HGF-independent Potentiation of EGFR Action by c-Met

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Abstract

The c-Met receptor is a potential therapeutic target for non-small cell lung cancer (NSCLC). Signaling interactions between c-Met and the mutant Epidermal Growth Factor Receptor (EGFR) have been studied extensively, but signaling intermediates and biological consequences of lateral signaling to c-Met in EGFR wild-type tumors is minimally understood. Our observations indicate that delayed c-Met activation in NSCLC cell lines is initiated by wild-type EGFR, the receptor most often found in NSCLC tumors. EGFR ligands induce accumulation of activated c-Met which begins at 8 h continues for 48 h. This effect is accompanied by an increase in c-Met expression and phosphorylation of critical c-Met tyrosine residues without activation of MAPK or Akt. Gene transcription is required for delayed c-Met activation; however, phosphorylation of c-Met by EGFR occurs without production of HGF or another secreted factor, supporting a ligand-independent mechanism. Lateral signaling is blocked by two selective c-Met tyrosine kinase inhibitors (TKIs), PF2341066 and SU11274, or with gefitinib, an EGFR TKI, suggesting kinase activity of both receptors is required for this effect. Prolonged c-Src phosphorylation is observed, and c-Src pathway is essential for EGFR to c-Met communication. Pre-treatment with pan-SFK inhibitors, PP2 and dasatinib, abolishes delayed c-Met phosphorylation. A c-Src dominant-negative construct reduces EGF-induced c-Met phosphorylation compared to control, further, confirming a c-Src requirement. Inhibition of c-Met with PF2341066 and siRNA decreases EGF-induced phenotypes of invasion by ~86% and motility by ~81%, suggesting that a novel form of c-Met activation is utilized by EGFR to maximize these biological effects. Combined targeting of c-Met and EGFR leads to increased xenograft anti-tumor activity, demonstrating that inhibition of downstream and lateral signaling from the EGFR-c-Src-c-Met axis might be effective in treatment of NSCLC.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Information

Supplementary information is available at Oncogene's website.

Keywords

c-Met; c-Src; EGFR; cross-talk

Introduction

EGFR is a receptor tyrosine kinase (RTK) that exhibits cellular action not only through ligands such as epidermal growth factor (EGF), transforming growth factor-alpha (TGF-α), and amphiregulin, but also through interactions with other RTKs and G-protein coupled receptors (Daub et al., 1997; Knowlden et al., 2005; Liu et al., 2007; Thomas et al., 2006). EGFR regulates well-characterized signaling cascades that modulate invasive growth, a program requiring precise coordination of growth, motility, and angiogenesis that is unregulated in human cancers (Ritter & Arteaga, 2003). Because of this, EGFR has been a strong candidate for therapeutic targeting with tyrosine kinase inhibitors such as gefitinib and erlotinib in many cancers, including non-small cell lung cancer (NSCLC). However, despite preclinical evidence demonstrating high efficacy of EGFR inhibitors, these expectations were unmatched in clinical settings (Molina et al., 2006). One reason that these EGFR inhibitors have not met expectations is due to compensatory signaling through mechanisms involving additional RTKs. Specifically, there has been strong evidence linking EGFR to activation of another prominent RTK involved in invasive growth, c-Met (Engelman et al., 2007).

C-Met interacts with the Hepatocyte Growth Factor (HGF) ligand, also identified as Scatter Factor, in a paracrine, endocrine, or autocrine manner (Bottaro et al., 1991; Nakamura et al., 1986; Naldini et al., 1991c; Stoker et al., 1987). Canonical activation of c-Met occurs when two c-Met monomers interact, inducing trans-autophosphorylation on tyrosine residues Y1230, Y1234, and Y1235 within the kinase domain. This activation leads to subsequent phosphorylation on tyrosine residues Y1349 and Y1356, which comprise the multi-substrate docking site, and allows recruitment of adaptor molecules responsible for downstream signaling such as Gab1 and c-Src (Ma et al., 2003; Naldini et al., 1991a; Naldini et al., 1991b; Ponzetto et al., 1994). Additionally, phosphorylation of Y1365 regulates cell morphogenesis, and Y1003 is required for internalization and degradation of c-Met through c-Cbl interaction (Peschard et al., 2001). The HGF-c-Met interaction is critical in a number of tightly controlled physiological processes such as tissue regeneration, in contrast to uncontrolled HGF/c-Met activation in tumorigenesis (Trusolino & Comoglio, 2002). Like EGFR, dysregulated HGF/c-Met signaling has been implicated in many cancers including NSCLC, and disruption of this pathway by c-Met-directed inhibitors decreases tumorigenic potential (Ma et al., 2005; Stabile et al., 2004; Stabile et al., 2008).

C-Met is frequently co-expressed with EGFR family members in human tumors, and it has been demonstrated that these RTKs can signal to one another (Fischer et al., 2004; Jo et al., 2000; Nakajima et al., 1999; Shattuck et al., 2008). Presnell *et al.* first showed that c-Met could be trans-activated by EGFR in rat liver epithelial cells constitutively expressing TGF-α (Presnell et al., 1997). Similar studies have since observed this signaling in NSCLC tumor models where EGFR contains activating mutations such as L858R or E746-A750del (Guo et

al., 2008). Additionally, lateral signaling to c-Met from EGFR has been identified in EGFR wild-type models, and much of this data suggests that cross-communication from EGFR to c-Met is dependent on EGFR or c-Met expression levels (Bergstrom et al., 2000; Ponzetto et al., 1991). To further validate this mechanism, Xu *et al*. recently demonstrated that induction of c-Met phosphorylation required upregulation of c-Met through EGFR-activated HIF-1α in both EGFR wild-type and mutant cell lines (Xu et al., 2010). Despite these increasing reports of EGFR –c-Met lateral signaling, the underlying mechanism of EGFR-induced c-Met phosphorylation is not well understood, and the extent of involvement of c-Met in carrying out EGFR-initiated phenotypes has not been well documented. Because c-Met signaling acts as an acquired resistance mechanism to EGFR TKI therapy, preclinical efforts are ongoing to augment c-Met targeted therapies with EGFR inhibitors to facilitate tumor growth inhibition (Engelman et al., 2007; Tang et al., 2008; Yano et al., 2008).

