Parallel RNA Interference Screens Identify EGFR Activation as an Escape Mechanism in FGFR3-Mutant Cancer

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ABSTRACT

Activation of fibroblast growth factor receptors (FGFR) is a common oncogenic event. Little is known about the determinants of sensitivity to FGFR inhibition and how these may vary between different oncogenic FGFRs. Using parallel RNA interference (RNAi) genetic screens, we show that the EGF receptor (EGFR) limits sensitivity to FGFR inhibition in FGFR3-mutant and -translocated cell lines, but not in other FGFR-driven cell lines. We also identify two distinct mechanisms through which EGFR limits sensitivity. In partially FGFR3-dependent lines, inhibition of FGFR3 results in transient downregulation of mitogen-activated protein kinase signaling that is rescued by rapid upregulation of EGFR signaling. In cell lines that are intrinsically resistant to FGFR inhibition, EGFR dominates signaling via repression of FGFR3, with EGFR inhibition rescued by delayed upregulation of FGFR3 expression. Importantly, combinations of FGFR and EGFR inhibitors overcome these resistance mechanisms in vitro and in vivo. Our results illustrate the power of parallel RNAi screens in identifying common resistance mechanisms to targeted therapies.

SIGNIFICANCE: Our data identify a novel therapeutic approach to the treatment of FGFR3-mutant cancer, emphasizing the potential of combination approaches targeting both FGFR3 and EGFR. Our data extend the role of EGFR in mediating resistance to inhibitors targeting a mutant oncogene, showing that EGFR signaling can repress mutant FGFR3 to induce intrinsic resistance to FGFR targeting. Cancer Discov; 3(9); 1058–71. © 2013 AACR.

INTRODUCTION

Activating mutations of the fibroblast growth factor receptors (FGFR) are found in multiple cancer types, with the highest prevalence occurring with FGFR3 mutations in bladder cancer (1) and FGFR2 mutations in endometrial cancer (2, 3). In other cancer types, activation of FGFRs occurs predominantly through receptor gene amplification, with FGFR1 amplification in squamous lung and breast cancer (4, 5), and FGFR2 amplification in gastric and breast cancers (6, 7).

Further mechanisms of activation include activating translocations involving the FGFRs, described initially in hematologic malignancies although recently also described in solid tumors (8, 9), and FGF ligand–mediated signaling (10). Preclinical studies have suggested that activated FGFRs are potential therapeutic targets (2, 3, 6, 11–13), and multiple FGFR inhibitors have entered clinical trial with early evidence of efficacy in FGFR1-amplified breast and lung cancer (14, 15). Yet, it is not clear what determines whether cancers will respond to FGFR inhibitors, what the mechanisms of
resistance will be, and how this may vary between different oncogenic receptors and cancer types. This presents a major limitation to the clinical development of FGFR inhibitors, as it is unclear which of the diverse mechanisms of activation of the FGFRs are most likely to translate to clinical efficacy.

RNA interference (RNAi) screens have substantial potential to elucidate the determinants of sensitivity to cancer therapies (16–18), identifying both mechanisms of resistance (17) and key pathways that determine sensitivity (18). Here, we use parallel siRNA screens to identify determinants of sensitivity and mechanisms of resistance to FGFR inhibition in the protein kinome/phosphatome, along with a panel of amplified and mutant cancer cell lines to identify mechanisms specific to different FGFR mutations and amplifications. Through this approach, we identify the EGF receptor (EGFR) as a major factor limiting the efficacy of targeting FGFR3 mutations.

RESULTS

High-Throughput Kinome/Phosphatome Screens

To identify the determinants of sensitivity to FGFR inhibitors, we conducted high-throughput parallel siRNA screens using a library targeting all known protein kinases and phosphatases in a panel of 11 FGFR-amplified, -mutant, or -translocated cell lines (Fig. 1A). Such parallel siRNA screens allow for comparison between different oncogenic aberrations and have the potential to identify key mutation- or subtype-specific mechanisms of resistance. The screening panel represented the most common FGFR aberrations observed in carcinomas, including cell lines with FGFR amplification (JMSU1 and H1581), FGFR2 amplification (MFM223, SUM52, SNU16, KATOIII, and OCUM2M), FGFR2 mutation (AN3CA), and FGFR3 activation (FGFR3 point-mutated 97-7 and MGHU3, and RT112M that has an activating FGFR3-TACC3 fusion; Supplementary Table S1). Cell lines were transfected with the siRNA library in triplicate, and 48 hours later, half of the plates were treated with the cell line EC50 dose of the pan-FGFR inhibitor PD173074 and half with vehicle for 72 hours (Fig. 1A and C). Vehicle control plates were used to examine the effect of siRNA on cell survival/growth, and the relative growth in plates exposed to PD173074 versus vehicle was used to identify siRNA that altered sensitivity to PD173074 (Fig. 1A).

