is well established that transmembrane EGFR interacts with and activates STAT3, resulting in STAT3 nuclear translocalization and gene activation (Coffer and Kruijer, 1995; Park et al., 1996), and EGFR has been shown to be located in the nucleus and to associate with transcriptional activity (Lin et al., 2001; Lo et al., 2005), we asked whether STAT3 and EGFR might interact with each other to exert their transcriptional function in the nucleus. To this end, we found EGFR colocalized and physically associated with STAT3 in the nucleus (Figure 1). The association between EGFR and STAT3 in the nuclear compartment was first demonstrated by coimmunoprecipitation/Western blot (coIP/WB) analyses in EGFR-overexpressing A431 cells (Figure 1A). The level of nuclear EGFR/STAT3 complex was significantly enhanced by 30 min of EGF treatment (Figure 1A, left). As a positive control, Figure 1B (left) shows that cytoplasmic EGFR interacted with STAT3, and the interaction was enhanced following 30 min of EGF treatment. Lack of PARP in the cytosolic fraction indicates that it is free of nuclear contamination (Figure 1B, right). In the immunofluorescent microscopy/deconvolution analyses (Figure 1C), colocalization of EGFR (red) and STAT3 (green) occurred near the cell surface 10 min after EGF stimulation (yellow signals). After 30 min of EGF treatment, EGFR and STAT3 colocalized predominantly in the nucleus. In these studies, approximately 20–30 cells were analyzed for each treatment and the majority (70%–75%) of them behaved similarly. A few cells representative of those examined are shown in Figure 1C.

To further ensure that nuclear interaction occurs between EGFR and STAT3, we used electron microscopy (EM) using another EGFR-overexpressing cell line, MDA-MB-468 human breast carcinoma cells (Figures 1D and 1E). As shown in Figure 1D, inset a, nuclear EGFR (solid arrows) interacts with STAT3 (dashed arrows). The observed colocalization pattern resembles patterns previously reported in which 15 nm (EGFR) and 5 nm (STAT3) gold particles were detected in close proximity (Violot et al., 2003). Also noticeable is that a portion of nuclear EGFR did not colocalize with nuclear STAT3 (inset b) and vice-versa (inset c). This suggests that both EGFR and STAT3 may remain in an unbound form or associate with other nuclear proteins. In contrast, negative controls shown in Figure 1E did not

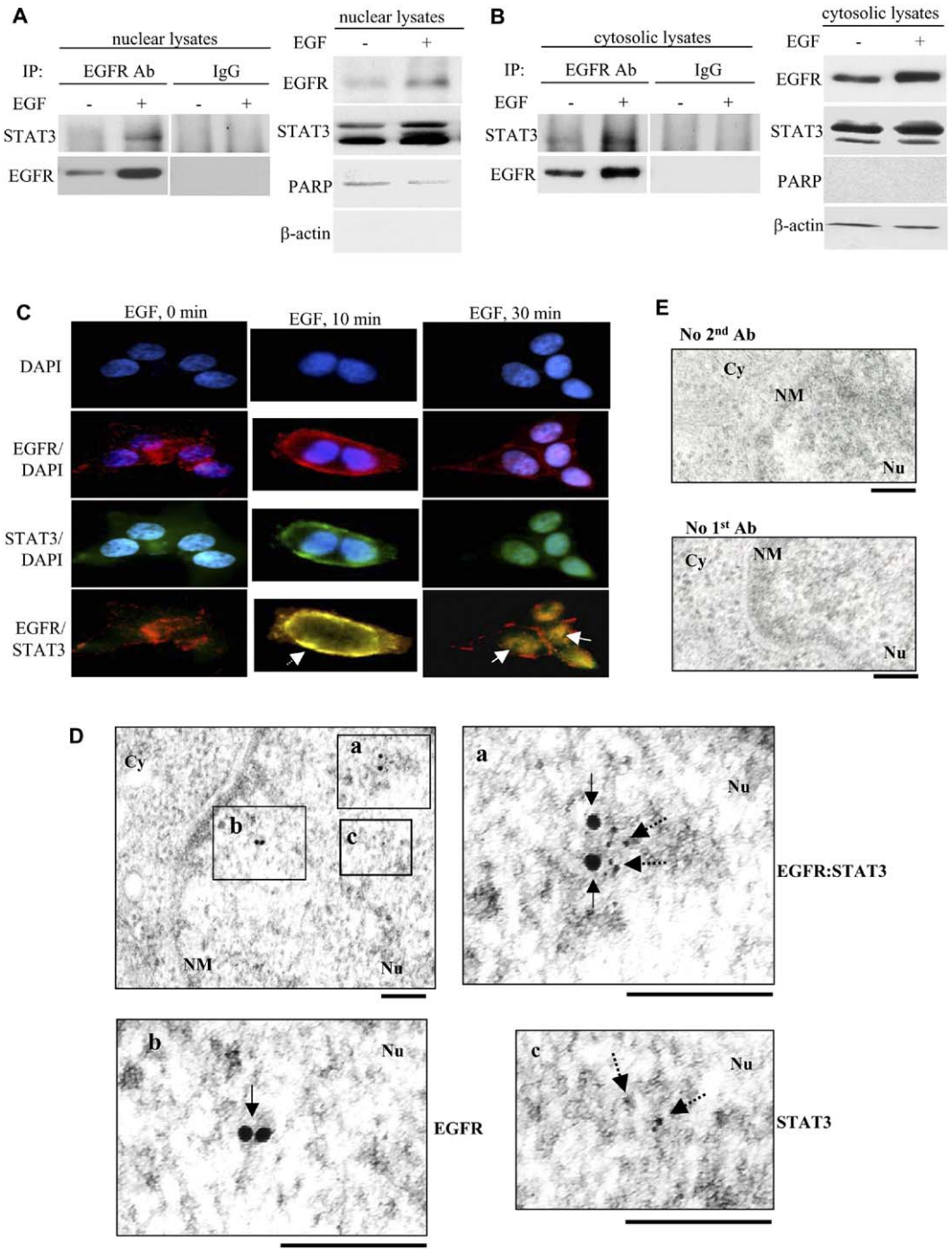


Figure 1. Nuclear interaction of EGFR and STAT3

Tumor cells were analyzed for colocalization of EGFR and STAT3. Cells were serum starved for 24 hr prior to EGF treatment.

A and B: Co-IP/WB analyses. A431 cells without (−) or with 30 min EGF (+) treatment were harvested and fractionated into nuclear (**A**) and cytosolic (**B**) fractions. Co-IP/WB (left) or WB (right) was carried out using indicated antibodies.

C: Immunofluorescent microscopy/deconvolution analyses. A431 cells were immunostained for EGFR (red) and for STAT3 (green), and their nuclei were stained with DAPI (blue). Arrows indicate colocalization of EGFR and STAT3 (yellow).

D and E: EM analysis. (**D**) EGF-stimulated MDA-MB-468 cells were stained for EGFR (large particles, solid arrows) and for STAT3 (small particles, dashed arrows). Insets **a**, **b**, and **c** are higher-resolution images showing the EGFR/STAT3 complex, EGFR, and STAT3, respectively. (**E**) Negative controls using EGFR Ab only (top) or secondary Ab only (bottom). Scale bar represents 100 nm. Cy, cytosol; NM, nuclear membrane; Nu, nucleus.

reveal any signals, indicating no nonspecific cross-reactivity. As expected, we also observed, using EM analysis, EGFR/STAT3 interaction near the plasma membrane and the cytoplasm (See Figures S1A and S1B in the Supplemental Data available with this article online). We also observed the existence of nuclear STAT3 prior to EGF treatment and the level enhanced by EGF (Figure S1C). This observation is consistent with previous reports showing that STAT3 is constitutively activated in MDA-MB-468 cells (Garcia et al., 2001). EGF-activated EGFR nuclear transport and nuclear interaction of EGFR and STAT3 were further detected in nontransformed immortalized HaCaT human keratinocytes that express both proteins (Figure S1D) (Sriuranpong et al., 2003). Together, these findings demonstrate a nuclear interaction of EGFR and STAT3 that has not been described previously.