We previously demonstrated that exogenous PGE_2 stimulated invasion in our NSCLC model system through an intricate signaling axis requiring EGFR ligand production, c-Met, and the Src family kinases (SFK) (Siegfried et al., 2007). Here, we extend our studies to show that EGFR activation of c-Met in NSCLC cells is a delayed event that occurs not through HGF ligand production, but through an intracellular signaling cascade requiring gene transcription as well as tyrosine kinase activities of EGFR, the c-Src, and c-Met. We also found evidence for formation of a complex between c-Met and c-Src that was maximal at 24 h after EGF stimulation. Moreover, c-Met pharmacologic inhibition and siRNA knockdown reduced EGF-induced invasion and motility confirming that EGFR requires c-Met to maximize invasive phenotypes. In combining EGFR (gefitinib) and c-Met (PF2341066) tyrosine kinase inhibitors, we observed a statistically significant reduction in xenograft tumor growth of a NSCLC cell line expressing wild-type EGFR, compared to individual targeting. These results indicate that EGFR-induced, HGF-ligand independent delayed c-Met activation requires c-Src, and suggest that EGFR utilizes lateral signaling to c-Met for maximal stimulation of processes of invasive and motility that promote lung cancer.

Results

EGFR Ligands Induce Prolonged Phosphorylation and Increased c-Met Protein Levels in NSCLC Cells

It has been demonstrated that EGFR activation contributes to c-Met tyrosine phosphorylation in a variety of cell models, with varying kinetic parameters (Fischer et al., 2004; Xu et al., 2010) Since NSCLC commonly express both receptors, we examined whether EGFR ligands induce c-Met phosphorylation in human NSCLC cell lines. 201T and A549 cells were selected because of moderate expression levels of wild-type EGFR and wild-type c-Met, which is representative of the majority of NSCLC tumors found in patients (Fig. S1). C-Met also is not amplified in these cell lines (data not shown). Analysis of activated c-Met was performed following stimulation with ligands EGF, TGF-α, or HGF. Time-course analysis of EGF and TGF-α treated 201T cells revealed that c-Met phosphorylation first appears at 8 h, is sustained at maximal levels for 48 h, and decreases thereafter, while EGFR phosphorylation begins at 5 minutes and is completed by 1 h (Fig.

1a, Fig. S2, and Data not shown). In contrast, HGF stimulation induced c-Met phosphorylation that begins within 5 minutes and quickly terminates. (Fig. 1a). Similar results were obtained in A549 and H1435 cells (Fig. S3). EGFR ligand stimulation also resulted in a 1.5- (EGF) to 2.0- (TGF-α) fold increase in total c-Met protein levels (p<0.0005) and a 2–4 fold up-regulation of c-Met transcript levels at 24 h (p<0.005) (Fig. 1b and S4).

Pre-incubation with the pan-transcription inhibitor, Actinomycin D, prior to EGF administration abolished the increase in total c-Met protein and corresponding delayed phosphorylation of c-Met, while having no effect on direct activation by HGF (Fig. 1d). This demonstrates that a transcriptional component is required for EGFR trans-phosphorylation of c-Met and that the increase in c-Met expression is due to new c-Met transcription.

The individual c-Met tyrosine residues required for activation and cell signaling are well characterized. The initiating event for c-Met activation is phosphorylation at residues Y1230/Y1234/Y1235 followed by residues required for cell signaling such as Y1349, Y1365, and Y1003. To determine whether c-Met is being phosphorylated in response to EGFR ligands in a manner similar to HGF, we measured individual phospho-tyrosine levels of c-Met. It was confirmed that following EGF stimulation, all c-Met tyrosine residues analyzed were phosphorylated and followed a pattern similar to HGF stimulation, albeit at delayed time points (Fig. 1c). Together, these data suggest a novel mode by which EGFR can regulate a prolonged, full activation of c-Met through phosphorylation that persists for long periods and increased c-Met protein.

Kinase Activities of Both EGFR and c-Met are Necessary for c-Met Phosphorylation Induced by EGF

To ascertain the signaling cascade responsible for c-Met phosphorylation, use of the EGFR tyrosine kinase inhibitor (TKI), gefitinib, was employed to discern whether EGFR tyrosine kinase activity was required. Pretreatment with gefitinib abolished tyrosine phosphorylation of c-Met induced by EGF, while not inhibiting rapid c-Met tyrosine phosphorylation in response to HGF (Fig. 2a and 2c). Additionally, gefitinib also inhibited the accompanied increase in total c-Met protein following EGF treatment (Fig. 2a). No association was observed between EGFR and c-Met (Data not shown). These results confirmed that induction of an active EGFR by EGF was needed to facilitate c-Met phosphorylation.

We next addressed whether kinase activity of c-Met, itself, is required for delayed c-Met phosphorylation. To address this, 201T cells were pretreated with c-Met TKIs, SU11274 and PF2341066, and c-Met phosphorylation status was measured following EGF stimulation (Fig. 2b). These c-Met TKIs had no effect on EGFR autophosphorylation induced by EGF (Fig. 2d). C-Met inhibitors ablated all c-Met phosphorylation initiated by EGF, while having no effect on c-Met protein up-regulation, demonstrating that c-Met is not being utilized by EGFR as an adaptor-like molecule, but rather delayed activation of c-Met kinase activity is used to relay the EGFR signal (Fig. 2b).

C-Src is a Key Biphasic Mediator in Signal Transmittance from EGFR to c-Met

The Src family kinases (SFK) are a group of non-receptor tyrosine kinases including c-Src, which regulate downstream tumorigenic phenotypes (Klinghoffer et al., 1999) This family, particularly c-Src, has been identified as a key intermediary in regulating multiple steps for lateral RTK-RTK signaling (Popsueva et al., 2003). Because of these data and observations of c-Met tyrosine phosphorylation, we hypothesized that the SFKs are critical signaling molecules in delayed EGFR activation of c-Met in NSCLC. To identify the presence of SFK members, we assessed protein expression in the lung cancer cell lines. It was observed that c-Src was ubiquitously expressed across our NSCLC cells (Fig. S1). Activation of c-Src is known to be downstream of EGFR (Goi et al., 2000). Therefore, we assessed the response to EGF stimulation at delayed time points corresponding with c-Met phosphorylation by measuring levels of activated c-Src in 201T cells. The results suggest that EGF stimulation activated c-Src in a biphasic, and a continually increasing, manner (Fig. 3a). The initial activation of c-Src was present 2 h post-EGF stimulation and was subsequently reduced to near baseline levels by 4 h, while the second wave of greater activation was initiated 8 h post-treatment and lasted 48 h. This pattern of c-Src phosphorylation suggests a temporal association between delayed c-Src activation and delayed c-Met activation following EGF stimulation. PF2341066 treatment does not block delayed c-Src phosphorylation at 8 h–24 suggesting that all c-Src activation is upstream of c-Met (Fig. S5). C-Src plays a central role in mediating invasive phenotypes, and c-Src inhibition blocks all EGF-induced migration of NSCLC cells at 48 h (Fig. S6).