Across the panel of FGFR-driven cell lines, FGFR1-amplified and FGFR2-amplified/mutated cell lines were selectively sensitive to the corresponding siRNA (Fig. 1B), with FGFR2-amplified cell lines, in particular, being strongly addicted to FGFR2. Similarly, across the panel of cell lines, silencing of FGFR1 or FGFR2 was epistatic to FGFR inhibition in the corresponding cell lines (Supplementary Fig. S1A). Unexpectedly, a similar effect was not seen with FGFR3 siRNA in the FGFR3-activated cell lines, with FGFR3 siRNA having little
High-throughput siRNA kinome/phosphatome to identify genes required for the growth of FGFR-driven cell lines. **A**, schematic of siRNA screen. Cells were reverse transfected in 384-well plates with siRNA SMARTpools targeting all known protein kinases and phosphatases, and 48 hours later exposed to PD170374 at EC50 or control, and survival assessed after 72 hours of exposure. The effect of siRNA on survival was assessed on the vehicle plates (survival effect), and the effect of siRNA on sensitivity to PD173074 was assessed as the differential effect of siRNA according to the panel of screened cell lines to a range of concentration of PD173074. **B**, survival effect of FGFR2 amp, FGFR2 mut, FGFR3 mut, and FGFR1 amp. Black bars indicate those siRNA with a significant effect in cell survival, defined as a Z score < -2. **C**, relative growth of FGFR3 mutant cell lines compared with vehicle (sensitivity to PD173074). Both analyses were expressed as a Z score. **D**, schematic of siRNA screen. Cells were reverse transfected in 384-well plates with siRNA SMARTpools targeting all known protein kinases and phosphatases, and 48 hours later exposed to PD170374 at EC50 or control, and survival assessed after 72 hours of exposure. The effect of siRNA on survival was assessed on the vehicle plates (survival effect), and the effect of siRNA on sensitivity to PD173074 was assessed as the relative growth in PD173074 plates compared with vehicle (sensitivity to PD173074). Both analyses were expressed as a Z score. **E**, survival effect of FGFR1, FGFR2, and FGFR3 siRNA according to FGFR gene mutation/amplification; left, FGFR1-amplified; middle, FGFR3 mutation; right, FGFR2-amplified and -mutated (AN3CA). Black bars indicate those siRNA with a significant effect in cell survival, defined as a Z score < -2. **C**, relative growth of the panel of screened cell lines to a range of concentration of PD173074, **B**, supervised clustering of the siRNA effect on sensitivity to PD173074, with the differential effect of siRNA according to FGFR3 mutational status. Displayed are siRNA with a permutation P value less than 0.05 ordered by mean effect in the FGFR3-activated cell lines. **E**, top, EGFR siRNA increased sensitivity to PD173074 specifically in FGFR3-activated cell lines, P = 0.001, Student t test. Bottom, survival effect of EGFR siRNA. EGFR siRNA significantly reduced the survival of the 97-7 cell line.
or no effect on cell survival (Fig. 1B). FGFR3 cell lines were also noted to be relatively insensitive to PD173074 (Fig. 1C), potentially suggesting the existence of alternative drivers of proliferation in the FGFR3-activated cell lines, and here we focus on identifying the alternative drivers and mechanisms of resistance in these cell lines.

**FGFR Is a Key Mediator of Resistance in FGFR3-Activated Cell Lines**

We set out to identify what determined sensitivity in FGFR3-activated cell lines and the factors that limited the sensitivity of these cell lines to FGFR inhibition. To identify features common to all three FGFR3-activated cell lines, we conducted supervised hierarchical clustering to identify the siRNA that differentially modulated sensitivity to PD173074 in FGFR3-activated cell lines compared with the other FGFR-driven cell lines (Fig. 1D). *EGFR* siRNA was the top siRNA that differentially increased sensitivity to PD173074 in the FGFR3-activated cell lines (Fig. 1D). This illustrated the potential power of parallel siRNA screens to identify common subgroup-specific resistance mechanisms, as *EGFR* siRNA was obscured by a multiplicity of effects and noise in individual screens (Supplementary Fig. S1B). A number of additional hits identified were potentially linked to FGFR signaling in network analysis (Supplementary Fig. S1C). *EGFR* siRNA sensitized all three of the FGFR3-activated bladder cell lines screened to PD173074, but none of the other cell lines, identifying a clear subtype/mutation-specific event (Fig. 1E). *EGFR* siRNA also reduced the survival of the PD173074-insensitive 97-7 cell line, suggesting that this cell line may be primarily EGFR-dependent (Fig. 1E).

The effect of silencing EGFR on sensitivity to PD173074 was validated with two independent siRNA-targeting *EGFR* (Fig. 2A and B), and the selectivity of the effect to FGFR3-activated cell lines was also confirmed with EGFR inhibitors (lapatinib and gefitinib) in short-term survival assays (Supplementary Fig. S2A). To understand why *EGFR* siRNA seemed to sensitize only FGFR3-activated cell lines, we examined EGFR expression and phosphorylation by Western blotting. The FGFR3-activated bladder cancer cell lines expressed high levels of both total and phosphorylated EGFR (Fig. 2C), with EGFR either not expressed or phosphorylated in the other cell lines. This suggested that the specificity of the effect seen with *EGFR* siRNA (Fig. 1E) in part reflected expression and intrinsic activation of EGFR in the FGFR3 cell lines. In the blots, FGFR3 was expressed at a number of different molecular weights (Fig. 2C). RT112M cells are driven by an *FGFR3*-TACC3 fusion of higher molecular weight than wild-type FGFR3. Wild-type FGFR3 is expressed as two forms, a lower molecular weight unglycosylated form and a higher molecular weight glyco-ylated form indicated by arrows, with the glycosylated form, in particular, expressed at low level in 97-7 cells (Fig. 2C).

We conducted clonogenic assays in the FGFR3-activated cell lines to further examine the potential role of EGFR in these cell lines. Colony formation was either abolished, or substantially decreased, by the combination of PD173074 and gefitinib (Fig. 2D). To confirm that this observation extended beyond the cell lines used in the screen, we treated additional FGFR3 point-mutant cell lines, again with substantial combination efficacy in 94-10 and 639V (Fig. 2D). Similar synergy was also seen between the clinical FGFR inhibitor AZD4547 and gefitinib, or cetuximab and PD173074 in the RT112M cell line (Supplementary Fig. S2C and S2D).

Two distinct patterns of response to PD173074 and gefitinib were apparent in the clonogenic and short-term survival assays (Figs. 1C and 2D; Supplementary Fig. S2A and S2B). The RT112M, MGHU3, and 639V cell lines were partially FGFR-dependent: PD173074 modestly reduced colony formation, although combination with the EGFR inhibitor gefitinib further reduced clonogenic survival, suggesting that in these cell lines EGFR limited sensitivity to FGFR inhibition. In contrast, the 97-7 and 94-10 cell lines were principally EGFR-dependent: gefitinib alone substantially reduced colony formation, with PD173074 alone having no effect on the growth of these cell lines, suggesting that EGFR mediated the intrinsic resistance of these cell lines to PD173074. In the presence of gefitinib, clonogenic survival was further reduced by PD173074, suggesting that reciprocally in 97-7 and 94-10 cells, FGFR signaling limited the sensitivity to EGFR inhibition.