In vivo binding of nuclear EGFR and STAT3 to the iNOS promoter

The identification of a nuclear interaction between EGFR and STAT3 prompted us to search for candidate target genes that are regulated by the nuclear EGFR/STAT3 complex. Since both are known to bind to specific DNA sequences to activate transcription, we performed both database (TFSEARCH, http://www.cbrc.jp/research/db/TFSEARCH) and literature searches to identify cancer-related genes that contain binding sites for both EGFR and STAT3. We found five gene promoters that contain both binding sites: namely, iNOS, c-*fos*, cyclin D1, human telomerase reverse transcriptase (hTERT), and VEGF. VEGF and c-*fos* can be up-regulated by STAT3 (Niu et al., 2002; Wei et al., 2003; Yang et al., 2003). The promoter of hTERT is activated by EGF via Ets (Maida et al., 2002). It should be noted that the functionality of the two ATRS motifs in the cyclin D1 gene promoter (–74 to –70 and –31 to –27) and the STAT3 binding sites in the promoters of the c-*fos* (–348 to –339) and VEGF (–842 to –849) genes have been previously characterized (Lin et al., 2001; Niu et al., 2002; Wei et al., 2003; Yang et al., 2003). We selected promoters of cyclin D1, c-*fos*, and iNOS for binding analysis. Among them, the EGFR binding site (ATRS) of the cyclin D1 promoter and STAT3 binding site of the c-*fos* promoter have been shown experimentally (Lin et al., 2001; Yang et al., 2003). The other ATRS and STAT3 sites in these three promoters are identified on the basis of sequence homology predicted from the computer-based analysis.

To examine whether these genes are targets for both nuclear EGFR and STAT3, we initially performed an in vivo binding assay, chromatin immunoprecipitation (ChIP). As summarized in Figure 2A, we observed significant and specific EGF-induced binding of both nuclear EGFR and STAT3 to the iNOS promoter. Consistently, quantitative real-time PCR confirmed in vivo EGF-activated binding of nuclear EGFR and STAT3 to the iNOS promoter by 3.3 ± 1.8-fold and 3.6 ± 0.5-fold, respectively. For the cyclin D1 promoter (Figure 2B), we observed EGF-activated binding of nuclear EGFR, as previously reported (Lin et al., 2001). Nuclear STAT3, however, binds to the cyclin D1 promoter independently of EGF stimulation (Figure 2B). Supporting the previous finding that STAT3 regulates the c-*fos* promoter via its STAT3 binding site (–348 to –339), Figure 2B shows that STAT3 binds to the c-*fos* promoter as expected, and the binding is significantly enhanced by EGF. Interestingly, we also found the nuclear EGFR to associate with the c-*fos* promoter following EGF activation (Figure 2B). These observations were further confirmed by quantitative real-time PCR analysis (data not shown). Together, these data demonstrate that both nuclear EGFR and STAT3 in vivo bind to all three promoters and that EGF enhanced such binding, except for that of STAT3 to the cyclin D1 promoter.

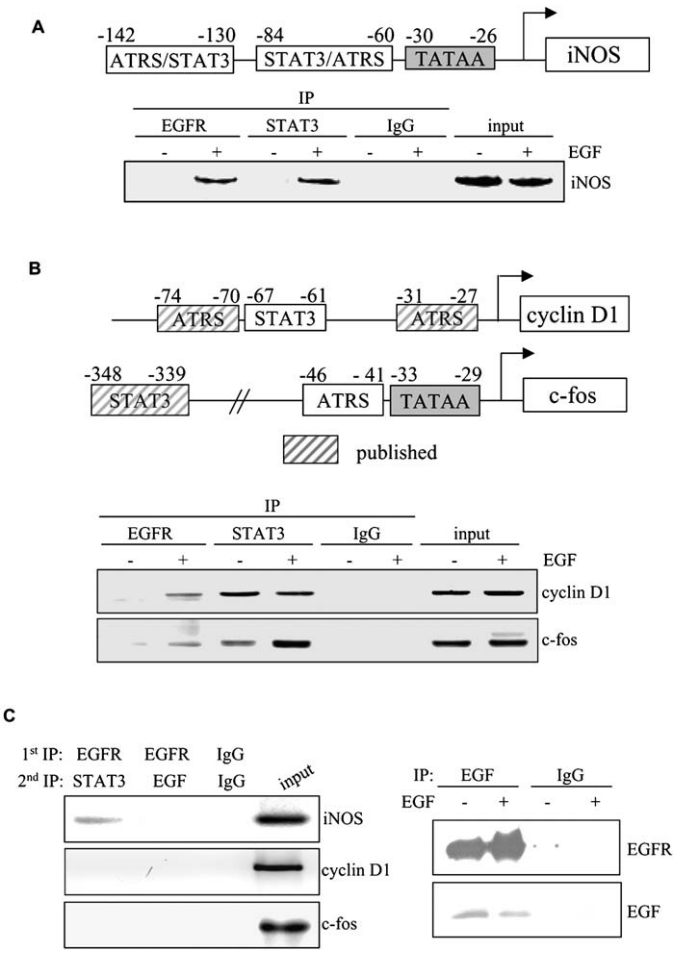


Figure 2. ChIP analysis of EGFR/STAT3 complex binding at promoters of iNOS, cyclin D1, and c-*fos* in vivo

MDA-MB-468 cells were serum starved for 24 hr then left without (−) or stimulated with EGF (+) for 30 min.

A and B: Association of EGFR and STAT3 with promoters of iNOS (**A**), cyclin D1, and c-*fos* (**B**) detected using indicated antibodies.

C: Association of EGFR/STAT3 complex with the iNOS promoter was detected using sequential IP. Following the first IP using EGFR Ab, EGFR:chromatin complex was eluted and subjected to second IP using Abs for STAT3 or EGF. The IP efficiency of the EGF Ab was tested by IP/WB using whole cell lysates (right).

In vivo binding of nuclear EGFR/STAT3 complex to the iNOS promoter

Next, we examined whether the nuclear EGFR/STAT3 complex associates with the iNOS, cyclin D1, and c-*fos* gene promoters, using sequential ChIP analyses. This investigation is particularly important given that EGFR forms a complex with STAT3 in the nuclear compartment (Figure 1). In these studies, we immunoprecipitated nuclear EGFR-associated chromatins with EGFR Ab, eluted the protein:chromatin complex, and then re-

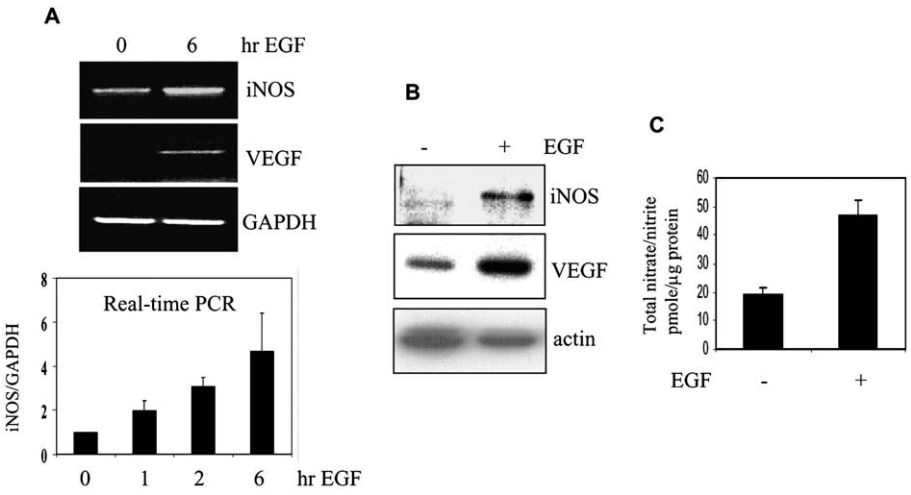


Figure 3. EGF/EGFR enhances iNOS gene expression

A and B: The expression of iNOS and VEGF genes in MDA-MB-468 cells without and with EGF for 6 hr was examined using (**A**) RT-PCR and (**B**) WB analyses. Lower panel in **A**: Quantitative real-time PCR method. The levels of iNOS transcripts were normalized against those of GAPDH, and all numbers (mean ± SD) were calculated from three independent experiments.

