Published in final edited form as:

Mol Cancer Ther. 2016 March ; 15(3): 503–511. doi:10.1158/1535-7163.MCT-15-0548-T.

EGFR Is Regulated by TFAP2C in Luminal Breast Cancer and Is a Target for Vandetanib

James P. De Andrade*, Jung M. Park*, Vivian W. Gu, George W. Woodfield, Mikhail V. Kulak, Allison W. Lorenzen, Vincent T. Wu, Sarah E. Van Dorin, Philip M. Spanheimer, and Ronald J. Weigel

Department of Surgery, University of Iowa, Iowa City, IA, USA

Abstract

Expression of TFAP2C in luminal breast cancer is associated with reduced survival and hormone resistance, partially explained through regulation of RET. TFAP2C also regulates EGFR in HER2 breast cancer. We sought to elucidate the regulation and functional role of EGFR in luminal breast cancer. We used gene knockdown (KD) and treatment with a tyrosine kinase inhibitor (TKI) in cell lines and primary cancer isolates to determine the role of RET and EGFR in regulation of p-ERK and tumorigenesis. KD of TFAP2C decreased expression of EGFR in a panel of luminal breast cancers and ChIP-seq confirmed that TFAP2C targets the *EGFR* gene. Stable KD of TFAP2C significantly decreased cell proliferation and tumor growth, mediated in part through EGFR. While KD of RET or EGFR reduced proliferation (31% and 34%, $p < 0.01$), combined KD reduced proliferation greater than either alone (52% reduction, $p < 0.01$). The effect of the TKI vandetanib on proliferation and tumor growth response of MCF-7 cells was dependent upon expression of TFAP2C and dual KD of RET and EGFR eliminated the effects of vandetanib. The response of primary luminal breast cancers to TKIs assessed by ERK activation established a correlation with expression of RET and EGFR. We conclude that TFAP2C regulates EGFR in luminal breast cancer. Response to vandetanib was mediated through the TFAP2C target genes EGFR and RET. Vandetanib may provide a therapeutic effect in luminal breast cancer, and RET and EGFR can serve as molecular markers for response.

Keywords

Breast Cancer; TFAP2C; EGFR; RET; Luminal; Vandetanib

INTRODUCTION

An estimated 235,000 patients will be diagnosed with breast cancer annually in the United States, and while many advances in treatment have been made, breast cancer remains the second leading cause of cancer-related death in women (1). Molecular analysis and clinical data have made it clear that breast cancer is a heterogeneous disease with distinct molecular

Contact: Ronald J. Weigel, MD, PhD, Department of Surgery, University of Iowa, 200 Hawkins Drive, 1516 JCP, Iowa City, IA 52242, USA, Ronald-Weigel@uiowa.edu.

*Authors contributed equally to this manuscript

CONFLICT OF INTEREST: The authors declare that they have no conflict of interest.

subtypes, the most common of which is the luminal subtype (2). Luminal breast cancer is usually characterized by expression of the hormone receptors estrogen receptor- α (ER α) and/or progesterone receptor (PR). Anti-estrogen therapy has been an important treatment strategy for luminal breast cancer. Unfortunately, while this subtype typically has been correlated with a better prognosis than other forms of breast cancer, recurrence and hormone resistance remain significant clinical problems.

A promising avenue of investigation in cancer treatment has been to understand the pathways that play a key role in driving the growth and progression of the various subtypes of breast cancer to provide novel targets for directed therapy. Members of the AP-2 transcription factor family have been shown to play a critical role in multiple subtypes of breast cancer (3). The TFAP2C family member is involved in regulation of the luminal mammary compartment during development and maintains the luminal differentiated phenotype of luminal breast cancer (4). TFAP2C regulates the expression of several key luminal breast cancer markers including ER α and FOXA1 (5, 6). Overexpression of TFAP2C is associated with reduced survival and hormone resistance (7–9). Further work is necessary to better understand the mechanisms by which TFAP2C drives breast cancer progression and hormone response.