In both 97-7 and RT112M cells, combined inhibition of FGFR and EGFR with PD173074 and gefitinib induced a greater loss of S-phase compared with either inhibitor given alone (Fig. 2E and Supplementary Fig. S3A), concomitant with relatively increased p27 levels with the combination (Fig. 2F). Gefitinib, and in particular the combination, increased PARP cleavage in 97-7 cells (Fig. 2F), although only a relatively minor increase in Annexin V staining was observed (Supplementary Fig. S3B). This suggested that the combined effect of the inhibitors was mediated predominantly through greater cell-cycle arrest, along with a minor increase in apoptosis.

**Inhibition of FGFR3 Results in Upregulation of EGFR Signaling in Partially FGFR3-Dependent Cell Lines**

We identified that EGFR signaling limited sensitivity to FGFR3 targeting in all five FGFR3-activated cell lines examined. We described two groups of FGFR3-activated cell lines: one group principally FGFR3-dependent with EGFR signaling limiting sensitivity to FGFR inhibitors; and the other group primarily EGFR-dependent with intrinsic resistance to FGFR inhibitors, although in these cell lines FGFR signaling limited sensitivity to EGFR inhibition. We investigated the link between EGFR and FGFR3 signaling in these cell lines: how one receptor compensated for inhibition of the alternative receptor, and whether the same mechanisms were involved in the partially FGFR3- and EGFR-dependent cell lines.

We first focused on the cell lines that were partially FGFR3-dependent. In the RT112M and 639V cell lines, PD173074 completely suppressed extracellular signal-regulated kinase (ERK)1/2 phosphorylation at 1 hour, confirming that FGFR3 was the dominant receptor in these cell lines (Fig. 3A). However, at later time points, ERK1/2 phosphorylation was partially restored, reaching steady state by 4 hours. The increase in ERK1/2 phosphorylation was accompanied by upregulated EGFR Tyr1068 phosphorylation (Fig. 3A). A similar pattern was also seen in the PD173074-sensitive MGHU3 cell line (Supplementary Fig. S4A). The restoration of ERK1/2 phosphorylation was blocked by the addition of gefitinib both at 6 hours and chronically at 24 hours (Fig. 3B), and
Figure 2. EGFR is intrinsically active in FGFR3-activated cell lines with combination efficacy from targeting both EGFR and FGFR3. A, Western blot analyses of RT112M cells transfected 72 hours earlier with siCON or individual siRNA targeting EGFR (siEGFR-A and B) and 48 hours posttransfection exposed to a fixed dose of PD173074 (+) or vehicle (−) for 72 hours. B, growth of RT112M cells transfected with siCON and two individual siRNA targeting EGFR (A and B) and 48 hours posttransfection exposed to a fixed dose of PD173074 (PD+) or vehicle (−) for 72 hours. C, Western blot analysis of indicated cell lysates blotted for FGFR3, EGFR, and phosphorylated EGFR (P-EGFR), with actin loading control. The three FGFR3-activated cell lines are indicated. D, clonogenic survival assays in indicated cell lines treated continuously with 500 nmol/L PD173074 (PD), 250 nmol/L gefitinib (Gef), the combination, or vehicle alone. Activating aberration in FGFR3 is indicated for each cell line, with cell lines classified as either partially FGFR3-dependent or primarily EGFR-dependent. Right, quantification of three independent experiments. E, analysis of S-phase fraction from propidium iodide fluorescence-activated cell sorting profiles of indicated cell lines treated for 24 hours with 500 nmol/L PD173074, 250 nmol/L gefitinib (Gef), the combination, or vehicle alone. * P < 0.05, Student’s t test. F, expression of p27 increases with combination therapy at 24 hours, with a minor increase in cleaved PARP in 97-7 cells. DMSO, dimethyl sulfoxide.
**Figure 3.** EGFR signaling is upregulated in response to FGFR3 inhibition in partially FGFR-dependent cell lines. **A,** Western blot analysis of FGFR3-dependent (RT112M and 639V) and EGFR-dependent (97-7 and 94-10) cell lysates treated for the indicated times with 500 nmol/L PD173074 blotted for phosphorylated (P) and total EGFR, ERK1/2, and AKT Ser 473. **B,** Western blot analysis of RT112M cell lysates treated for the indicated times with 500 nmol/L PD173074 (PD), 250 nmol/L gefitinib (Gef), combination, or vehicle, blotted for phosphorylated and total EGFR and ERK1/2. **C,** Western blot analysis of serine 473 phosphorylated and total AKT in RT112M cells treated as indicated. **D,** clonogenic survival assay of RT112M cells with PD173074 500 nmol/L, MEK inhibitor CI-1040 100 nmol/L, or the combination. Right, quantification of three independent experiments. **E,** Western blot analysis of RT112M cells treated with PD173074, gefitinib, or CI-1040 100 nmol/L as indicated for 6 hours. DMSO, dimethyl sulfoxide.

the combination of PD173074 with gefitinib led to greater suppression of AKT–Ser473 phosphorylation in RT112M cells than was seen with PD173074 alone (Fig. 3C). To ascertain whether the reactivation of mitogen-activated protein kinase (MAPK) signaling explained the relative insensitivity to FGFR inhibition, we examined the combination of PD173074 and the MAP–ERK kinase (MEK) inhibitor CI-1040. In RT-112M cells, CI-1040 increased sensitivity to PD170374 in clonogenic assays, suggesting that in part reactivation of MEK–ERK1/2 signaling by EGFR reduced sensitivity to PD173074 (Fig. 3D).
Mechanism of EGFR Activation by FGFR Inhibitors

We set out to establish how EGFR gets activated and subsequently upregulated by FGFR3 inhibition. EGFR was wild-type in all five FGFR3-activated cell lines, and the EGFR gene was not amplified (data not shown). The FGFR3-activated bladder cancer cell lines expressed high levels of TGF-α, with silencing of TGF-α in RT112M cells increasing sensitivity to PD173074, suggesting an autocrine loop (Supplementary Fig. S4B). There was no change in the expression of TGF-α in response to PD173074 (Supplementary Fig. S4C), suggesting that upregulation of EGFR signaling in response to PD173074 did not reflect increased TGF-α ligand expression. We were also unable to show coimmunoprecipitation between EGFR and FGFR3, suggesting that the two receptors did not interact (Supplementary Fig. S4D).