We explored whether inhibition of the SFKs could block EGFR-induced c-Met phosphorylation through the use of pan-SFK inhibitors, PP2 and dasatinib. Pretreatment with either PP2 or dasatinib blocked all EGF-induced c-Met phosphorylation while having minimal effect on increased total c-Met protein (Fig. 3b). In contrast, neither PP2 nor dasatinib showed any inhibitory effect on c-Met autophosphorylation (Fig. 3b) demonstrating that the SFKs are an intermediary needed for signaling from EGFR to c-Met. To discern whether c-Src, specifically, is a key mediator, 201T cells were stably transfected with either a dominant-negative c-Src construct (DN-Src) that reduced c-Src activity in both DN-Src-4 and DN-Src-9 clones by 28% and 66%, respectively compared to an empty vector (EV) plasmid as published previously (Data not shown) and (Liu et al., 2007). Stimulation of DN-Src cells for 24 h with EGF resulted in a 2 to 3-fold decrease in total c-Met phosphorylation compared to 201T EV cells (Fig. 3b). Through co-immunoprecipitation studies following 24 h EGF stimulation, we found that there is no evidence of a c-Src/c-Met or a c-Src/EGFR association up to 24 h suggesting that no persistent EGFR/c-Src/c-Met signaling complex is formed and that c-Src activity at 8 h–48 h may indirectly induce c-Met phosphorylation (Data not shown). This does not rule out the possibility that c-Src might directly phosphorylate c-Met through a transient interaction.

It has been hypothesized that c-Met dimerization and activation can result from increased c-Met density (Bergstrom et al., 2000; Ponzetto et al., 1991). Therefore, we determined whether c-Src activation through EGFR was required for elevated c-Met mRNA levels. Surprisingly, blockade of c-Src by PP2 had no significant effect on EGF-induced c-Met transcript levels suggesting that c-Src is not upstream of the modest increase in c-Met

protein, but may be upstream of other critical transcriptional events (Fig. 3c and Fig. 4d). These experiments advocate that c-Src is a candidate molecule that is likely involved at multiple points leading to initialization of EGFR –c-Met trans-activation, although the exact role remains unclear and participation of other SFKs in some of the effect cannot be excluded.

HGF Autocrine Signaling is not Responsible for EGFR-induced c-Met Phosphorylation

HGF autocrine loops have been observed in many tissue types; therefore, analysis of HGF production and secretion was performed at times corresponding with c-Met phosphorylation induced by EGF. 201T and A549 cells were treated with EGF under serum-free conditions and tissue culture media was harvested for HGF ELISA. No HGF secretion was detected at any time point, suggesting that autocrine signaling is not occurring (Fig. 4a). To confirm this observation, mRNA was harvested from 201T cells following exposure to EGF, and HGF levels were measured. The results from the qRT-PCR assay detected no presence of the HGF transcript produced in unstimulated or stimulated NSCLC cells. (Fig. 4b). It was also observed that media harvested from 24 h EGF-treated 201T cells did not induce c-Met phosphorylation on serum-deprived 201T cells up to 48 h, suggesting that no secreted factor is responsible for c-Met activation (Data not shown). A neutralizing antibody to HGF did not block EGF-induced phosphorylation of c-Met (Fig. 4c). Based on these data, HGF is not involved in EGF-initiated c-Met lateral signaling. Taken together, our findings favor an intracellular lateral signaling cascade from EGFR to c-Met that involves, and transcription of an as-yet unidentified protein that initiates delayed c-Met phosphorylation (Fig. 4d).

C-Met Inhibition Abrogates EGF-induced Invasive Growth Phenotypes

It is well-documented that both EGFR and c-Met initiate signaling cascades for invasive growth, and downstream signaling from each RTK involves similar molecules (Trusolino & Comoglio, 2002). Based on our observations of delayed c-Met activation by EGF, we hypothesized that c-Met mediates at least part of the EGFR-induced invasion and motility that leads to tumor progression. PF2341066 treatment significantly reduced EGF-induced 201T and A549 cellular invasion by 86% at 48 h (Fig. 5a). Cell motility through EGFR, as assessed by 48 h wound healing assay, was also inhibited with PF2341066 by 81% and 57% in 201T and A549 cells, respectively (Fig. 5b).

To confirm these findings, invasion and wound healing experiments were repeated with c-Met specific siRNA. Knockdown of c-Met by siRNA resulted in a 92% specific reduction of c-Met compared to the non-targeting siRNA (Fig. 5d). This knockdown resulted in similar results to PF2341066 in EGF-induced wound healing and invasion experiments, where a 77% and 53% reduction in EGF-induced invasion and wound healing was observed in 201T cells, respectively (Fig. 5c). Cell proliferation in response to EGFR ligand only increases cell number by about 25% at 24 h when maximal migration is inhibited. Knockdown of c-Met does inhibit this modest growth response, so it is possible that c-Met does participate in some EGFR-induced growth responses (Data not shown). Despite c-Met being phosphorylated at all tyrosines and required for EGFR phenotypes, delayed c-Met activation by EGF was not associated with MAPK and Akt phosphorylation, suggesting that these normal elements of c-Met signaling are not re-activated by c-Met at delayed time points

(Fig. 5e). These results indicate that EGFR utilizes lateral activation of c-Met to maximize cell motility and invasion through non-classical, delayed signaling mechanisms.