RET is a receptor tyrosine kinase (RTK) that is a TFAP2C target gene, and RET expression was shown to be associated with ER α expression and was further correlated with hormone resistance (10, 11). Recently, it has been shown that inhibiting the RET pathway augmented estrogen response, and the ability of the tyrosine kinase inhibitor (TKI) sunitinib to block ERK activation in primary breast cancers correlated with RET expression (12). Response to the TKI vandetanib was also found to be significantly reduced with knockdown of RET; however, TKIs such as vandetanib have activity against several RTKs that may drive breast cancer growth. For example, vandetanib also has TKI activity against EGFR (13). Interestingly, it has recently been shown that EGFR is regulated by TFAP2C in HER2 breast cancer and *neu*-activated mouse mammary cancer (14). These findings generate several interesting questions. First, it is important to establish whether TFAP2C regulates EGFR expression in luminal breast cancer and to determine whether EGFR contributes to the ability of TFAP2C to induce proliferation and tumor growth. In addition, since RET and EGFR are both RTKs and both regulated by TFAP2C, it would be important to understand whether there are cooperative effects by these two pathways in regulating proliferation and tumor growth in luminal breast cancer. The current study was undertaken to answer these critical questions on the role of TFAP2C and its target genes EGFR and RET in regulating cell proliferation and tumor progression in luminal breast cancer.

METHODS

Cell Culture

MCF-7, ZR-75-1, and T-47D cell were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA) and maintained in DMEM medium supplemented with 10% FBS, 1% 100X Pen/Strep antibiotics, and 1% 100X GlutaMAX (all components from Life Technologies: Madison, WI, USA) in a standard humidified incubator at 37° C and 5.0% carbon dioxide (5). The cells were not tested and authenticated by the authors and were

passed for less than six months since obtaining the cells. Vandetanib was added to appropriate experiments at a final concentration of 10 μ M.

Gene Knockdown

Cells were transfected with siRNA directed towards non-targeting (NT), TFAP2C, RET, and EGFR (Life Technologies) with lipofectamine (Life Technologies) according to the manufacturer's recommendations for 96 hours to achieve transient gene knockdown. Clones of MCF-7 with stable gene knockdown of NT or TFAP2C were created using lentivirus-mediated shRNA transduction (4).

Expression Analysis

mRNA from cell lysates was converted to cDNA by polymerase chain reaction (PCR) using random hexamers (Life Technologies) method. Using the delta-delta CT method of quantitative PCR (qPCR), relative gene expression was calculated using TaqMan primers to TFAP2C, RET, and EGFR with 18s rRNA subunit (Life Technologies) used as an endogenous control. Western blots were performed using antibodies to TFAP2C, EGFR, ERK (Santa Cruz Biotechnology: Dallas, TX, USA), RET, and phosphorylated ERK (p-ERK) (Cell Signaling: Beverly, MA, USA) with GAPDH (Santa Cruz Biotechnology) used as a loading control. Relative protein levels were quantified from western blots using ImageJ (<http://rsb.info.nih.gov/ij/download.html>) per standard protocol. Either GAPDH or total ERK were used to normalize the relative densities.

Immunohistochemistry

Xenografts were formalin fixed and paraffin-embedded. Sections were evaluated by hematoxylin & eosin (H&E) staining and immunohistochemistry (IHC) was performed for Ki-67 (Dako, Denmark) and cleaved caspase 3 (CC3; Cell Signaling Technology, Danvers, MA) with appropriate positive and negative controls by the University of Iowa Animal Pathology Core Laboratory. Quantitative data for Ki-67 and CC3 were obtained by counting five high power fields in biologic triplicates.

MTT Viability Assay

Cells were plated on 48 well plates in at least technical triplicate. After treatment and/or siRNA transfection, cells were incubated with MTT (Life Technologies) according to manufacturer's recommendations for 4 hours. Crystals were dissolved in 10% SDS in 0.01 M HCl for an additional 4 hours and then read on an Infinity 200 Pro (Tecan: Switzerland) plate reader at an absorbance of 570 nm.

Chemicals and Treatments

For in vitro experiments, vandetanib (SelleckChem: Germany) was used at a final concentration of 10 μ M and was added 72 hours after transfection with siRNA for 24 hours of drug treatment. For animal experiments, vandetanib stock was initially dissolved in DMSO and then water to a final concentration of (10 μ M) and administered by oral gavage at 10 mg/kg. Parallel experiments with corresponding concentrations of DMSO in water were used as a vehicle control gavage.