C: EGF enhances NO production. Levels of total NO (nitrite and nitrate) were determined in MDA-MB-468 cells without (−) or with 24 hr EGF treatment (+). Means ± SD (error bars) were derived from three independent experiments.

immunoprecipitated EGFR-associated chromatins using STAT3 Ab. Simultaneously, chromatins were reimmunoprecipitated with EGF Ab to determine whether nuclear EGFR remains bound to EGF while associating with the promoters. The sequential ChIP assay suggests that the nuclear EGFR/STAT3 complex in vivo binds to the iNOS promoter, but not that of the cyclin D1 or c-*fos* genes (Figure 2C, left). In contrast, in the ChIP experimental conditions, nuclear EGFR did not maintain its association with its ligand, EGF, when interacting with the promoters (Figure 2C, left), although the EGF Ab used for re-IP was effective in pulling down both EGF and EGFR, as indicated by coIP/WB analysis (right).

EGF/EGFR enhances iNOS gene expression

Detection of the EGFR/STAT3 complex interacting with the iNOS promoter prompted us to address whether EGF may regulate iNOS expression. Using reverse transcriptase-polymerase chain reaction (RT-PCR), we observed that EGF increased the iNOS mRNA level by ~7-fold in MDA-MB-468 cells (Figure 3A). Using quantitative real-time PCR, we observed an immediate early activation of iNOS transcription at 1 hr after EGF treatment, and the level increased to approximately 5-fold at 6 hr (lower panel, Figure 3A). The protein synthesis inhibitor, cycloheximide, did not significantly affect the EGF effect (data not shown). Consistently, WB analysis also shows an approximately 7.5-fold increase in iNOS protein expression after EGF treatment (Figure 3B). As a positive control for EGF responsiveness, we also analyzed the ability of EGF to alter VEGF gene expression, as it has been shown that activated STAT3 enhanced its gene activity (Niu et al., 2002). As expected, EGF treatment significantly increased levels of both the transcripts and the proteins of VEGF. Furthermore, we observed an increase of approximately 2.4-fold in endogenous NO after 24 hr of EGF treatment (Figure 3C). The degree of NO-induction by EGF is comparable to previously reported 3- to 3.5-fold in colon and liver cancer cells (Cianchi et al., 2004; Fantappie et al., 2002). Thus, EGF/EGFR, indeed, induces iNOS gene expression.

Characterization of iNOS gene promoter for EGF responsiveness

We further characterized the elements responsible for EGFR/STAT3 regulation in the iNOS promoter by examining responses to EGF of luciferase reporters driven by various deletion mutants of a 7.2 kb iNOS promoter. As indicated in Figure 4A, we observed the highest EGF responsiveness from the 0.3 kb iNOS promoter, and this construct was used in subsequent reporter studies.

Next, we aimed to identify the response elements in the iNOS gene promoter recognized by nuclear EGFR and STAT3. The iNOS promoter contains two clusters of putative ATRS/STAT3 sites that are highly homologous to the consensus sequences (Figure 4B). EGF enhanced the binding of nuclear EGFR and STAT3 to both NOS-1 and NOS-2 probes, with more profound stimulation for the NOS-1 probe (Figure 4B, left). As positive binding controls, nuclear EGFR and STAT3 bind to the cyclin D1 ATRS (CYD-ATRS) and a functional STAT3 binding site (APRE, acute-phase response element) known to bind to the STAT3/APR factor (Wegenka et al., 1993; Yu et al., 1995), respectively, following EGF stimulation (Figure 4B, mid-panel). Using an electrophoresis mobility shift assay (EMSA), we found significant EGF-activated binding of nuclear protein to both NOS-1 and NOS-2 (left, Figure 4C). Analysis of the components binding to the iNOS probes, via EMSA/WB, revealed the high-molecular-weight signals (arrowed) to contain both EGFR and STAT3 (left, Figure 4C). When the Abs for EGFR and STAT3 were included in the binding reactions, we observed disappearance of the high molecular band, marked by arrows (right, Figure 4C), indicating the interference of protein-DNA complexes by specific Abs recognizing EGFR and STAT3 or indicating the inability of the large EGFR/STAT3/Ab complex to enter the gels. Similar band disappearance was observed when binding reactions included Abs raised against the N- and C-terminal EGFR regions, but not the β-actin Ab (Figure S2). Collectively, these findings suggest that both nuclear EGFR and STAT3 interacted with the NOS-1 and NOS-2 regions in the iNOS promoter.

To further identify the critical nucleotides within the NOS-1 and NOS-2 regions that are required for the binding to nuclear

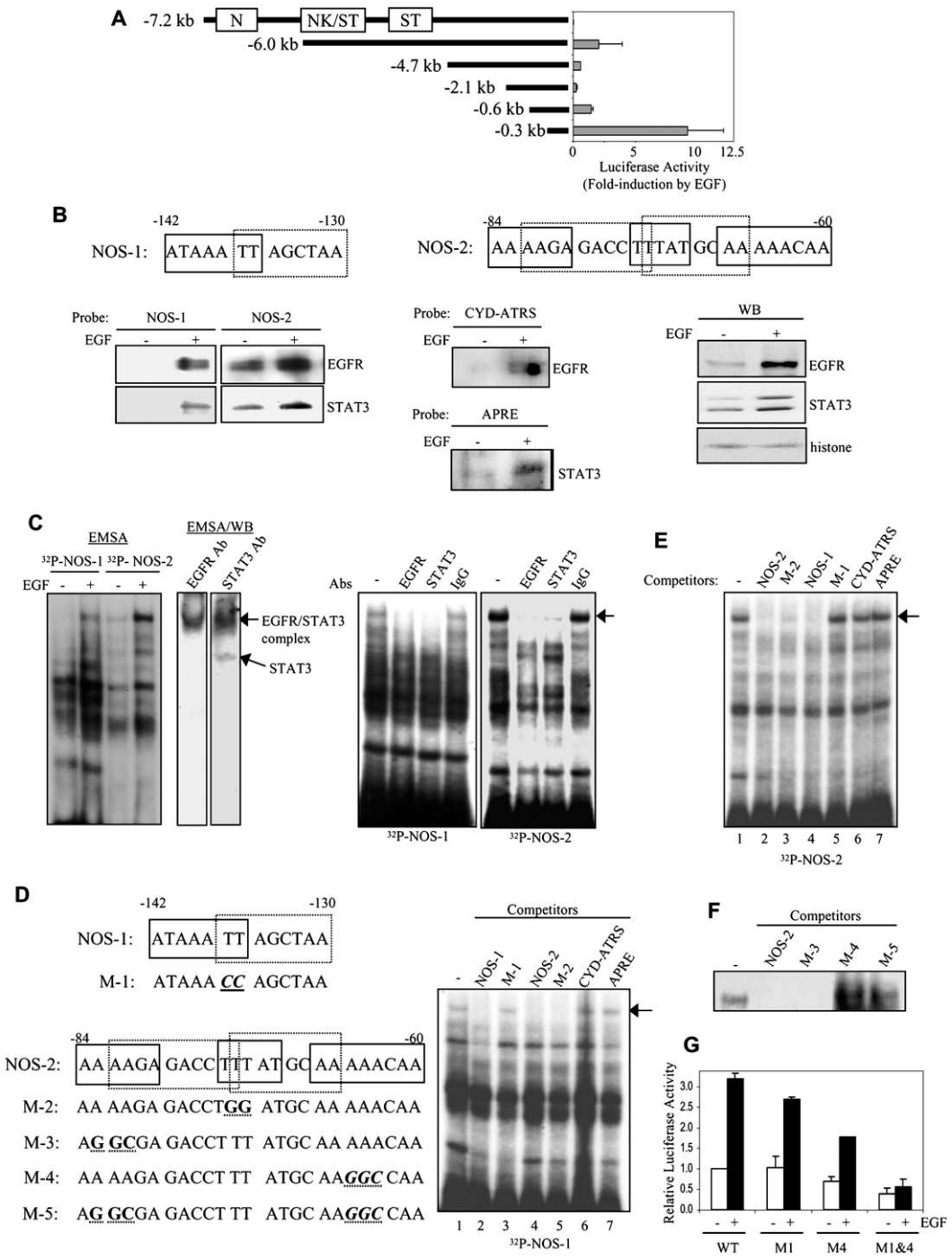


Figure 4. Characterization of iNOS gene promoter for EGF responsiveness

A: Activities of the 7.2 kb iNOS promoter and its deletion mutants were analyzed using a luciferase reporter system in MDA-MB-468 cells. N-box: NRE at −6749 to −6739. NK/ST-box: NF-kB/STAT1 elements at −5.8 kb. ST-box: STAT1 binding site at −5.2 kb.