In the EGFR-dependent cell lines 97-7 and 94-10, PD170374 had no effect on downstream signaling and no effect on EGFR phosphorylation (Fig. 3A), suggesting that the upregulation of EGFR seen with PD173074 in FGFR3-dependent cell lines did not reflect EGFR inhibition per se, but the resulting loss of downstream signaling. We hypothesized that the decrease in ERK1/2 activity that occurred with PD173074 resulted in the upregulation of EGFR signaling, and inhibition of MEK with CI-1040 in RT112M cells decreased ERK1/2 phosphorylation and substantially upregulated EGFR Tyr1068 phosphorylation (Fig. 3E). This therefore suggested a model whereby FGFR3-mediated MAPK signaling suppressed signaling from EGFR, and that this negative feedback was subsequently released by FGFR3 inhibition.

Multiple mechanisms of regulation of EGFR signaling through negative feedback loops mediated by MEK–ERK1/2 have been described, such as the ERK1/2-mediated phosphorylation of CBL to promote receptor internalization and degradation, and to promote expression of the sprouty proteins (19). In addition, the CDC25 family has been identified as potential EGFR phosphatases (20) that have been previously suggested to mediate a feedback loop between inhibition of mutant BRAF, loss of MEK–ERK1/2 signaling, and upregulation of EGFR (17, 21). Silencing CDC25C with multiple different siRNAs increased EGFR phosphorylation and downstream signaling (Supplementary Fig. S4E). However, we were unable to show a decrease in CDC25C Thr48 phosphorylation with PD170374 in RT112M cells, the site proposed to be regulated by ERK1/2 (ref. 21; Supplementary Fig. S4F), suggesting that although CDC25C is a phosphatase that regulates EGFR phosphorylation and signaling, it is unlikely to be involved in the feedback loop mediated by PD170374 in these cell lines. Moreover, the siRNA against CDC25C did not affect sensitivity to PD173074 in either RT112M or MGHU3 cells (Supplementary Fig. S4G).

Receptor internalization and trafficking is a major factor regulating the signaling from receptor tyrosine kinases, with internalization ultimately leading to signal termination (19). After internalization, EGFR continues to signal from early endosomes, with signaling only terminated later due to subsequent lysosomal degradation, or in late recycling endosomes (19). Silencing of CBL with multiple different siRNAs decreased sensitivity to PD173074 (Supplementary Fig. S4H), potentially suggesting a role for receptor trafficking in sensitivity to PD173074. To examine whether there was a defect in receptor trafficking that may contribute to EGFR upregulation, we examined EGFR localization by immunofluorescence. In RT112M cells, PD173074 treatment led to EGFR accumulation at the plasma membrane (Fig. 4A and B), in regions characterized by aberrant dense cortical filamentous actin (F-actin; Fig. 4A). EGFR also accumulated in giant actin-coated rings (Fig. 4C) that stained with EEA1 (Fig. 4D), indicating accumulation of EGFR in giant early endosomes (Fig. 4C and D). These results suggested that the activation of EGFR following FGFR inhibition was mediated by both loss of ERK1/2-induced negative feedback, and impaired receptor internalization and sorting that would contribute through impaired signaling termination.

EGFR Mediates Intrinsic Resistance to FGFR3 Targeting through Repression of FGFR3 Expression

We next focused on the cell lines that were primarily EGFR-dependent, to understand why these cell lines were EGFR-dependent and intrinsically resistant to FGFR targeting (Fig. 2D and Supplementary Fig. S2A and S2B). Both the EGFR-dependent cell lines, 97-7 and 94-10, had a S249C FGFR3-activating mutation identical to germline FGFR3-activating mutations found in a proportion of people with the skeletal dysplasia syndrome thanatophoric dysplasia (22, 23). S249C constitutively activates the kinase through constitutive receptor dimerization as a result of an aberrant extracellular intermolecular disulphide bridge (22, 24). Expression of the FGFR3 mutation was confirmed by real-time PCR (RT-PCR; Supplementary Fig. S5A). In the EGFR-dependent cell lines 97-7 and 94-10, gefitinib acutely blocked both ERK1/2 and AKT–Ser473 phosphorylation, confirming that EGFR dominated downstream signaling in these cell lines and not the mutant FGFR3 receptor (Fig. 5A). Gefitinib alone had no effect on signaling in the FGFR3-dependent cell lines (Fig. 5A), an inverse of the effect seen with PD173074 (Fig. 3A). Therefore, in any one cell line either FGFR3 or EGFR was the dominant receptor controlling signaling, and this directly reflected sensitivity to the corresponding inhibitor.

Although in the FGFR3-dependent cell lines PD173074 led to relatively rapid upregulation of EGFR and restoration of ERK1/2 phosphorylation (Fig. 3), in the EGFR-dependent cell lines the restoration of ERK1/2 phosphorylation was much delayed, occurring only after 24 hours of exposure to gefitinib (Fig. 5A). The delayed restoration of ERK1/2 phosphorylation was blocked by PD173074, both at 24 and 48 hours after gefitinib addition (Fig. 5B and C), confirming that in these cell lines FGFR signaling compensated for the loss of EGFR signaling. The very substantial difference in the kinetics of the restoration of downstream signaling by the non-dominant EGFR or FGFR3 (Figs. 3A and 5A) suggested that the mechanism(s) leading to the upregulation of FGFR3 signaling were distinct. Concurrent with the increase in ERK1/2 phosphorylation, we observed substantial upregulation of FGFR3 protein, in particular the fully glycosylated form (Fig. 5A), with consequent upregulation of FRα2 phosphorylation (adapter protein of FGFRs; Fig. 5D).