Tumor xenografts

Female nu/J athymic nude mice (Jackson Laboratory, Bar Harbor, ME, USA) were implanted with a 1.7-mg estrogen pellet (Innovative Research). 8×10^6 cells were suspended in serum free media/Matrigel (1:1 volume) and sKD-NT cells were injected into the right flank of eight mice, and an equal number of sKD-C cells were injected into the left of the same mice. Four mice were then randomized to receive vandetanib oral gavage, with the remaining four receiving vehicle oral gavage, as noted above. In separate experiments, seven nude mice were right flank injected with 8×10^6 MCF-7 cells transfected with siRNA to EGFR, with the contralateral flank injected in parallel with an equal number of cells transfected with NT siRNA. Tumor length, width, and depth were measured by calipers at least five times weekly. Volumes were calculated according to formula for an ellipsoid, $(A \times B^2)/2$, where A is the longest dimension and B is the length of the tumor perpendicular to A. All animal experiments performed were approved by the University of Iowa Institutional Animal Care and Use Committee.

Primary human tumors

Banked breast tumors for H&E and IHC were obtained through the University of Iowa Breast Molecular Epidemiology Resource (BMER), an institutional review board approved tumor tissue bank. IHC was performed with EGFR (Cell Signaling Technology, Danvers, MA) and RET (Cell Signaling Technology, Danvers, MA) antibodies as previously described (15). Fresh primary breast cancer samples were obtained from BMER with the University of Iowa Tissue Procurement Center. Fresh tumors were minced finely, dissociated overnight with gentle collagenase/hyaluronidase (Stemcell Technologies: Vancouver, BC, Canada), and treated with control media, vandetanib at a final concentration of 10 μ M, or PD153035, which is a selective EGFR inhibitor PD153035 (SelleckChem), at a final concentration of 10 μ M before harvesting for protein as described previously (12, 16).

Statistical analysis

Parametric data were analyzed using Student's t-test, nonparametric data by Fisher's exact test, and tumor-free survival curves by log-rank using R (17). Tumor growth curves were compared by performing Student's t-test of the areas under the curve between cohorts.

RESULTS

TFAP2C Regulates EGFR in Luminal Breast Cancer Cell Lines

Previous work has established that TFAP2C regulates EGFR expression in both human and mouse models of the HER2-amplified breast cancer subtype (14). To determine whether similar mechanisms of regulation occur in luminal breast cancer, three ER α -positive luminal A cell lines were examined. TFAP2C expression was knocked down by siRNA in MCF-7, T-47D and ZR75-1 breast cancer cell lines and EGFR expression was assessed by RNA and protein compared to NT siRNA transfection. As seen in Figure 1A, knockdown of TFAP2C resulted in a significant reduction of EGFR RNA and protein in all three luminal A cell lines. Using ChIP-seq analysis in MCF-7 cells, several TFAP2C peaks were identified within the *EGFR* gene and upstream of the transcriptional start site (Figure 1B).

Furthermore, the bc-GenExMiner database reported a significant positive correlation between TFAP2C and EGFR expression in primary breast cancer samples including an analysis of ER α -positive cancers (18). These data support the conclusion that *EGFR* is a target gene of TFAP2C in luminal breast cancer cells.

TFAP2C Controls Proliferation and Tumor Growth in MCF-7 Cells

Transient knockdown can result in variations of effect due to transfection efficiency, timing of analysis and cell density. To better assess the role of TFAP2C in proliferation and tumor growth, an MCF-7 cell clone with stable knockdown of TFAP2C using lentivirus-mediated shRNA (sKD-C) was compared in parallel to a cell clone created with non-targeting shRNA (sKD-NT) (4). As seen in Figure 1C, sKD-C cells were found to have significantly repressed expression of EGFR compared to sKD-NT cells. Relative cell viability was assessed by MTT assay; sKD-C cells demonstrated a 38% reduction in relative viability compared to sKD-NT cells (Figure 1D). Parallel experiments with transient knockdown using siRNA demonstrated a similar effect on cell viability with a 16.4% reduction in viability with knockdown of TFAP2C compared to NT siRNA transfection (Figure 1E). The greater effect on cell viability seen with the more complete silencing of TFAP2C expression with stable knockdown suggests a dose-dependent effect of gene silencing. Similarly, knockdown of TFAP2C in ZR-75-1 (Figure 1F) and T-47D (Figure 1G) reduced cell viability by 48% and 51%, respectively.