Combinational Targeting of c-Met and EGFR have Enhanced Anti-tumor Activity in a Xenograft Model of NSCLC

There is increasing evidence that c-Met compensatory signaling acts as a resistance mechanism against EGFR TKIs. In particular, Engelman, *et al.* has established that c-Met signaling was a driver of acquired EGFR TKI resistance in EGFR mutant lung cancer cells (Engelman et al., 2007). NSCLC with wild-type EGFR are also inherently resistant to EGFR TKIs. The identification of delayed c-Met activation in NSCLC cells with wild-type EGFR, as in the case of a majority of NSCLC patients, provides a rationale for combining therapies to improve response to EGFR TKIs. To address whether combinational targeting of EGFR and c-Met pathways leads to enhanced anti-tumor effects, athymic nude mice bearing 201T flank tumors were treated with either gefitinib, PF2341066, combination, or vehicle for 5 d/wk for 3 weeks. PF2341066 alone at a dose of 50 mg/kg had no significant effect on inhibiting tumor xenograft growth, whereas gefitinib significantly reduced tumor volume by 51%. Combining TKI therapies produced a 66% decrease in tumor volume; a significantly greater effect compared to PF2341066 and gefitinib groups, individually (Fig. 6a). EGFR and c-Met targets were confirmed in treatment groups by immunohistochemistry (Fig. S7). Ki-67 staining of tumors at 28 days revealed that the combination group had a significantly greater effect compared to individual PF2341066 and gefitinib treatments (Fig. 6b). Although minimal inhibition of xenograft tumor volume was observed with PF2341066 treatment alone, a significant decrease in Ki-67 staining was measured (Fig. 6a and 6b). This is likely due to stromal tissue comprising 40% of tumor volume in the PF2341066 treated group. Stromal cells appear not to be actively proliferating as shown in Figure 6b. Because remodeling of tumor architecture follows PF2341066 treatment, volume measurements likely underestimate the true anti-tumor effect.

Discussion

We present findings characterizing a novel mode of delayed lateral communication from EGFR to c-Met that is dependent on c-Src and mediates invasion and cell movement. These findings provide an alternative-working model of c-Met activation by EGFR in NSCLC cell lines expressing wild-type, non-amplified EGFR and c-Met. Here, EGFR ligands induce prolonged c-Met tyrosine phosphorylation after a pronounced delay that is not attributable to an HGF-ligand autocrine production. Additionally, we have ruled out the secretion of other factors that might trigger c-Met phosphorylation because conditioned media experiments could not replicate the EGF-induced effect. While increased c-Met transcription was observed following EGF treatment of NSCLC cells, along with a modest increase in total c-Met protein, this increase in c-Met protein levels appears not to be solely responsible for the delayed c-Met phosphorylation because it is independent of c-Src activity. Based on a recent report, the rise in protein level may be attributed to induction of new c-Met transcripts through EGFR activation of the HIF-1α pathway (Xu et al., 2010). Moreover, studies in thyroid carcinoma and human gastric carcinoma cells suggested that increased c-Met levels alone might lead to receptor activation, but conclude that there was a requirement of

additional factors to achieve phosphorylation (Bergstrom et al., 2000; Ponzetto et al., 1991). Although it has been shown that c-Src can signal to HIF-1α, c-Src is minimally involved in our model because PP2 had almost no effect on c-Met protein and mRNA levels following EGF stimulation (Karni et al., 2002). This suggests that in the NSCLC cell studies, c-Src-dependent c-Met phosphorylation is a distinct event from c-Met upregulation. We cannot, however, rule out that *de novo* c-Met molecules are also contributing to this novel pathway in some capacity. This is the first time c-Src has been implicated as a critical intermediary linking EGFR activation to c-Met phosphorylation.

Activation of invasion and motility are hallmarks of EGFR signaling that drive lung tumor progression (Ritter & Arteaga, 2003). We showed that EGFR activation of invasive phenotypes rely largely on c-Met signaling through an HGF-independent pathway. A possible explanation for this is that EGFR utilizes c-Met as a second-in-line molecule not to initiate response, but to amplify the complex phenotypic programs following early activation by EGFR. We also anticipate that an alternative c-Met cascade is being activated at delayed time points, as MAPK and Akt are not phosphorylated at time points corresponding to c-Met phosphorylation. This mode of signaling might occur through intracellular relocalization of a distinct population of c-Met molecules that do not associate with the Gab1 adaptor protein that is known to be required for prolonged MAPK activation (Cai et al., 2002). Early, but not yet reproduced confocal microscopy studies, point to an intracellular compartmentalization of c-Met molecules 24 h following EGF addition. Sequestering of c-Met might stabilize c-Met and allow for an altered mode of signaling. Future studies will aim to measure the levels of effectors responsible for the late-acting signaling from c-Met such as E-cadherin, focal adhesion kinase, and other potential candidates downstream of c-Met that regulate invasive phenotypes (Kim et al., 2009).

Our results suggest a model for lateral signaling from EGFR to c-Met that involves intracellular intermediates and requires both gene transcription and extended c-Src activation that is upstream of c-Met. The fully phosphorylated c-Met molecules that accumulate at delayed time points do not activate common downstream mediators such as MAPK and Akt. The observed temporal association between c-Met and c-Src activation suggests they might cooperate and lead to prolonged activation of regulatory molecules responsible for invasion and cell motility downstream of both c-Met and c-Src (Fig. 4d). We hypothesize that after EGFR stimulation, c-Src activates a signaling cascade that leads to transcription of an unknown intermediate accompanying the c-Met protein increase. This intermediary might also be required for late c-Src activation. It does not appear that a complex between c-Met and c-Src forms at these late time points. Having ruled out secreted factors, a probable scenario for late c-Src activation involves the production of molecules that shift c-Src toward increased phosphorylation status such as increased association with Src homology-2 domain-containing proteins or increased phosphatase activity to remove inhibitory phosphorylation. It is possible that increased integrin production or clustering might account for both c-Met and c-Src events as both kinases demonstrate increased phosphorylation in response to integrin interaction with the substratum (Danilkovitch-Miagkova et al., 2000; Moro et al., 2002; Wang et al., 1996). The c-Met and c-Src events might be linear or parallel with one another and future studies will aim to determine whether

integrins have a role in delayed c-Met phosphorylation by EGFR ligand (Pawson & Scott, 1997).

An alternate model for c-Met activation was considered, where c-Src might directly phosphorylate c-Met. Here, we showed that although delayed c-Src activation is coupled with delayed c-Met phosphorylation, no stabilized association between c-Src and c-Met was observed. These data conflict with such a model, and suggest that c-Src is not directly phosphorylating c-Met, implying an additional mediator is needed for increased c-Met phosphorylation. It is still possible that c-Src might regulate more than one event in this pathway due to prolonged c-Src phosphorylation.

Clinical responses to EGFR tyrosine kinase inhibitors (TKI) in NSCLC patients have produced variable results (Molina et al., 2006). In East Asian populations, where there is a high rate of EGFR mutation (~40%), EGFR targeted therapies give a good response. In contrast, patients from western countries tend to have a low frequency of EGFR mutations (3–8% of all lung tumors) and respond poorly to EGFR TKIs (Wu et al., 2008). In studies reported here, we focused our efforts on EGFR –c-Met communication in NSCLC cell lines expressing wild-type EGFR and c-Met. Through identification of a dual-regulation paradigm of tumorigenic signaling from EGFR to c-Met, we further show that combining EGFR and c-Met TKIs provided an enhanced inhibition of tumor growth and a concurrent reduction in Ki-67 staining in 201T xenograft tumors. These results suggest a rationale for simultaneous targeting of EGFR and c-Met to short-circuit signaling cascades responsible for invasive growth initiated and maintained through both receptors as well as trans-activation from EGFR to c-Met.