To confirm that EGFR signaling could repress FGFR3 expression, we hyperactivated EGFR in the partially
FGFR3-dependent cell line RT112M. Supplementation of media with TGF-α resulted in RT112M cells becoming resistant to PD173074 (Fig. 5E), accompanied by near-complete repression of FGFR3–TACC3 expression (Fig. 5F). In the EGFR-dominant cell lines, compared with cell lines in which FGFR3 signaling dominated, EGFR was expressed at substantially higher levels relative to FGFR3 (Figs. 2C and 5G and H), and, similarly, EGFR was phosphorylated at substantially higher levels in 97-7 cells compared with RT112M or MGHU3 cells (Fig. 2C). This suggested that the relative expression and activation level of EGFR may have dictated whether the cell line became EGFR-dependent.

To investigate how EGFR signaling led to repression of FGFR3, we first examined FGFR3 mRNA expression. Gefitinib led to substantially increased FGFR3 mRNA levels in the EGFR-dependent 97-7 cell line (Fig. 6A), with the increase in mRNA seen not at 6 hours but at 24 hours, mirroring the timing of increased FGFR3 protein (Fig. 5A). We examined which signaling pathways downstream of EGFR might regulate FGFR3 mRNA expression, treating 97-7 cells with a panel of signal transduction inhibitors (Fig. 6B). Inhibition of phosphoinositide 3-kinase (PI3K) with GDC0941, or AKT with AZD5363, did not result in upregulation of FGFR3 mRNA (Fig. 6B) nor FGFR3 protein (Fig. 6C). Inhibition of AKT–Ser473 phosphorylation by GDC0941, and stimulation by AZD5363, confirmed target inhibition by the respective inhibitors. Therefore, although PI3K/AKT inhibition results in upregulation of multiple receptor tyrosine kinases, for example of the EGFR/ERBB family (25, 26), PI3K/AKT signaling alone did not repress FGFR3 expression. Similarly, solitary inhibition of MEK, Janus-activated kinase 2 (JAK2), protein kinase C (PKC)α/β, or ROCK had no effect on, or further repressed, FGFR3 mRNA expression (Fig. 6B).

Across the panel, dasatinib (used at 100 nmol/L), which is relatively specific to the SRC family of kinases along with ABL/KIT (27), resulted in clear FGFR3 mRNA upregulation (Fig. 6B).

**Figure 4.** Localization of EGFR in response to FGFR inhibition in partially FGFR3-dependent cell lines. A, immunofluorescence of RT112M cells treated with 500 nmol/L PD170374 for indicated times, or dimethyl sulfoxide (DMSO) vehicle, stained for F-actin (red) and EGFR (green). White arrows indicate areas of EGFR accumulation. B, quantification of three independent immunofluorescence experiments of RT112M cells treated with 500 nmol/L PD170374 for 4 hours, proportion of cells with EGFR membrane staining. *, P < 0.001, Student t test. C and D, immunofluorescence of RT112M cells treated with 500 nmol/L PD170374 for 4 or 6 hours. C, stained for actin (red) and EGFR (green) and (D) at 4 hours stained for EEA1 (red) and EGFR (green).
along with upregulated FGFR3 protein (Fig. 6D). Hyperactivation of EGFR with TGF-α in RT112M cells, which repressed FGFR3 expression, also resulted in substantial SRC-Tyr416 phosphorylation (Fig. 5F). SRC signaling has previously been suggested to regulate FGFR3 expression by regulation of microRNA (miRNA) miR-99a (28). However, RT112M cells have an FGFR3-TACC3 translocation that removes the 3’untranslated region and is consequently not targeted by miR-99a (29), or other FGFR3 regulatory miRNA, implying that the repression of FGFR3 was not due to the effects of miRNA (Fig. 5F). Similarly, the transfection of miRNA inhibitors against miR-99a and miR-100 in 97-7 cells, miRNA that have been shown to regulate FGFR3 expression in bladder cancer (30), did not alter FGFR3 expression (Supplementary Fig. SSB).

EGFR and SRC interact, cross-phosphorylate each other, and mutually reinforce downstream signaling (31, 32). As we were

Figure 5. EGFR mediates intrinsic resistance to FGFR3 targeting through repression of FGFR3 expression, with delayed upregulation of FGFR3 expression in response to EGFR inhibition. A, Western blot analysis of partially FGFR3-dependent cell lines (MGHU3, RT112M, and 639V) and EGFR-dependent cell line (97-7 and 94-10) lysates treated for the indicated times with gefitinib (Gef). Black arrows indicate glycosylated and unglycosylated FGFR3.

B and C, Western blot analysis of 97-7 cell lysates treated for the indicated times with PD173074 (PD), gefitinib, combination, or vehicle.

D, Western blot analysis of FRS2 following treatment with PD173074, gefitinib, or the combination.

E, RT112M cells grown for 72 hours in increasing concentrations of PD173074 (μmol/L) or vehicle. Partially FGFR3-dependent RT112M cells acquire resistance to PD173074 in the presence of TGF-α.

F, Western blot analysis of RT112M lysates after 24 hours of exposure to TGF-α 10 ng/mL or vehicle, blotted with indicated antibodies.

G, Western blot analysis of cell lysates from FGFR3-activated cell lines blotted for FGFR3, EGFR, and actin loading control.