To assess the effect of TFAP2C on tumorigenesis, the outgrowth of sKD-C and sKD-NT tumor xenografts was examined. Athymic nude mice were dual flank inoculated with tumor cells; 8×10^6 sKD-C or sKD-NT cells were dual flank injected in parallel. sKD-C cells formed smaller tumor xenografts compared to sKD-NT with a mean of 44 mm^3 vs. 828 mm^3 ($p = 0.006$) at 15 days post-inoculation, after which mice began requiring euthanasia due to tumor burden of the sKD-NT xenografts (Figure 2A, B). Several markers of proliferation have been described, with Ki-67 being most commonly used in clinical assessment (19). Immunohistochemistry of the sectioned xenografts revealed significantly higher relative staining of the proliferation marker Ki-67 in sKD-NT-derived tumors compared to sKD-C-derived tumors (1.0 vs. 0.66, $p < 0.05$) (Figure 2C). There was a trend for increased relative staining of the apoptosis marker cleaved caspase 3 (CC3), which failed to reach statistical significance (1.0 vs. 1.13, $p = \text{not significant}$, Figure 2C).

Tumorigenesis Effect of TFAP2C Is Partially Attributed to EGFR

To assess the role of EGFR in the tumorigenesis model, tumor growth was compared with transient knockdown of EGFR. Previous studies have shown that knockdown of EGFR by siRNA reduced outgrowth of tumor xenografts (20); using a similar model, nude mice were dual flank injected with 8×10^6 MCF-7 cells transfected with siRNA to EGFR or an equal number of MCF-7 cells transfected with NT siRNA. NT cells formed palpable tumor xenografts in a median of 5.0 days versus 7.0 days for EGFR knockdown cells ($p = 0.007$) (Figure 3A, B). Tumors were also smaller in the EGFR knockdown group (Figure 3C), with mean volumes of 128 mm^3 at twelve days post-inoculation compared to 322 mm^3 in the NT group ($p = 0.002$) (Figure 3D). The findings support an important role of EGFR, which at least partially accounts for the effect of TFAP2C on tumorigenesis.

TFAP2C Regulates Proliferation through Cooperative Effects of EGFR and RET

As previously described, RET is a downstream target of TFAP2C in luminal breast cancer (10). In the luminal MCF-7 and BT-474 cell lines, transient knockdown of RET reduced *in vitro* cell proliferation, which was implicated to occur through changes in the MAP kinase pathway, as knockdown of RET led to reduced levels of phosphorylated extracellular-signal-regulated kinases (p-ERK) (12). We compared the effect of knockdown of RET and EGFR alone and in combination. Transient knockdown of RET and EGFR both led to decreased ERK activation in MCF-7 cells (Figure 4A). Importantly, these pathways are not completely redundant, as knockdown of both RET and EGFR together reduced levels of p-ERK more than either alone, with no changes in expression of TFAP2C. Furthermore, these molecular changes bear out differences in cellular proliferation. Compared to normalized NT siRNA transfection, knockdown of RET in MCF-7 produced a 31% decrease in cell proliferation ($p < 0.01$), and knockdown of EGFR reduced proliferation 34% on MTT ($p < 0.01$) (Figure 4A, bottom panel). The combined knockdown of RET and EGFR resulted in a 52.3% reduction in proliferation compared to NT ($p < 0.01$). The effect on proliferation in the combined knockdown was statistically reduced compared to either RET or EGFR knockdown alone ($p=0.003$ and $p=0.009$, respectively). Parallel experiments were performed in ZR-75-1 and T-47D (Figures 4B & 4C); similar to data in MCF-7 cells, combined knockdown of RET and EGFR demonstrated cumulative effects on reductions in p-ERK and relative viability. The level of RET expression was below routine detection by western blot in ZR-75-1 and T-47D; however, knockdown of RET expression was demonstrated by RT-PCR (See Supplemental Figure 1).

EGFR and RET are Markers for Response to Vandetanib in MCF-7

We have previously reported that vandetanib treatment of the luminal MCF-7 and BT-474 cells *in vitro* reduced cell proliferation (12). However, vandetanib has TKI activity that targets several RTKs including RET and EGFR. The individual contribution of RET and EGFR to vandetanib sensitivity was evaluated in MCF-7 cells. Knockdown of RET or EGFR significantly reduced the response to vandetanib but did not completely eliminate it (Figure 5A). However, knockdown of both RET and EGFR eliminated a significant response to vandetanib.