In summary, we present findings identifying a signaling requirement for c-Met to achieve maximal EGFR-induced invasion and motility, that occurs through delayed events involving c-Src. Delayed c-Met signaling occurs through a non-canonical pathway. Targeting c-Met in addition to EGFR leads to an enhanced anti-tumor effects in a wild-type, non-amplified EGFR and c-Met cell line. The findings presented in this study provide insight regarding the manner by which the EGFR–c-Src–c-Met axis regulates lung tumor progression through varied temporal modulation. Future studies will seek to clarify the exact role of c-Src and which other potential signaling intermediaries are active in EGFR-c-Met lateral signaling as well as to identify what effector molecules downstream of delayed c-Met signaling regulate invasion and motility in NSCLC in response to EGFR ligands.

Materials and Methods

Reagents and Cell Culture

NSCLC cell lines 201T were established in our laboratory from primary tissue and, A549 and H1435 were obtained from American Type Culture Collection (Manassas, VA) and maintained at 37°C in 5% CO_2 (Siegfried et al., 1999). All cells were grown in Basal Medium Eagle (BME) with 10% fetal bovine serum and 2 mM L-Glutamine. Rabbit polyclonal anti-pY1349-Met, anti-pY1365-Met, anti-Y1003-Met, and anti-pY418-Src as well as mouse monoclonal anti-c-Src were obtained from Invitrogen (Carlsbad, CA). Rabbit anti-c-Met (C-28), mouse anti-c-Src (B-12) and mouse anti-pY99 were purchased from

Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-c-Met, rabbit anti-EGFR, rabbit anti-Y1234/35-Met and anti-rabbit and anti-mouse-HRP linked secondary antibodies were obtained from Cell Signaling (Beverly, MA). Anti-EGFR, mouse, antibody was purchased BD Transduction Laboratories (San Jose, CA). Dasatinib and gefitinib were from Chemitek (Indianapolis, IN). Actinomycin D, SU11274, and PP2 were purchased from Calbiochem (San Diego, CA). The c-Met and non-targeting siRNA ON-TARGET*plus* SMARTpools were purchased from Dharmacon (Lafayette, CO). EGF, TGF-α, HGF, HGF neutralizing antibody, and non-immune IgG control were purchased from R&D Systems (Minneapolis, MN).

Cell Treatments, Immunoprecipitation, and Immunoblotting

Cells were washed with phosphate-buffered saline (PBS) and lysed with NP-40 buffer as described previously (Stabile et al., 2008). The insoluble fraction was cleared by centrifugation at $10000 \times g$ for 15 min at 4°C. Protein concentrations were measured by Bradford Assay (Pierce). For immunoprecipitation, equal protein was subjected to pre-clearing and immunoprecipitation with antibodies overnight at 4°C then incubated with Protein A-Agarose Beads (Thermo Fisher Scientific, Rockford, IL) for 2 h at 4°C. Immunoblotting was performed as described previously (Stabile et al., 2008). For reprobing, blots were stripped using IgG Elution Buffer (Thermo Fisher Scientific). Blots were quantified by densitometry and ImageJ analysis (National Institutes of Health, Bethesda, MD). For immunoprecipitations, all phospho-proteins were normalized to respective total immunoprecipitated protein, and whole cell lysate proteins utilized β-actin for quantitation.

Human HGF ELISA

Cell culture media was harvested and analyzed in duplicate by Quantikine Human HGF enzyme-linked immunosorbent assay (ELISA; R&D Systems) according to the manufacturer's instructions. Conditioned media from fibroblasts was used as a positive control for HGF.

Quantitative Real Time-PCR

Total RNA was extracted using the RNAeasy kit (Qiagen, Valencia, CA) according to manufacturer's directions. cDNA was synthesized by a single reverse transcription reaction in a thermocycler. The primers and probes used for detection of HGF, c-Met and β-glucuronidase are provided in supplementary data.

Wound Healing Assay

Cells were plated on 12-well plates, grown to a confluent monolayer, and serum-deprived for 24 h. Wounds were created and washed twice with 1X PBS. All treatments were applied every 24 h over the experimental time course. Three wells per experimental treatment were examined 10X magnification by light microscope. Migration distance was assessed by measuring the ability of cells to close the wound area.

Matrigel Invasion Assay

Growth Factor Reduced Matrigel-coated Transwell chambers (BD Biosciences) were activated in serum-free media at 37°C for 2 h. NSCLC cells (1×10^4) were serum-deprived for 24 h and plated on transwell chambers in 1% charcoal-stripped FBS containing media in the top chamber containing indicated inhibitor treatments. In the bottom chamber, growth factors and inhibitors were refreshed every 24 h. Non-invading cells in the top chamber were removed by cotton swab, and invading cells were fixed and stained using Diff-Quik staining solutions according to the manufacturer's instructions (VWR International). The number of invading cells was counted at 10X magnification.

siRNA Transfection

201T cells were transfected with 50 pmol of either c-Met or non-targeting siRNA pools using Oligofectamine (Invitrogen) for 8 h. Transfection medium was replaced overnight followed by experimental treatments.

In vivo Tumor Xenograft Model

Female athymic nude-Foxn1nu mice were obtained from Harlan (Somerville, NJ). 201T cells were suspended in serum-free PBS supplemented with 50% Matrigel (BD Biosciences). Cells (2×10^6) were injected in the hind flank region of each mouse and allowed to develop. Ten days after tumor implantation the mice were divided into 4 treatment groups (10 mice per group): (a) placebo, (b) PF2341066 (c) gefitnib (d) PF2341066 plus gefitnib. Treatment with PF2341066 (50 mg/kg) or vehicle control (0.9% saline/1% Tween-80) was administered daily by oral gavage and gefitnib (150 mg/kg) was administered twice a week by oral gavage for 3 weeks. Tumor size was measured weekly and reported as an average relative tumor volume calculated as ($length \times width \times height \times$ p)/2 (mm^3). At the end of the treatment period, animals were sacrificed; tumors were removed, and fixed in 10% buffered formalin for immunohistochemical analysis. Animal care was in strict compliance with the institutional guidelines established by the University of Pittsburgh.