H, quantitative RT-PCR assessment FGFR3 mRNA expression in RT112M and 97-7 cell lines. DMSO, dimethyl sulfoxide.
FGFR-Driven Cell Line siRNA Screens

Figure 6. EGFR signaling regulates FGFR3 expression in part through SRC family signaling and the effects of multiple downstream signal transduction pathways. A, 97-7 cells were treated for 6 or 24 hours with gefitinib (Gef), or dimethyl sulfoxide (DMSO), with quantitative RT-PCR assessment of FGFR3 mRNA expressed relative to the level in DMSO treated cells. B, 97-7 cells were treated for 24 hours with DMSO vehicle, 250 nmol/L gefitinib (EGFR inhibitor), 250 nmol/L GDC-0491 (PI3K inhibitor), 200 nmol/L AZD5363 (AKT inhibitor), 500 nmol/L CI-1040 (MEK inhibitor), 100 nmol/L dasatinib (SRC family inhibitor), 1 μmol/L BMS-911543 (JAK2 inhibitor), 100 nmol/L Go-6976 (protein kinase C inhibitor), or 150 nmol/L H1152 (ROCK inhibitor). FGFR3 mRNA expression was assessed by quantitative RT-PCR relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and expressed relative to DMSO-treated cells. C and D, Western blot analysis of 97-7 lysates after 24 hours of exposure to indicated compounds, or a combination of gefitinib and PD173074 (PD), blotted with indicated antibodies.

unavailable to show that inhibition of a single downstream signal transduction pathway repressed FGFR3 expression (Fig. 6B), we examined combination pathway inhibition. Combined inhibition of MEK and PI3K resulted in substantial derepression of FGFR3 (Fig. 6E), suggesting that EGFR signaling repressed by SRC repressed FGFR3 expression through the combined action of multiple downstream pathways. Therefore, these data suggest that EGFR signaling repressed FGFR3 transcription, and gefitinib released this repression, resulting in restoration of FGFR3 expression and signaling. Similar to that observed with EGFR, FGFR3 also accumulated in the cytoplasmic membrane after gefitinib treatment (Fig. 6F and Supplementary Fig. S5C), whereas in untreated cells FGFR3 was predominantly cytoplasmic, suggesting that upregulation of FGFR3 was also reinforced by impaired receptor internalization.

In Vivo Efficacy of Combined EGFR and FGFR Inhibition

To investigate efficacy in vivo, we established xenografts of RT112M cells in nude mice. The combination of gefitinib and PD173074 was poorly tolerated at the doses used, with rapid weight loss (Supplementary Fig. S6A). Nevertheless, the short duration of 3 days of combination therapy reduced tumor volume to a greater extent than either treatment given alone, including two complete responses (Fig. 7A), confirming that the in vitro observations predicted for greater tumor control with combination treatment in vivo.

We repeated experiments with the combination of cetuximab and PD173074 given for 2 weeks of treatment, which was well tolerated (Supplementary Fig. S6B). The combination of cetuximab and PD173074 reduced tumor size to a significantly greater extent than either agent given alone (P < 0.0001, log-rank test; Fig. 7B). Treatment with PD173074 led to tumor stasis, whereas combination therapy led to substantial tumor reduction (mean relative tumor volume at end of 2-week treatment period: 1.28 for PD173074 vs. 0.51 for combination; P = 0.006, Student t test). On stopping treatment, PD173074 tumors rapidly resumed growth, whereas the combination-treated animals had sustained tumor control (median time to tumor doubling: control, 6 days; cetuximab, 13 days; PD173074, 17 days; combination group median not reached after 41 days of follow-up; Fig. 7C). These data suggested substantial synergistic efficacy in vivo between dual targeting of EGFR and FGFR3 in FGFR3-activated cell lines.

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Figure 7. Inhibition of EGFR and FGFR3 has combination efficacy in vivo. A, RT112M xenografts were established in nude mice, and divided randomly into four groups treated with vehicle, PD173074 (PD) 20 mg/mg intraperitoneal (i.p.), gefitinib (Gef) 110 mg/kg per os, or combination of both inhibitors for 3 days. Tumor growth was assessed 7 days following commencement of therapy. Comparison between groups with Student t test. B, RT112M xenografts were established in nude mice, and divided randomly into four groups treated with vehicle, PD173074 15 mg/mg i.p. (days 0–3 and 7–10), cetuximab (Cet) 40 mg/kg i.p. [days 0, 3, 7, and 10], or combination of both inhibitors with solid bar indicating 2-week treatment interval. *, P < 0.01 compared with all other groups, Student t test. C, Kaplan–Meier plot of time to tumor doubling from experiment described in B. Combination-treated animals have substantially increased tumor control, P < 0.01, log-rank test comparing combination-treated animals with all other groups. D, expression of TGF-α mRNA in a publicly available gene expression dataset of normal bladder, invasive bladder cancers, and superficial-type bladder cancers. Comparison between groups with Student t test.

DISCUSSION

In this study, we have used parallel RNAi screens in multiple FGFR-dependent cell lines to dissect mechanisms of resistance to FGFR inhibition. We show that the sensitivity of FGFR3-activated cancers is limited by intrinsic activation of EGFR in all the cell lines studied, and we described two distinct mechanistic groups. In partially FGFR3-dependent cell lines, EGFR signaling is upregulated following FGFR inhibition through release from negative feedback, and this partially compensates for loss of FGFR signaling. In EGFR-dependent cell lines, despite the presence of an activating FGFR3 mutation, EGFR dominates downstream signaling through repression of FGFR3 expression. In the EGFR-dependent cell lines, EGFR inhibition resulted in delayed upregulation of FGFR3 expression, restoring FGFR3 signaling, and partially compensating for loss of EGFR signaling.