B: EGF stimulates binding of nuclear EGFR and STAT3 to the iNOS promoter. Top panel: sequences of NOS-1 and NOS-2. Solid-line box, ATRS; dashed-line box, STAT3 binding site. Biotinylated oligonucleotides as indicated were used in the binding assay (left and middle panels). Levels of EGFR, STAT3, and histone in A431 nuclear lysates were examined using WB analysis (right).

C: EGF-activated binding of nuclear EGFR and STAT3 to the iNOS promoter. Left: EMSA was performed using ^{32}P-labeled probes and detected using autoradiography, whereas EMSA/WB was performed using an unlabeled probe and detected using indicated Abs; these reactions were run on the same gel. Right: EMSA analyses performed in the presence of indicated Abs. The arrow marks the band containing both nuclear EGFR and STAT3.

D, E, and F: Nucleotides critical for NOS-1 and NOS-2 binding the EGFR/STAT3 complex. (**D**, left) Sequences of NOS-1 and NOS-2 and their mutants; mutated nucleotides are shown in bold, and critical nucleotides are shown in italicized bold. (**D–F**) EMSA competition experiments carried out without (−) or with 200-fold molar excess of indicated unlabeled probes. Arrows mark the EGFR/STAT3-containing complex.

G: Transcription activity of mutant iNOS promoters. Luciferase activity from MDA-MB-468 cells transfected with indicated reporter plasmids without (−) or with (+) EGF treatment was determined the means ± SD (error bars) were derived from three independent experiments.

EGFR/STAT3 and for EGF-mediated activation, a series of mutant oligonucleotides were generated (Figure 4D). The unlabeled M-1 mutant failed to compete for EGFR/STAT3 binding, indicating its inability to interact with nuclear EGFR/STAT3 and the functional importance of the two nucleotides TT (–137 and –136) for NOS-1 (Figures 4D and 4E). In contrast, unlabeled NOS-1 and NOS-2 probes effectively outcompeted the binding signal, indicating binding specificity and suggesting that the same DNA binding protein was involved in the binding to both NOS-1 and NOS-2 (Figures 4D and 4E). Moreover, the M-2 mutant remains the function of wild-type NOS-2, indicating that the two nucleotides TT (–73 and –72) are not important for the functionality of NOS-2. Interestingly, neither cyclin D1 ATRS (CYD-ATRS) nor the STAT3 binding site, APRE, was able to outcompete the binding signals, suggesting that the complex structural requirement may lie within the promoter element that is specific for EGFR/STAT3 recognition (Figures 4D and 4E).

As the M-2 mutant retains its binding function as the wild-type NOS-2, we further designed and tested three additional mutants: M-3, M-4, and M-5 (Figure 4D and Table S2). As indicated by Figure 4F, the binding of the nuclear EGFR/STAT3 complex to the NOS-2 probe was outcompeted by the M-3 mutant, but not by the M-4 and M-5 mutants, indicating that the nucleotides AAA at positions -65 to -63 are critical bases for NOS-2 functionality. Using reporters containing 0.3-kb iNOS promoters mutated at M-1or M-4 as well as both M-1 and M-4 positions, we further examined the functionality of the EGFR/STAT3 binding elements in the iNOS promoter. We found double mutations at both M-1 and M-4 positions significantly reduced the activity of the iNOS promoter (Figure 4G). Interestingly, the iNOS promoter with mutations at either M-1 or M-4, within NOS-1 and NOS-2 regions respectively, did not significantly alter promoter activity, suggesting that one cluster of wild-type EGFR/STAT3 elements is sufficient for EGF-responsiveness. Taken together, these analyses indicate the importance of EGFR/STAT3 elements within both NOS-1 and NOS-2 regions in EGF responsiveness and in the basal activity of the iNOS promoter.

EGFR and STAT3 expression/activity are important for EGF-induced iNOS promoter activity

In addition to STATs, EGF can activate multiple signaling pathways such as Ras/MAPK and PI3K/Akt. We then asked whether EGF activates the iNOS promoter via the EGFR/STAT3 module and whether nuclear EGFR is important during such activation. AG490 (Jak2/STAT kinase inhibitor) and PD158780 and AG1478 (EGFR kinase inhibitors) inhibited the phosphorylation and activity of their respective targets as expected (Figure 5A, right, and Figure S3). AG490 reduced EGF-induced iNOS promoter activity by 95%, whereas PD158780 and AG1478 reduced it by 45%–74% (Figure 5A). Consistently, 1 hr pretreatment of AG490, AG1478, AG490/1478, and gefitinib (Iressa) inhibited NO production, with the combination of AG490 and AG1478 producing the highest rate of suppression (Figure 5B). The reduction of NO synthesis by gefitinib was not due to cell death, as the MTT cell proliferation assay showed only approximately 10% cell-kill after 24 hr treatment of 2.5 μM gefitinib (data not shown). It was also evident that gefitinib pretreatments abolished EGF-induced EGFR activation/phosphorylation and, to a lesser degree, that of STAT3 while the total endogenous levels of EGFR and STAT3 remained constant (Figure 5C).

To determine whether EGF-induced iNOS expression requires nuclear EGFR. We generated an EGFR mutant that is defective in nuclear entry but retains its cell-surface location/functions by mutating a putative nuclear localization signal (pNLS; ^{645}RRR^{647} to AAA) located within the juxtamembrane domain (Lin et al., 2001; Waugh and Hsuan, 2001). A similar NLS is also found in the juxtamembrane domain of HER-2 and ErbB4, the deletion/mutation of which rendered these molecules unable to enter the cell nucleus (Wang et al., 2004; Williams et al., 2004). Three stable lines were then generated from the parental EGFR-null CHO cells and designated CHO-NEO, CHO-EGFR, and CHO-EGFR-pNLS to express the parental vector, wild-type EGFR, and EGFR-pNLS mutant, respectively. The EGFR mutant in CHO-EGFR-pNLS cells is not present in the nucleus (Figure 5D, left) and therefore did not interact with nuclear STAT3 (Figure 5D; right), whereas significant amounts of EGFR were contained in both nuclear and nonnuclear fractions of CHO-EGFR cells, and the interaction between nuclear EGFR and STAT3 was readily detectable (Figure 5D; right). In contrast to enhanced iNOS promoter activity and iNOS expression in CHO-EGFR cells, EGF did not increase iNOS promoter activity or iNOS expression in CHO-NEO and CHO-EGFR-pNLS cells (Figure 5E).

Additionally, we found that the wild-type EGFR and the pNLS mutant interacted with STAT3 to a similar degree in the whole-cell lysates, as shown by Figure 5D (middle), indicating that the pNLS mutation does not affect the ability of EGFR to interact with STAT3. We also detected nuclear STAT3 in all three CHO lines, suggesting that the intact STAT3 pathway alone is not sufficient for activating the iNOS promoter following EGF stimulation and that STAT3 nuclear import can occur independently of EGFR subcellular location (Figure 5D, left). Moreover, the EGFR-pNLS mutant retains its cell-surface function (Figure 5F, left) that transduces the classical EGFR-Ras-ERK pathway and activates the elk-target promoter (Rao and Reddy, 1993). In addition, the EGFR-pNLS mutant retains its cell-surface localization (data not shown) and undergoes EGF-induced autophosphorylation at Y1045 (Figure 5F, right). Together, these data indicate that the pNLS region within EGFR is required for its nuclear import and that nuclear EGFR is important for the activation of the iNOS promoter and subsequent NO production.