Statistical Analysis

All values are expressed as the mean ± S.E.M. Student's *t* test except for the tumor xenograft experiment when an unpaired *t-test* with *Welch's correction* was utilized. Significance tests were performed with a two-sided significance level of 0.05.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations

EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
HGF	hepatocyte growth factor
NSCLC	non-small cell lung cancer
SFK	Src Family Kinase
TKI	tyrosine kinase inhibitor
TGF-α	transforming growth factor-alpha

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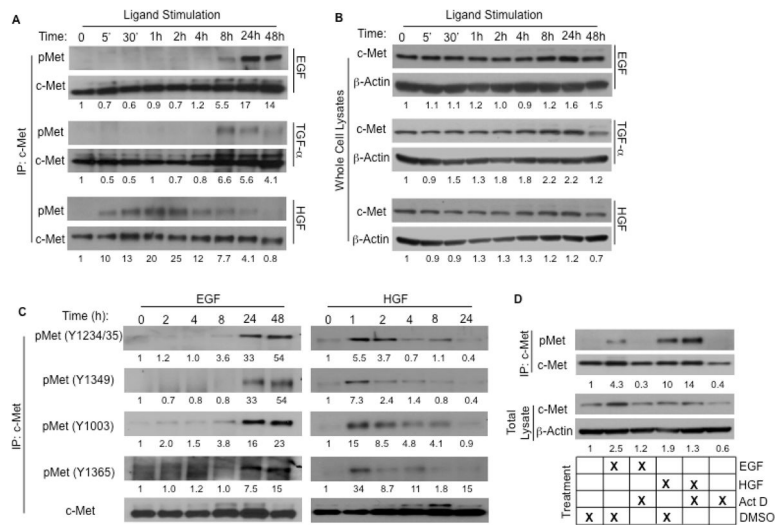
Figure 1.
EGFR ligands induce c-Met activation. (A and B) 201T cells were serum-deprived for 2 days prior to stimulation with EGF and TGF-α (10 nM) for 0–48 h followed by immunoprecipitation (IP) with c-Met and western blotting with pY99, total c-Met, and β-Actin antibodies. (C) Specific c-Met phosphorylation sites were measured by c-Met IP then immunoblotting with phospho-c-Met antibodies: Y1003, Y1234/35, Y1349, and Y1365. All blots were stripped and reprobed with a total c-Met antibody to confirm equal loading. (D) 201T cells were serum starved 2 days, pretreated with actinomycin D (0.01 mg/mL), and treated with either EGF (10 nM) for 24 h or HGF (10 ng/mL) for 5 min. Cell lysates were analyzed for c-Met tyrosine phosphorylation and expression.
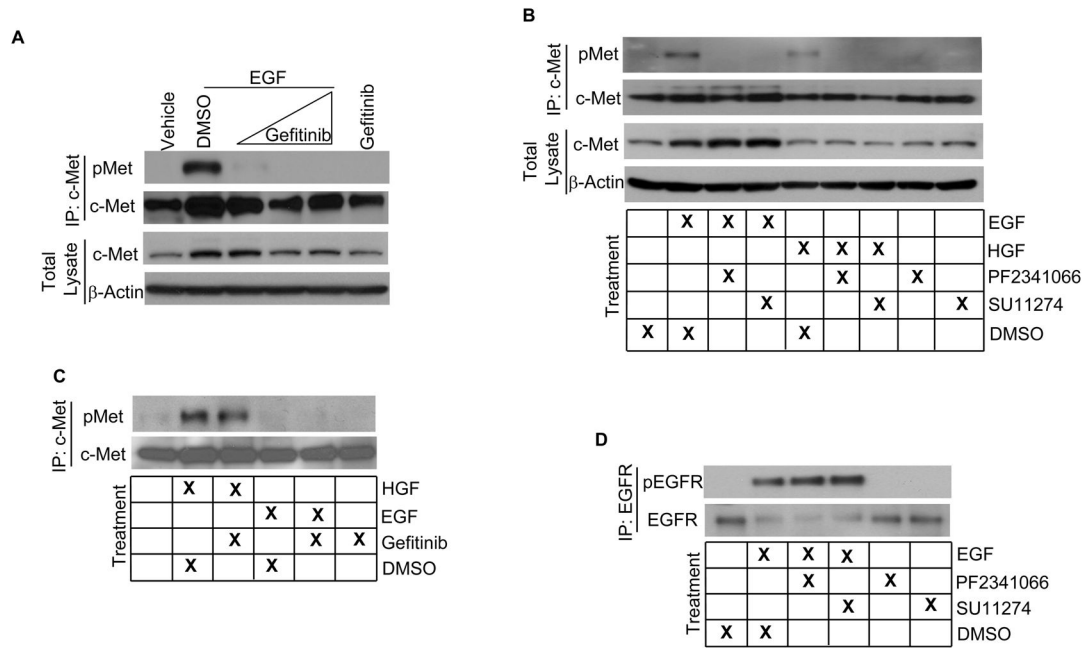
A

B

C

D

Figure 2.