Since sKD-C cells have lost expression of RET and EGFR, we hypothesized that knockdown of TFAP2C would induce insensitivity to vandetanib in tumorigenesis assays. Tumorigenesis studies were performed with sKD-C compared to sKD-NT cells in animals gavaged with vandetanib or vehicle. When treated with vandetanib, sKD-NT cells formed smaller tumor xenografts than untreated mice receiving vehicle (335 mm^3 vs. 828 mm^3 , $p < 0.05$) (Figure 5B, C). Vandetanib appeared to exhibit an anti-proliferative effect, as tumors from mice in the treatment group exhibit a 29% decrease in relative Ki-67 staining ($p < 0.05$, Figure 5D). This effect was dependent on functional TFAP2C; although baseline tumor growth was reduced, sKD-C xenografts exhibited no change in tumor volume with vandetanib treatment (44 mm^3 vs. 91 mm^3 , $p>0.1$, NS, Figure 5B, C) and no longer demonstrated statistically significant differences in Ki-67 (Figure 5D).

Co-expression of EGFR and RET in Luminal Breast Cancer

The findings suggest an important cooperative effect of the TFAP2C target genes RET and EGFR in controlling cell proliferation. Furthermore, the cell line models indicate that the two genes share a common mechanism of regulation by TFAP2C, thus suggesting the coordinate expression of these RTKs. Primary human luminal breast cancers were assessed for RET and EGFR expression by immunohistochemistry. As seen in Figure 6A, three examples of luminal breast cancers were readily identified that demonstrate co-expression of RET and EGFR protein.

Nine fresh primary human breast cancers from chemoradiation-naïve patients were obtained from surgically excised breast specimens. From the final operative pathology report, tumors 1, 2, 3, 5, 7, 8 and 9 were ER/PR-positive; whereas, tumors 4 and 6 were ER/PR-negative. There were no HER2-amplified tumors. Fresh tumor tissue from these tumors was incubated *ex vivo* in media with either DMSO vehicle control, vandetanib (with TKI activity against RET and EGFR) or the EGFR-specific TKI, PD153035 (PD). After 20 minutes of treatment, western blots were performed on these cell lysates for total ERK, p-ERK, and GAPDH (Figure 6B). In addition, *RET* and *EGFR* mRNA expression relative to the MCF-7 cell line was quantified (Figure 6C). A table summarizing the relative change in p-ERK with vandetanib or PD treatment is shown in Figure 6D. As expected, the TNBC Tumors 4 and 6 had two of the highest levels of EGFR expression with relatively lower levels of RET. Neither of these two tumors showed significant reductions of p-ERK with TKI treatment. Of the seven hormone receptor-positive tumors, decreased levels of p-ERK were seen with EGFR-specific inhibition by PD153035 in five tumors (Tumors 2, 5, 7, 8 and 9) (Figure 6B & 6D). Similar or more pronounced reductions in ERK phosphorylation were seen in most of these tumors with vandetanib treatment, indicating that vandetanib—which targets both RET and EGFR—appears to reduce p-ERK greater than EGFR inhibition alone. Tumors 2, 5, 8 and 9 are examples with approximately equal response to both TKIs, and suggested that the effect is mediated by EGFR. This conclusion is consistent with relatively low expression of RET in most of these tumor samples. Tumor 7 had a marginal response to PD153035 of only 17% and the response to vandetanib failed to reach significance; the response of tumor 7 to TKIs was consistent with the relatively low expression of RET and EGFR. Tumors 1 and 3, which had relatively high levels of RET expression, were responsive to vandetanib but had minimal response to PD153035, indicating that the vandetanib response was likely driven by RET inhibition.