We next attempted to demonstrate the requirement of STAT3 in EGF-mediated iNOS gene activation, as EGF/EGFR has been shown to activate, in addition to STAT3, other members of the STAT family of proteins, such as STAT1 and STAT5. Figure 5G shows that a dominant-negative STAT3 (STAT3-DN) mutant, which carries the mutation Y705F (Nakajima et al., 1996), reduced the ability of EGF to activate the iNOS promoter. In addition, iNOS promoter activity was increased in cells expressing a constitutively active STAT3 (Stat3-C) (Bromberg et al., 1999) with and without EGF. Furthermore, STAT3 siRNA, which reduced the protein level of STAT3 but not of STAT1, suppressed the 0.3 kb iNOS promoter activity by approximately 75% in CHO-EGFR cells and by 50% in CHO-EGFR-pNLS cells transiently expressing wild-type EGFR (Figure 5H). Consistently, STAT3 siRNA, but not nonspecific (NS) siRNA, reduced iNOS expression (Figure 5H, right). Together, these data indicate that STAT3 expression/activity is important for the activation of the iNOS gene promoter.

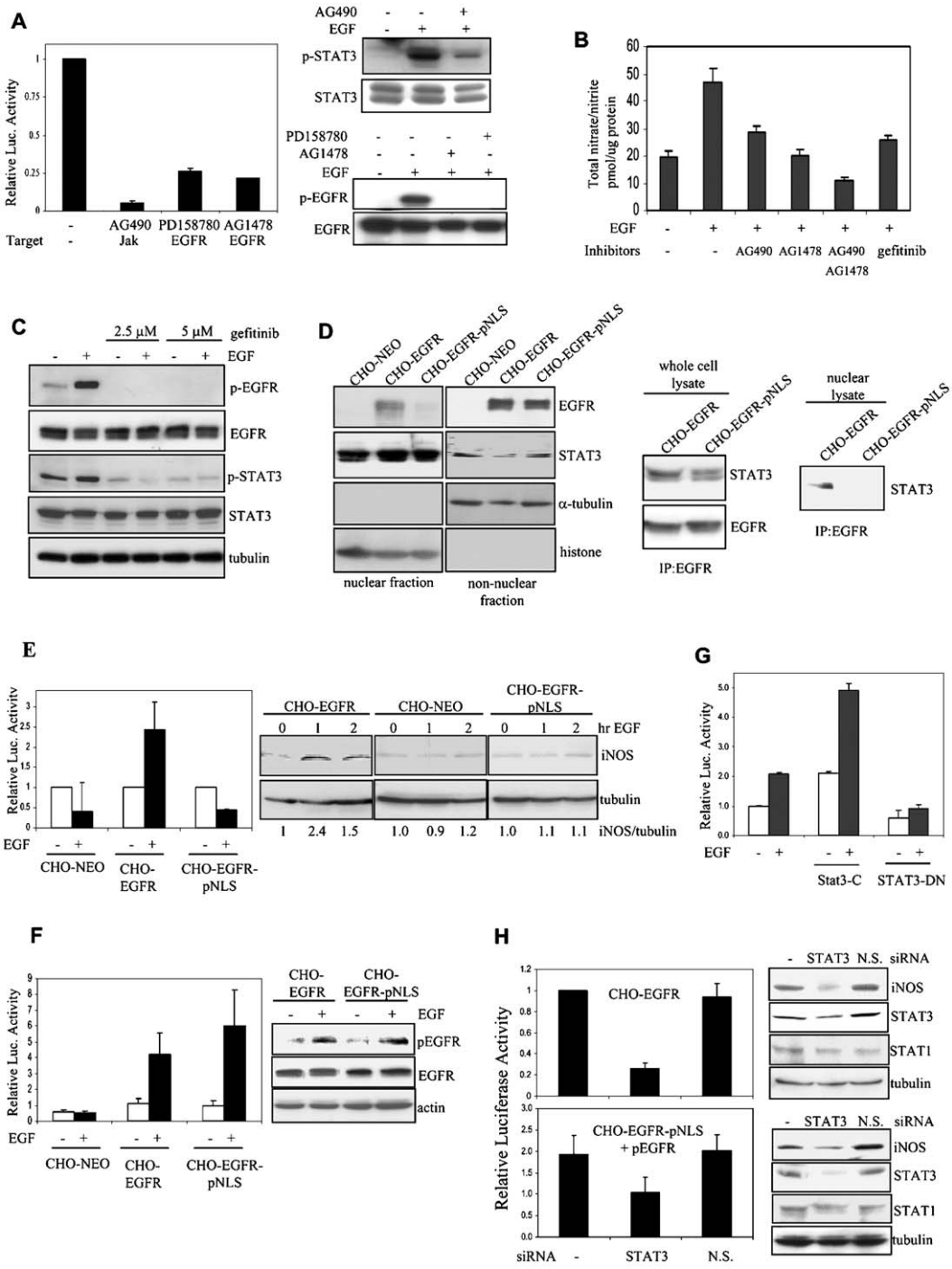


Figure 5. EGFR and STAT3 are important for EGF-induced iNOS promoter activity

Means ± SD (error bars) were derived from at least three independent experiments.

A: Reduction of iNOS promoter activity by inhibitors to Jak2 and EGFR. Luciferase activity of MDA-MB-468 cells transfected with piNOS(0.3)Luc and then treated with indicated inhibitors for 30 min prior to 4 hr EGF stimulation was determined and compared to that of cells not treated with inhibitor. Inhibition of phosphorylation of EGFR or of STAT3 in cells treated with indicated inhibitors for 1 hr prior to 30 min EGF stimulation was determined using WB (right).

B: Reduction of EGF-induced NO synthesis by 10 μM AG490, 10 μM AG1478, and 2.5 μM gefitinib. MDA-MB-468 cells were pre-treated with indicated inhibitors for 1 hr prior to 24 hr EGF stimulation. Total nitrate/nitrite content (pmol/μg protein) was determined.

C: Gefitinib effectively inhibits EGFR activation/autophosphorylation. MDA-MB-468 cells were treated without and with gefitinib for 1 hr prior to 30 min EGF stimulation.

D: EGFR-pNLS mutant fails to enter the nucleus, but retains a functional STAT3 signaling pathway. Left: Expression of EGFR and EGFR-pNLS. Middle: EGFR and EGFR-pNLS bind to STAT3 in whole-cell lysate. Right: EGFR binds STAT3 in nuclear lysate.

E: Nuclear EGFR is important for EGF-induced iNOS gene activity. The EGF-induced iNOS promoter activity was determined by either reporter assay (left) or expression of iNOS (right) in indicated CHO cell lines.

F: EGFR-pNLS mutant activates elk-targeted promoter (left) and undergoes autophosphorylation in response to EGF (right).

G: STAT3 activation is important for iNOS promoter activity. Luciferase activity in Hela cells transfected with piNOS(0.3)Luc alone or together with plasmids expressing STAT3-DN or Stat3-C was determined.

H: STAT3 expression is important for iNOS promoter activity. Luciferase activity (left) and levels of indicated proteins (right) in cells transfected with piNOS(0.3)-Luc alone or with indicated siRNA. Left: Luciferase activity derived from piNOS(0.3)Luc. Right: WB analysis.

Synergistic/additive cytotoxic effect of anti-EGFR and anti-STAT agents

STAT3 has been associated with EGFR-independent activity (Fernandes et al., 1999; Sriuranpong et al., 2003). Constitutively activated STAT3 can be attributed to increased Src and Jak activities (Garcia et al., 2001). Consistent with these reports, STAT3 interacts with the cyclin D1 promoter in an EGF-independent manner (Figure 2B). Furthermore, our results (Figure 5C) indicate that gefitinib was not completely effective in inhibiting STAT3 activation as measured by p-STAT3. Thus, we sought to investigate whether there was a therapeutic advantage in combining agents targeting EGFR and STAT3. We found that a combination of AG490 and AG1478 was more potent than either inhibitor alone in killing A431 and MDA-MB-468 cells in both MTT and clonogenic assays (Figure 6A).