EGFR and c-Met kinase activity is required for EGF-induced c-Met phosphorylation. 201T cells were serum deprived for 2 days, pretreated with indicated inhibitors, and stimulated with EGF (10 nM) for 24 h or HGF (10 ng/mL) for 5 min. Cell lysates were analyzed for c-Met tyrosine phosphorylation and expression. (A) Pretreatment with gefitinib (100 nM, 1 μM, 10μM) occurred for 2 h, and cells were stimulated with respective ligands. (B) After 2 h pretreatment with the c-Met inhibitors, SU11274 (1 μM) or PF2341066 (1 μM), cells were growth factor treated. (C) Cells were pretreated with gefitinib (10 μM) followed by stimulation with either HGF (10 ng/mL) or EGF (10 nM) for 5 min. Cell lysates were immunoprecipitated for c-Met and probed for pY99 and total c-Met. (D) 201T cells were pretreated with either SU11274 or PF2341066 (1 μM) followed by stimulation with EGF (10 nM) for 5 min. Cell lysates were immunoprecipitated for EGFR and probed for pY99 and total EGFR.
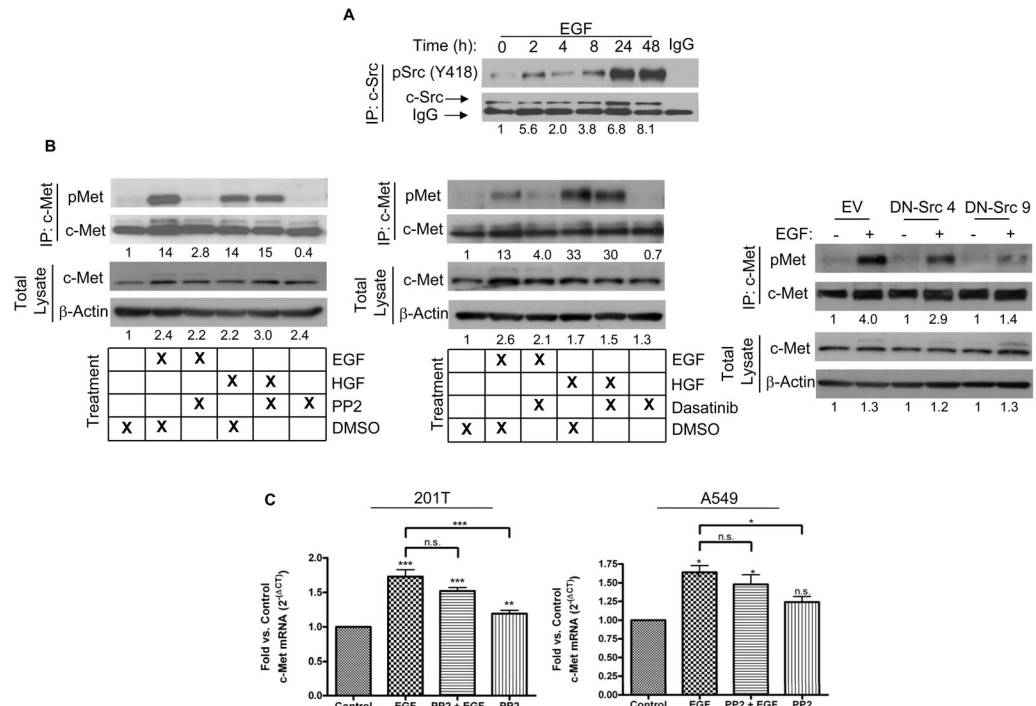
Figure 3.
C-Src mediates EGFR-induced c-Met phosphorylation. 201T cells were serum deprived for 2 days followed by addition of EGF (10 nM) for 0–48 h. (A) Cell lysates were immunoprecipitated for total c-Src and analyzed for phospho-c-Src (Y418). (B) 201T cells were pretreated for 2 h with SFK inhibitors PP2 (500 nM) or dasatinib (50 nM) prior to 24 h EGF (10 nM) or 5 min HGF (10 ng/mL) stimulation, or were stably transfected with either a dominant-negative c-Src construct (DN-Src) or empty vector (EV) and stimulated with EGF. Cell lysates were prepared and analyzed for phospho- and total c-Met levels. (C) A549 and 201T cells were pretreated for 2 h with PP2 (500 nM) prior to 24 h EGF stimulation, mRNA was harvested, and subjected to c-Met quantitative RT-PCR using β-Gus as an internal control.
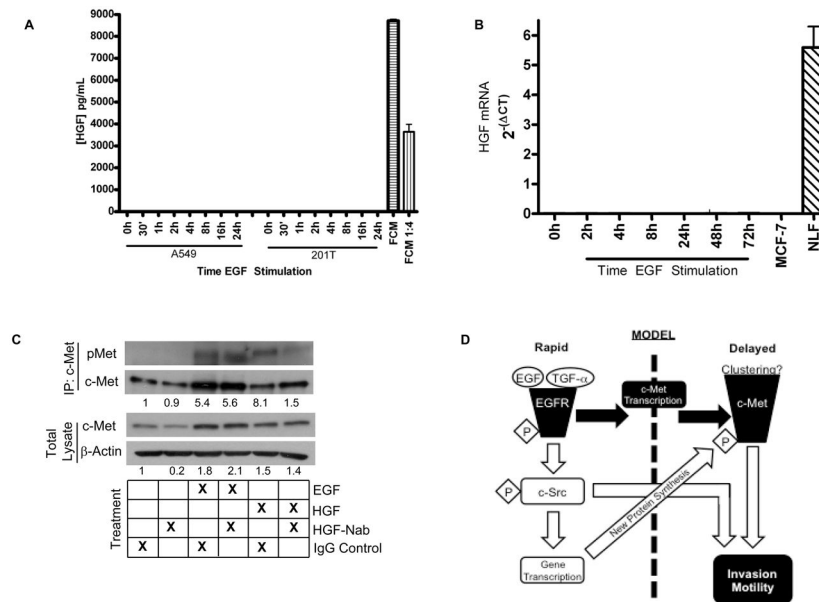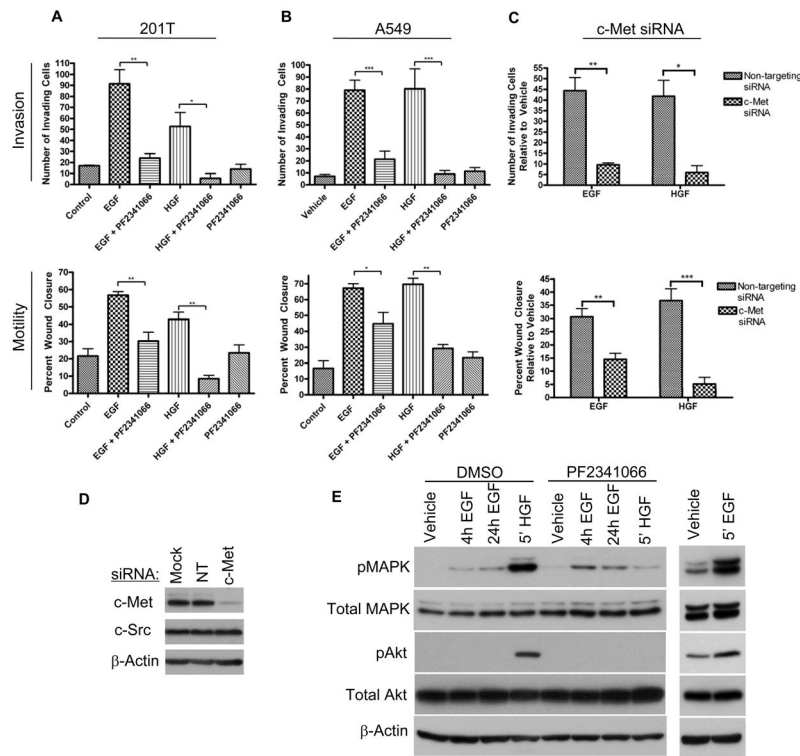
Figure 4.
EGFR activation of c-Met does not require HGF production or secretion. A549 and 201T cells were serum starved 2 days prior to stimulation with EGF (10 nM). (A) Tissue culture media was harvested for 0–24 h time points and subjected to HGF ELISA with fibroblast-conditioned media (FCM) as a positive control. (B) Cells were treated, mRNA was harvested, and subjected to HGF quantitative RT-PCR using β-Gus as an internal control. Normal lung fibroblast (NLF) mRNA was utilized as a positive control while the breast cancer cell line, MCF-7, was used as a negative control for HGF expression. (C) EGF (10 nM) or HGF (10 ng/mL) was pretreated for 2 h with HGF neutralizing antibody or IgG control (300 ng/mL) prior to addition to 201T cells for 24 h. Cell lysates were prepared and analyzed for phospho- and total c-Met levels. (D) Proposed model of delayed c-Met activation. In NSCLC cells, EGFR ligands induce a delayed accumulation of tyrosine phosphorylated c-Met receptors as well as an increase in total c-Met levels. EGFR ligands activate EGFR tyrosine kinase activity, which leads to rapid c-Src signaling and new gene transcription that is required for EGFR-c-Met lateral communication. At delayed time points, activated c-Src continues to accumulate, and c-Met is phosphorylated to maximize EGFR-activated phenotypes of invasion and motility. Production of unidentified proteins might cooperate with c-Src to cause c-Met phosphorylation. This could occur by promoting the clustering of c-Met molecules.