We show a reciprocal relationship between targeting the dominant oncogene and rescue mediated by an alternative receptor tyrosine kinase, which is emerging as a major mechanism of resistance to cancer therapies (33–36). In FGFR3-dependent cell lines, EGFR signaling is upregulated relatively rapidly following the loss of MAPK signaling-mediated negative feedback induced by PD173074 (Fig. 3E). FGFR inhibition in FGFR3-activated cell lines did not induce an apoptotic response (Fig. 2F and Supplementary Fig. S3), and potentially such a
cytostatic response allows for upregulation of the alternative receptor to subvert the effect of inhibition of the dominant receptor. In contrast, in EGFR-dependent cell lines, rescue from EGFR inhibition is reliant on delayed derepression of FGFR3 expression. Impaired receptor trafficking reinforces the upregulation of both receptors. In response to FGFR3 inhibition, EGFR accumulates at the membrane and in early endosomes, and, likewise, in response to EGFR inhibition, FGFR3 accumulates at the membrane. FGFR3 and EGFR were noted to accumulate in the membrane in areas of abnormally dense actin filament formation, and it will be interesting in future research to examine the possible role of aberrant regulation of the cytoskeleton in receptor accumulation (37).

EGFR dominates downstream signaling in some FGFR3-mutant cell lines through repression of mutant FGFR3 expression, resulting in intrinsic resistance to FGFR inhibitors. Although FGFR3 mutation presumably initiated cancer development in these cancers, at some point in tumor development EGFR signaling increased to a level where it repressed mutant FGFR3 and dominated downstream signaling. Although prior research has emphasized the role of PI3K/AKT signaling in repression of receptor tyrosine kinases (25, 26), neither inhibition of PI3K nor AKT alone derepressed FGFR3 expression in the EGFR-driven dominant-cell lines (Fig. 6B and C). In contrast, inhibition of SRC family kinases with dasatinib resulted in substantial FGFR3 derepression. Although EGFR signals strongly to SRC (Fig. 5F), mutant FGFR3 has been shown to signal only weakly to SRC (24), and the difference in downstream signaling between EGFR and mutant FGFR3 likely provides an explanation as to why FGFR3 signaling does not itself repress FGFR3 expression.

FGFR3 mutation is one of the most common oncogenes in transitional cell carcinomas of the bladder, also described at relatively high frequency in cervical cancer (1). Recent data have confirmed that FGFR3 is also activated by translocations in cancer that generate a fusion protein between FGFR3, deletion of the last exon of FGFR3, and a number of partner proteins in glioblastoma (8), bladder cancer (38), and upper aerodigestive tract squamous cancers (9). The clinical translational challenge in tackling the upregulation of receptor tyrosine kinases in resistance to targeted therapies is the identification of the receptor that drives resistance. Our data partially overcome this challenge for FGFR3-driven bladder cancer by showing that EGFR is active and mediates resistance in all five FGFR3-driven cell lines examined. The MET receptor has also been shown to be an important receptor mediating resistance, potentially through paracrine stromal release (33) or autocrine production of hepatocyte growth factor (HGF) ligand (39). Our results are potentially conflicting with recent data that suggested MET as the mediator of resistance to FGFR3 targeting in RT112M cells (33, 39). However, we show that EGFR is the receptor that compensates for FGFR inhibition, and that this translates to substantial combination efficacy in vivo (Fig. 7). Examining a publicly available gene expression series, we find that TGFα mRNA is expressed at high levels in bladder cancer (Fig. 7D), along with expression of EGFR (Supplementary Fig. S7A), whereas HGF mRNA is expressed at low levels (Supplementary Fig. S7B), suggesting that our observations may be clinically relevant. Our data are also consistent with the recently identified role of EGFR in mediating resistance to mutant BRAF targeting in colon cancer (17, 34).

However, our results do suggest caution in the combination of FGFR and EGFR inhibitors, with the combination of PD173074 and gefitinib poorly tolerated in mice at the doses used (Supplementary Fig. S6). The combination of PD173074 and the EGFR inhibitor antibody cetuximab had improved tolerance (Supplementary Fig. S6). In addition, FGFR tyrosine kinase inhibitors typically broadly inhibit FGFR1/2/3, and the increased specificity of FGFR3 therapeutic antibodies may potentially further mitigate toxicity (40, 41).

Our study illustrates the power of parallel RNAi genetic screens to identify key determinants of resistance to targeted therapies. Through simultaneous examination of multiple cell lines with related oncogenic aberrations, the key common determinants of sensitivity can be identified that may remain obscured by the multiplicity of effects and noise seen in any one individual screen (Supplementary Fig. S1B). This approach could be applied to multiple different targeted therapies to identify the key determinants of sensitivity and reveal the common shared mechanisms of resistance.

METHODS

Cell Lines and Antibodies

Bladder cancer cell lines (RT112M, MGHU3, 639V, 97-7, and 94-10) were from the laboratory of M.A. Knowles (13). Other cell lines were obtained from American Type Culture Collection or Asterand. Cell lines were banked in multiple aliquots on receipt to reduce risk of phenotypic drift, and identity confirmed by short-tandem repeat profiling with the PowerPlex 1.2 System (Promega). Cell lines were maintained in phenol red-free Dulbecco’s Modified Eagle Medium (DMEM), DMEM/F12, or RPMI with 10% FBS (PAA gold) and 2 mmol/L L-glutamine (Sigma-Aldrich). Antibodies used were: phosphorylated Akt-Ser473 (4058), Akt (4691), phospho-ERK1/2-Thr202/Tyr204 (4370), EGFR (2232), phospho-EGFR-Tyr1068 (3777), phospho-CDK2C5-Thr18 (9527), phospho-SRC-Y416 (2101), SRC (2109; all from Cell Signaling Technology), β-actin (A5441; Sigma), FGFR3 (sc-13121), EGFR (sc-03), TGFα (sc-37443S), p21 (sc-528), PARP (sc-7150), and FRS2 (sc-8318; Santa Cruz Biotechnology). PD173074 was from Sigma, gefitinib from Tocris, and CI-1040 from Selleckchem.

siRNA Screening

Screening was in 384-well plates with a Dharmacon siGENOME SMARTpools library targeting all known protein kinases and phosphatases essentially as described previously (18, 42). Briefly, cells were reverse transfected at final siRNA concentration of 20 nmol/L; at 48 hours posttransfection, half of the plates were treated with PD173074 at the EC50 and half with vehicle, and survival was assessed after 72 hours of exposure with CellTiter-Glo cell viability assay (Promega). Individual plates were median-normalized before combination. The siRNA library was supplemented with nontargeting siRNA, PLK1 siRNA as a viability control, and four individual siRNA against the FGFR1-4 receptor family (Supplementary Tables S2 and S3). Screens were only accepted with a Z’ factor more than 0.3.