Next, we examined whether a therapeutic synergy or additive effect exists between agents targeting STAT3 and those targeting EGFR by performing median-effect analyses as described previously (Chou and Talalay, 1984). In these studies, tumor cells were treated with AG490 and AG1478 for 24 hr and with AG490 and gefitinib for 48 hr. Using the combination index (CI) method/median-effect analysis, we determined the values of CI for combination regimens. Synergism is indicated when the derived CI value < 1.0, whereas CI = 1.0 reflects additive effects, and CI > 1 represents antagonism. For MDA-MB-468 cells, the CIs for up to IC75 were below 1.0 for both AG490/AG1478 combination regimens (Figure 6B, left). For A431 cells, a synergistic effect between AG490 and AG1478 was seen when a molar ratio of 1:2 (AG490/AG1478) was administered (data not shown). When we replaced AG1478 with gefitinib, a moderate synergy for up to IC50 was observed and an additive effect, for above IC50 (Figure 6B, right). These effects were observed when we administered AG490 and gefitinib with 2:1 and 4:1 in molar ratio. Collectively, these data indicate a therapeutic benefit for combining agents targeting EGFR and Jak/STATs.

Reduction of the killing effect of anti-EGFR and Jak2/STAT agents by increase of cellular NO content

We then rationalized that NO would protect tumor cells from the cytotoxic effect of AG490 and AG1478 if the iNOS/NO pathway is indeed an important downstream player in the mitogenic EGFR/STAT3 pathway. We also hypothesized that reduction of iNOS activity and NO content would provide a growth disadvantage to tumor cells if the proliferation of these cells is indeed facilitated by NO. As indicated in Figure 6C (left), preincubation of the tumor cells for 30 min with a slow-releasing NO donor, S-nitroso-N-acetylpenicillamine (SNAP), reduced the killing effect of AG490/AG1478 (24 hr) from 96% to 57% in A431 cells and from 79% to 42% in MDA-MB-468 cells. The efficacy of SNAP in increasing NO levels is demonstrated in Figure 6C (right). Moreover, MTT studies showed that specific iNOS inhibitors (1400W and S-methylisothiourea, MIU) and NO scavengers (2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, PTIO, and 2-(4-carboxyphenyl)-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide, c-PTIO) suppressed the growth of MDA-MB-468 cells by approximately 42%–79% (Figure 6D, left). More profound cell death was observed in treated cells when clonogenic growth was measured (Figure 6D, right). Consistently, SNAP pretreatment compromised the growth-suppressing effect of 1400W (Figure 6E). Collectively, these data suggest that NO facilitates the proliferation of tumor cells and protects profound cell death resulting from suppression of the EGFR/Jak/STATs and iNOS pathways by chemical inhibitors.

Positive correlation between levels of nuclear EGFR/STAT3 and iNOS in primary breast carcinomas

We established, thus far, a positive regulatory role for nuclear EGFR and STAT3 in iNOS expression in the cell culture system. We next examined whether such regulation exists in primary human tumors. Using immunohistochemical staining analysis, we analyzed a cohort of 111 human breast carcinomas, previously analyzed for EGFR (Lo et al., 2005), for the expression of iNOS and correlated iNOS expression with levels of EGFR. A positive correlation was found between levels of nuclear EGFR and iNOS (Figure 7A). EGFR in the nonnuclear compartment, cytoplasm, and membrane, however, did not significantly correlate with iNOS expression. In contrast, we did not find a correlation between levels of iNOS/EGFR and tumor grades.

To further investigate a possible correlation between activated STAT3 and nuclear EGFR/iNOS in primary tumor tissues, we selected 15 tumors of those that had stained negative for both nuclear EGFR and iNOS and 15 of those that had stained positive for both markers, and we subjected them to immunohistochemical staining for p-STAT3 (Y705), the activated nuclear form. Importantly, we found a significant positive correlation between levels of p-STAT3 and expression of nuclear EGFR and iNOS (Figure 7B). Representative tumors are shown in Figure 7B (top): the tumor in the upper panel stained negative for nuclear EGFR (left), p-STAT3 (Y705, middle), and iNOS (right) and that in the lower panel stained positive for all three markers. Chi-square analysis showed a positive correlation between nuclear p-STAT3 level and nuclear EGFR/iNOS expression ($p = 0.025$); tumors with nuclear p-STAT3 expression $\geq$20% were considered to have constitutively activated STAT3 (Hsiao et al., 2003; Khoury et al., 2003). As some reports consider p-STAT3 expression $\geq$50% to be strong/constitutive expression (Masuda et al., 2002), we thus regrouped the cohort into <50% and $\geq$50%, and the analysis showed that 40% (6/15) and 80% (12/15) of the nuclear EGFR/iNOS-negative and -positive tumors, respectively, contained constitutively activated STAT3. The difference was statistically significant ($p = 0.005$). Furthermore, we found a lack of correlation between nonnuclear and nuclear levels of EGFR (Figure 7C), which supports the concept that nuclear and nonnuclear EGFR pathways may lead to different cellular targets.

We further examined whether iNOS expression correlated with survival rate in this cohort. Interestingly, patients with medium/high iNOS in their tumors were found to have a worse survival rate compared to those with no/low expression (Figure 7D). For the classification of iNOS levels, we followed the system that was reported previously (Ekmekcioglu et al., 2000; Vakkala et al., 2000). Patients with tumors containing medium/high iNOS levels constituted approximately 57.6% (64/111) of the cohort with 43.5 ± 15.6 survival months, whereas those with no/low iNOS contents, 42.4% (47/111), survived 53.5 ± 10.9 months. Together, these data indicate that the levels of EGFR and STAT3 in tumor nuclei correlate with the level of iNOS in human breast carcinomas, and iNOS may serve as a prognostic indicator for patients with breast cancer.

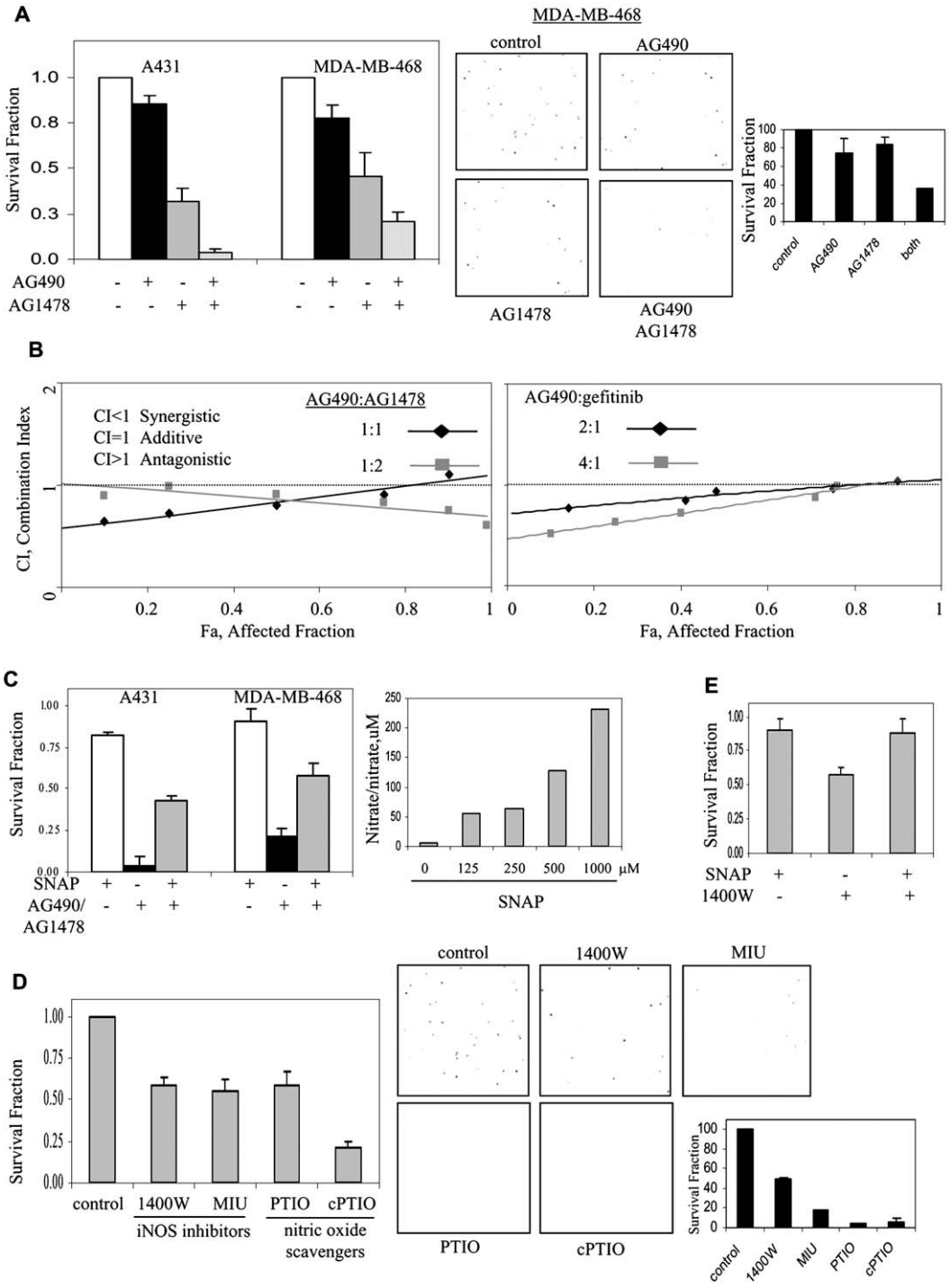


Figure 6. Cytotoxic effect of agents targeting EGFR, Jak2/STATs, and iNOS/NO pathways

Means ± SD (error bars) were derived from at least three independent experiments.