Figure 5.
EGFR relies on c-Met for maximal induction of EGFR phenotypes. 201T and A549 cells were serum deprived for 24 h prior to all 48 h phenotypic assays stimulated with EGF (50 ng/mL) or HGF (50 ng/mL). For experiments involving PF2341066 (1 μM), cells were pretreated with the c-Met inhibitor or DMSO for 2 h. For c-Met siRNA transfection experiments, 201T cells were plated and subjected to non-targeting siRNA or c-Met siRNA. Following transfection, cells were serum deprived in 24-well Matrigel invasion chambers or 12-well plates for wound healing assays. (A) Cells were plated in Matrigel invasion chambers with growth factors added to the lower chamber only. Invading cells of three independent experiments were counted. (B) Cells were grown on 12-well plates prior to serum starvation and wounding. Addition of ligands followed c-Met inhibitor pretreatment or siRNA knockdown. Wounds were imaged at 0 and 48 h by 10X light microscopy. Migrating cells were measured by comparing 48 h wound size to initial wound size and expressed as percent wound closure. Mean of three independent samples per treatment group. (C) Invasion and wound healing assays were repeated in 201T cells with c-Met or non-targeting siRNA knockdown as described previously. Mean of three independent samples per treatment group. (D) 201T cells were mock transfected or treated with either non-targeting siRNA (NT) or c-Met siRNA for 8 h. After 48 h, cell lysates were prepared and analyzed for total c-Met levels, c-Src, and β-Actin. (E) 201T cells were serum-deprived for 2 days prior to pretreatment with PF2341066 (1 μM) followed by stimulation with either EGF (10 nM) or HGF (10 ng/mL). In separate experiments, cell lysates were analyzed for MAPK and Akt phosphorylation and expression. ***, $P < 0.0005$; **, $P < 0.005$; *, $P < 0.05$ Student's *t* test.
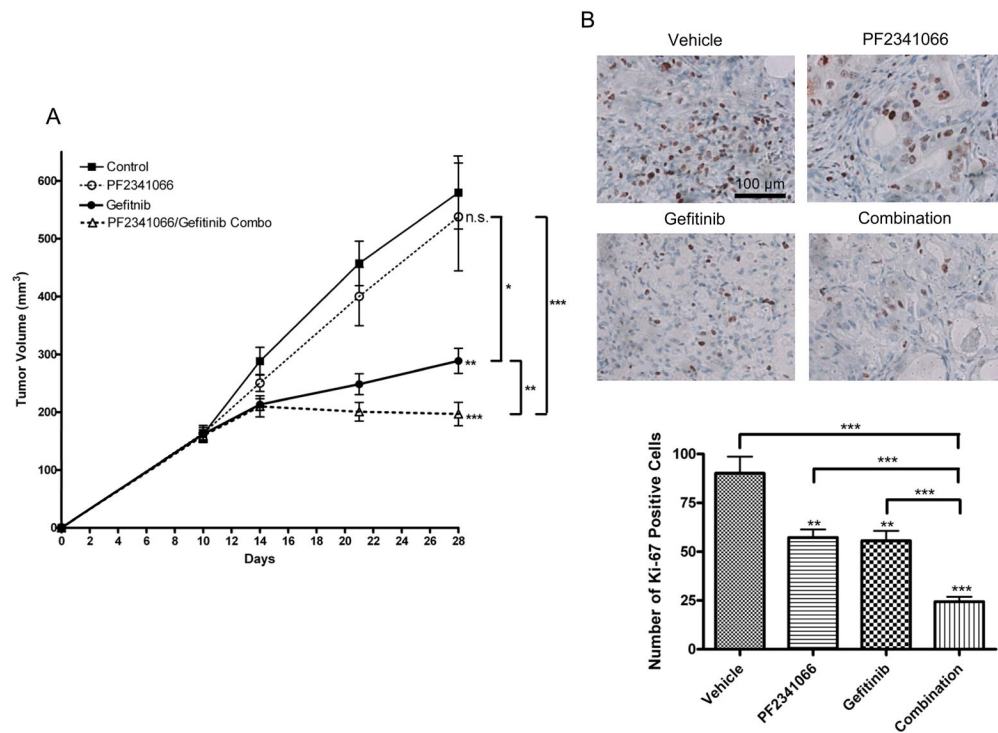
Figure 6.
Simultaneous targeting of EGFR and c-Met causes enhanced 201T xenograft tumor growth inhibition. (A) 201T cells (2×10^6) were injected into nude mice on day 0. On day 10, tumors were measured and experimental treatments were initiated. PF2341066 (50 mg/kg), gefitinib (150 mg/kg), and vehicle control (0.9% saline/1% Tween-80), were administered daily by oral gavage until day 28. (B) Representative Ki-67 stained sections of xenograft tumor imaged at 20X magnification. Quantitation of Ki-67 staining was performed by counting 5 fields at 40X magnification. $n = 5$; ***, $P < 0.0005$; **, $P < 0.005$; *, $P < 0.05$ Student's *t* test with Welch correction. Everything is compared to the vehicle except where indicated.