To assess the effect of siRNA on growth/survival, the effect of siRNA in the vehicle plates was expressed as a Z score, with the SD estimated from the median absolute deviation (MAD). A Z score of less than –2, approximately the 95% confidence interval, was considered as evidence of a significant decrease in survival with siRNA.

To assess the effect of siRNA on sensitivity to PD173074, the log2 ratio between growth in PD173074 plates and vehicle plates was assessed and expressed as a Z score. A Z score of less than 1.645 was not considered as evidence of a significant decrease in sensitivity with siRNA.

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considered as evidence of increased sensitivity to PD170374, and more than 1.645 as relative resistance (cutoffs equivalent to putative 90% confidence intervals). A lower cutoff was used for sensitivity score to reflect that multiple siRNAs altered sensitivity, affecting the null hypothesis in assessment of the SD from the MAD, and clear evidence that reproducible effects were seen with siRNA using this cutoff (Supplementary Fig. S1).

In Vitro Cell Line Assessment

Clonogenic assays were conducted in 6-well plates with 1,000 to 2,000 cells seeded per well, and 24 hours later, cells were exposed to 500 nmol/L PD173074, 250 nmol/L gefitinib, or the combination, followed by growth in media for 2 weeks, to allow colony growth. Colonies were fixed, stained with sulforhodamine B, and counted. For short-term survival assays, cells were exposed to fixed-ratio combinations of PD173074 and gefitinib, and survival was assessed after 72 hours of exposure with CellTiter-Glo cell viability assay (Promega). For EGFR siRNA short-term survival assays, RT112M cells were reverse transfected at final siRNA concentration of 20 nmol/L; at 48 hours posttransfection, plates were treated with PD173074 at the EC50 or vehicle, and survival was assessed after 72 hours of exposure.

siRNA Data Analysis

To compare siRNA results between two groups, we used supervised methods calculating the difference in the median effect of the siRNAs between the two groups, followed by estimation of a P value by permutation of labels to create a distribution for comparison with the actual differences (42). A significance cutoff of P < 0.05 was used.

Western Blotting and FACS

Cell lines were grown on 35-mm plates, treated as indicated, and lysed in NP-40 lysis buffer. Western blot analyses were conducted with precast TA or Bis-Tris gels (Invitrogen) as previously described. Fluorescence-activated cell sorting (FACS) analysis was conducted as previously described (43).

Immunofluorescence

Cells grown on coverslips were fixed with 4% paraformaldehyde, before incubation with primary antibodies against EGFR (1:100; sc-03), FGFR3 (1:100; sc-13121), EEA1 (1:1,000; sc-33585; Santa Cruz Biotechnology), Alexa Fluor-488 phallolidin (1:1,000; A12379; Invitrogen), and corresponding secondary Alexa Fluor-444 or Alexa Fluor-555 conjugated antibodies, with 4,6-diamidino-2-phenylindole (DAPI) nuclear stain. Cells were visualized with a Leica Confocal microscope.

Quantitative PCR

cDNA was synthesized from RNA using SuperScript III and random hexamers (Invitrogen). Quantitative PCR was conducted by absolute quantification with TaqMan chemistry on an ABI Prism 7900T System (Applied Biosystems) with FGFR3 (Hs00997400) expressed relative to control gene GAPDH (Hs 02758991). EGFR was sequenced with the cobas EGFR Mutation Test (Roche Diagnostics).

Xenografts

RT112M xenografts in nude mice were generated by transplantaion as previously described (13). In the first experiment, mice were treated with PD173074 20 mg/kg by intraperitoneal (i.p.) injection, gefitinib 100 mg/kg by oral gavage, or the combination, for 3 days. In the second experiment, mice were treated with PD173074 15 mg/kg on days 0 to 3 and 7 to 10, cetuximab 40 mg/kg day 0, 3, 7, and 10, or the combination by intraperitoneal injection. Tumor size was assessed at least three times a week. Time to tumor doubling was assessed by Kaplan-Meier methods as either doubling of relative tumor volume or tumor ulceration, censoring mice lost to follow-up through reasons not related to tumor progression. Experiments were carried out under a UK Home Office Project License assessed by the University of Bradford (Bradford, United Kingdom) ethical review committee.

Statistical Analysis

All statistical tests were conducted with GraphPad Prism version 5.0 or Microsoft Excel. Unless stated otherwise, P values were two-tailed and considered significant if P < 0.05. Error bars represent SEM of three experiments. Normalized gene expression data assessed on Affymetrix Human Genome U133A Arrays from Sanchez-Carbayo and colleagues (44) were downloaded as log2 median-centered data with probe 205016_at for TGFA (TGF-α) from Oncomine (45).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: M.T. Herrera-Abreu, M.A Knowles, N.C. Turner
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M.T. Herrera-Abreu, A. Pearson, S.D. Shnyder, M.A Knowles, N.C. Turner
Analysis and interpretation of data (e.g., statistical analysis, biosiatsistics, computational analysis): M.T. Herrera-Abreu, J. Campbell, S.D. Shnyder, N.C. Turner
Writing, review, and/or revision of the manuscript: M.T. Herrera-Abreu, M.A Knowles, A. Ashworth, N.C. Turner
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M.T. Herrera-Abreu
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Parallel RNA Interference Screens Identify EGFR Activation as an Escape Mechanism in FGFR3-Mutant Cancer

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