A: Increased cell-killing effect when both EGFR and Jak2/STATs activities were inhibited. Left: MTT assay. Right: clonogenic growth assay.

B: A therapeutic advantage with combined targeting of Jak2/STATs and EGFR.

C: NO protects tumor cells from AG490/AG1478-induced cell death. Growth of tumor cells was measured using MTT assay following treatment of SNAP (500 μM), AG490/AG1478 (10/20 μM), or all three agents for 24 hr (left). Total nitrate/nitrite content of SNAP-treated cells was measured (right).

D: Reduction of tumor cell growth by iNOS/NO inhibitors. Following treatment of 1400W (500 μM), MIU (1 mM), PTIO (10 μM), and cPTIO (500 μM) for 48 hr, MDA-MB-468 cell growth was determined using MTT (left) and clonogenic growth (right) assays.

E: SNAP compromises the growth-suppressing effect of 1400W. MTT assay was performed after a 48 hr treatment.

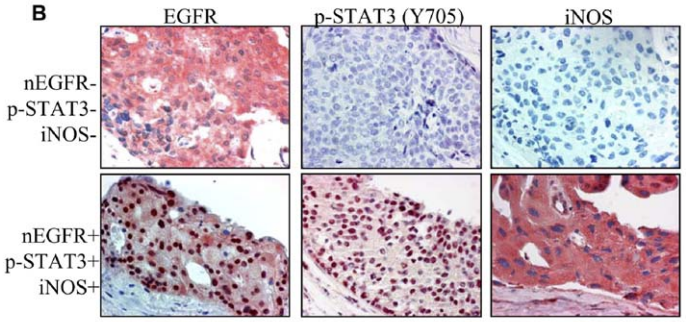
A

Chi-Square Analysis

EGFR	iNOS negative	iNOS positive	Total N	*P* value
Nuclear				
negative	34 (30.6%)[a]	39 (35.1%)	73	
positive	2 (1.8%)	36 (32.4%)	38	< 0.0001[b]
Total N	36	75	111	
Non-nuclear				
negative	11 (9.9%)	11 (9.9%)	22	
positive	25 (22.5%)	64 (57.6%)	89	= 0.05
Total N	36	75	111	

[a]The percent of entire cohort. [b]Statistically significant.

B

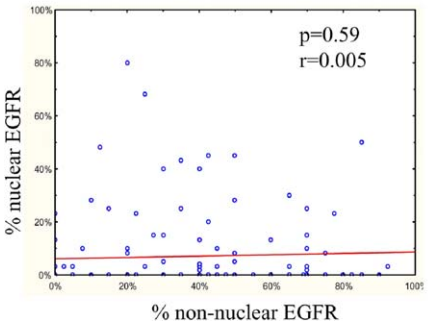


Chi-Square Analysis

STAT3 Activation	nEGFR-, iNOS-	nEGFR+, iNOS+	*P* value
p-STAT3-negative (<20%)	9 (30%[a])	3 (10%)	
p-STAT3-positive (≥20%)	6 (20%)	12 (40%)	p=0.025[b]
Total N	15	15	

[a]The percent of entire cohort. [b]Statistically significant.

C



D

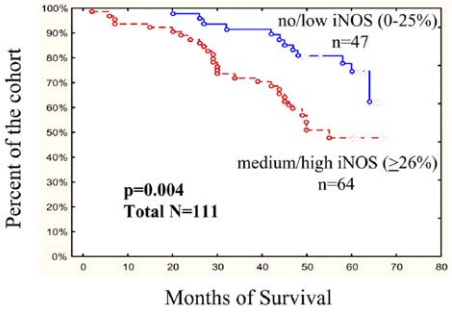


Figure 7. Positive correlation between levels of nuclear EGFR and iNOS in breast tumors and the level of iNOS as a prognostic marker for breast cancer patients

Levels of iNOS and p-STAT3 (Y705) in a cohort of primary breast carcinoma specimens, for which EGFR levels had been evaluated previously (Lo et al., 2005), were determined using immunochemical staining.

A: Positive correlation between nuclear EGFR and iNOS, calculated using the Pearson χ^2 test.

B: Correlation between the level of p-STAT3 and the levels of nuclear EGFR and of iNOS. A representative tumor that is negative for nuclear EGFR, p-STAT3, and iNOS (top) and a representative tumor that is positive for all three signals (middle) are shown. Bottom panel: χ^2 analysis of 30 tumors stained for all three proteins. Tumors with nuclear p-STAT3 ≥ 20% were considered to exhibit strong/constitutive staining.

C: Lack of correlation between levels of nuclear and nonnuclear EGFR by regression analysis.

D: High levels of iNOS as a prognostic indicator for poor survival. Kaplan-Meier survival analysis and the log-rank test were performed to correlate the levels of iNOS, no/low (0%–25%) and medium/high (≥26%), with overall patient survival.

Discussion

The findings of the current study advance our knowledge of the functions of nuclear EGFR. First, we showed that STAT3 physically associates and colocalizes with EGFR in the cell nucleus following EGF stimulation. This finding is of particular importance in understanding how nuclear EGFR, which lacks a putative DNA binding domain, regulates gene expression. Second, we found that the EGFR/STAT3 complex interacts with the human iNOS promoter and activates iNOS gene expression. As the role of EGF/EGFR in iNOS/NO pathway remains elusive, our findings demonstrate crosstalk between two cellular pathways, both involved in tumorigenesis and various processes of tumor progression. Third, via the structural and functional characterization of the iNOS promoter, we identified two regions within the iNOS promoter that contain binding elements for both EGFR and STAT3. Fourth, we observed in primary human breast carcinomas that expression of nuclear EGFR positively correlates with that of iNOS. We also found a significant inverse correlation between iNOS expression and patient survival in the cohort. Finally, we provided evidence showing the therapeutic advantage of suppressing tumor cell growth by combining agents targeting EGFR and Jak2/STATs.

Although it remains elusive whether EGF regulates expression of iNOS (Gallo et al., 2002; Liu and Neufeld, 2003; Salzman et al., 1996; Wang et al., 1999), our results provide conclusive evidence showing that the EGF/EGFR/STAT3 growth factor pathway activates iNOS gene expression and subsequent NO synthesis in human tumor cells. This finding is believed to be of particular importance, especially in view of a

new mode of transcriptional regulation that involves a nuclear oncogenic RTK, EGFR, and an oncogenic transcription factor, STAT3. This transcriptional machinery may potentially occur in other cell signaling modules composed of cell-surface receptors and their associating transcription factors that shuttle between the cell surface and the nucleus. In this context, other cell-surface receptors that are capable of interacting with transcription factors and of translocating into the nucleus may utilize similar mechanisms to regulate gene expression. The known receptor/transcription factor modules include IFNγR/STAT1, IL receptors/STATs, and TGF-βRI/Smads (Darnell et al., 1994; Larkin et al., 2000; Reguly and Wrana, 2003; Zwaagstra et al., 2000). Interestingly, IFNγR subunit I, receptors to IL-1 and IL-5, and the TGF-β type I receptor have also been found in the nucleus (Bader and Weitzerbin, 1994; Curtis et al., 1990; Jans et al., 1997; Larkin et al., 2000; Subramaniam and Johnson, 2002; Zwaagstra et al., 2000). Similar to the EGF/EGFR pathway, nuclear translocalization of IFN-γR occurs following IFN-γ stimulation (Bader and Weitzerbin, 1994; Larkin et al., 2000; Subramaniam and Johnson, 2002). IFN-γR subunit I colocalizes with STAT1α in the nucleus following IFN-γ treatment (Larkin et al., 2000). The primary nuclear function of IFN-γ has been shown to be involved in the enhancement of STAT1α transcriptional activity (Subramaniam et al., 2001). Taken together, our study certainly prompts an urgent need to investigate whether these receptor/transcription factor modules indeed have a direct physical interaction in the nuclear compartment leading to transcriptional regulation, as seen with EGFR/STAT3.

Our analyses of the cyclin D1 and c-*fos* promoters indicate that the EGFR/STAT3 complex may not be involved substantially in their activation, as the sequential ChIP studies did not detect an association of EGFR and STAT3 in the same DNA regions on these promoters (Figure 2C). However, the ChIP analysis indicates that EGFR and STAT3 individually associate with both promoters (Figure 2B). Together, the results suggest that EGFR and STAT3 do not form a complex while binding to these promoters. The current study suggests that the EGFR-induced activation of STAT3 is more complicated than thought previously. In addition to the traditional pathway in which EGFR activates STAT3 and activated STAT3, in turn, translocates into the nucleus to turn on gene expression, another mode of regulation could be derived from the EGFR/STAT3 complex, which coregulates transcription of genes such as the iNOS. These two modes of regulation may not be mutually exclusive and may be responsible for the activation of different genes (e.g., iNOS and cyclin D1). It is not yet clear how a gene promoter can distinguish regulation by STAT3, nuclear EGFR, and the EGFR/STAT3 complex. Several factors might contribute to this. For instance, close proximity of EGFR and STAT3 binding sites might be critical to allow formation of the EGFR/STAT3 complex (c-*fos* might not belong to this class). Alternatively, the location of EGFR and STAT3 on the same face of the DNA helix might be important for EGFR/STAT3 complex formation. In the case of the cyclin D1 promoter, STAT3 binding is constitutively activated and not responsive to EGF stimulation. Structural signatures/requirements for EGFR/STAT3-targeted promoters, such as the nucleotide sequences and the distance between the ATRS and STAT3 binding sites, will require further extensive studies to provide more detailed definitions.

Our results (Figure 5C) indicate insufficient suppression of STAT3 activity by the anti-EGFR agent gefitinib. Skin biopsy from gefitinib-treated patients was found to contain insufficient suppression of STAT3 activity despite complete inhibition of EGFR activity (Albanell et al., 2001). In addition, STAT3 is constitutively activated in many human cancers and can take place in both EGFR-dependent and -independent fashions (Fernandes et al., 1999; Sriuranpong et al., 2003). Constitutive activity of STAT3-activating kinases, Src and Jaks, contributes to the EGF-independent activation of STAT3 in breast cancers (Garcia et al., 2001). We therefore reasoned that combined usage of agents targeting EGFR and Jak/STATs would result in a better therapeutic effect. Our cytotoxicity results indeed suggest a therapeutic advantage for the combined use of AG490 and AG1478, as well as AG490 and gefitinib, compared to the use of single agents in MDA-MB-468 cells. In support of our finding, a recent report showed a better cytotoxic effect in A431 cells when AG490 and AG1478 were used in combination compared to treatment with either agent alone (Dowlati et al., 2004).

A recent study (Bild et al., 2002) reported the cotransit of EGF and STAT3 from the cell surface to the perinuclear region via endosomes. Inhibition of endocytosis blocked this movement and subsequent STAT3 nuclear activity. Although the presence of EGFR in the complex was not investigated, EGF presumably remains bound to EGFR, which implies that EGFR may cotransit with STAT3 from the cytoplasmic membrane to the perinuclear region and subsequently enter the cell nucleus. Our data shown in Figure 5D certainly do not exclude this exciting possibility, but only suggest that STAT3 nuclear translocation can occur independently of the nuclear transport of EGFR, which requires the putative NLS. Although we do not have evidence showing that the EGFR-pNLS mutant is not defective in EGF-activated endocytosis, based on the study by Bild et al. (Bild et al., 2002) and our current study showing that EGFR-pNLS mutant undergoes autophosphorylation (Figure 5F), this mutant may retain the wild-type ability to undergo EGF-activated endocytosis and allow STAT3 to enter the cytoplasm, perinuclear region, and the nucleus. Further investigation is needed to examine this possibility.

In summary, the identified transcriptional complex consisting of EGFR and STAT3 may potentially represent a class of transcription factors that functionally interact at both cytoplasmic and nuclear levels, resulting in a direct transduction of extracellular signals from the cell surface to the nucleus. As iNOS serves as a direct target of the identified EGFR/STAT3 transcriptional complex, the deregulated iNOS/NO pathway may, in part, contribute to the malignant biology of tumor cells with high levels of nuclear EGFR and STAT3.

Experimental procedures

Cell lines and cell culture

A431 human epidermoid carcinoma cells, MDA-MB-468 human breast carcinoma cells, EGFR-null Chinese Hamster Ovary (CHO) cells, HaCaT human keratinocytes, and Hela human cervical carcinoma cells were obtained from ATCC. CHO-NEO, CHO-EGFR, and CHO-EGFR-pNLS stable cells were derived from the parental CHO cells. All cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, except that, in addition, CHO-NEO, CHO-EGFR, and CHO-EGFR-pNLS stable lines were supplemented with 1 mg/ml G418.

Transmission electron microscopy

Serum-starved MDA-MB-468 cells were treated with 100 ng/ml EGF for 30 min and subjected to EM analysis. Briefly, following incubation with both mouse and rabbit IgGs, cell sections were treated with specific monoclonal EGFR Ab (Zymed Laboratories) and polyclonal STAT3 Ab (C-20; Santa Cruz Biotechnology, Inc.). Cells were then incubated with gold particle (5 or 15 nm) labeled goat anti-mouse and anti-rabbit secondary Abs (Amersham Biosciences, USA), diluted 1:20 for 45 min. Sections were washed, stained with uranyl acetate for 2 min and Reynolds's lead citrate for 1 min, and examined in a Jeol 1200EX microscope.

Generation of EGFR-pNLS mutant-expressing vector and stable cells

To generate the pNLS mutant of EGFR, specific alanine mutations were constructed by site-directed mutagenesis, using the wild-type EGFR cDNA template that was cloned into the pBluescript phagemid (Stratagene). The selection primer, 5′-ATCGATACCGTCGACCTCGCCGCGGGGCCCGGTAC CCAATTC-3′, was designed to replace the XhoI site in the pBluescript phagemid with a SacII site. The mutagenic primer, 5′-CCTTCACACATAC TGCGGCGCTGGATCCACAGG-3′, replaced the putative NLS (^{645}RRR^{647}) with alanine residues (^{645}AAA^{647}). Positive clones were obtained and their nucleotide sequences confirmed and they were designated pBS-EGFR-pNLS. The wild-type EGFR and EGFR-pNLS mutant cDNAs were then subcloned into XbaI and HindIII sites of pcDNA3.1 (Invitrogen) and positive clones designated pcDNA3.1-EGFR and pcDNA3.1-EGFR-pNLS, respectively. The authenticity of both constructs was confirmed via nucleotide sequencing. To generate cells stably expressing the wild-type EGFR and EGFR-pNLS mutant, pcDNA3.1-EGFR and pcDNA3.1-EGFR-pNLS, respectively, were transfected into the parental EGFR-null CHO cells. The empty vector pcDNA3.1 was transduced into CHO cells as a negative control. Following a 1 month selection period using 1 mg/ml G418, approximately 40 clones were selected and examined for levels of EGFR using WB analysis. Stable cells expressing the empty vector, wild-type EGFR, and EGFR-pNLS were designated CHO-NEO, CHO-EGFR, and CHO-EGFR-pNLS, respectively.

Supplemental data

Supplemental Data include three figures, two tables, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at http://www.cancercell.org/cgi/content/full/7/6/575/DC1/.

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