DISCUSSION

Previous work has shown that TFAP2C regulated the expression of luminal differentiation markers in breast cancer and knockdown of TFAP2C expression induced a change from a luminal to basal-like gene expression pattern that resembled epithelial-mesenchymal transition (EMT) (4). Clinical studies reported that overexpression of TFAP2C in primary breast cancers was associated with a worse prognosis and hormone resistance (7–9). We have sought to identify the TFAP2C target genes that mediate cancer progression and hormone resistance in luminal breast cancer. Prior work identified the *RET* gene as a TFAP2C target gene in luminal breast cancer and inhibiting RET signaling by either

knockdown of expression or TKI treatment augmented tamoxifen sensitivity (10, 12, 21). Herein, we have demonstrated that TFAP2C regulated the expression of EGFR in luminal breast cancer cell lines and ChIP-seq indicated that TFAP2C directly targets the *EGFR* gene. The potential that TFAP2C regulates EGFR through additional secondary effects may also be possible. Furthermore, the role of TFAP2C in regulation of breast cancer proliferation and growth of xenografts can be at least partially explained through regulation of EGFR. Inhibition of EGFR and RET had additive effects on ERK1/2 activation, cell proliferation and tumor progression, which was greater than the effects of knockdown of either RTK alone. Although knockdown of RET or EGFR significantly blunted the response to vandetanib, knockdown of both RTKs eliminated the anti-proliferative response to vandetanib. The findings support a model in which TFAP2C influences tumor progression and hormone response through signaling pathways mediated by the RTKs RET and EGFR.

Although EGFR expression is more commonly found in triple-negative/basal breast cancers, approximately 25% of the HER2 subtype and 6% of luminal cancers express EGFR (22). However, another study reported that EGFR was expressed in approximately 50% of ER α /PR+/HER2+ Luminal B breast cancers (23). Compared to luminal A breast cancers, the luminal B subtype tends to be hormone resistant and demonstrates a more aggressive clinical course with a greater likelihood of lung metastasis (24). Several lines of evidence suggest that EGFR is likely to play a significant role in hormone resistance and aggressiveness of luminal breast cancers. First, breast cancers that express EGFR are associated with a worse disease-free survival and resistance to systemic therapy (25). Second, the occurrence of lung metastases as the first distant site of disease was found to be strongly correlated with EGFR expression (26). Overexpression of EGFR in MCF-7 cells confers estrogen independent growth (27), and studies using an MCF-7 cell line model of hormone resistance showed that EGF-dependent activation of heterodimers of EGFR/HER1 and HER2 resulted in activation of the ERK pathway and increased cell proliferation (28). More recent studies confirmed the formation of EGF-dependent HER1/HER2 heterodimers in primary breast cancers and demonstrated that EGF-dependent HER1 and HER2 phosphorylation was inhibited by the TKIs lapatinib and erlotinib (29). Many other models of cancer growth and progression using several different cell types have similarly identified EGFR as a driver of cancer aggressiveness (20, 30–33). Hence, the clinical and experimental evidence strongly indicates that EGFR contributes to hormone resistance and cancer progression of luminal breast cancers (34).

The application of targeted therapy requires a deeper understanding of the mechanisms driving cancer progression and metastasis. The current study lends additional clarity to the application of TKIs in the treatment of breast cancer. Extrapolating the current experimental data to breast cancer treatment, one would conclude that TKIs with activity against both RET and EGFR are likely to be more effective than drugs with more specific mechanisms of action. Vandetanib is a TKI with significant activity against RET and EGFR as well as the vascular endothelial growth factor receptor (VEGFR) (13). However, earlier work suggested that VEGFR did not play a role in regulating the growth of luminal breast cancer cells (35). The findings further suggest that detection of RET and EGFR by IHC could be used as molecular markers predictive of response to vandetanib. As shown in Figure 6, luminal

breast cancers expressing both RET and EGFR were readily identified by IHC and further studies are needed to define the population of breast cancers expressing both RTKs. In addition, the level of RTK expression may be a critical factor in response to vandetanib, though MCF-7 cells, which have relatively low levels of EGFR (27), were clearly responsive. The development of hormone resistance continues to be an important clinical problem in ER α -positive breast cancer. The findings further suggest that vandetanib acting through inhibition of RET and EGFR may increase response to tamoxifen. Whereas many of the clinical trials of vandetanib have focused on advanced, metastatic disease (36, 37), there may be a role for TKI therapy to induce or augment hormone response (12). The current studies provide compelling pre-clinical data to investigate the use of TKIs such as vandetanib in the treatment of luminal breast cancer and further indicates that RET and EGFR may be used as molecular markers of response.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

FINANCIAL INFORMATION: This work was supported by a generous gift to R. J. Weigel from the Kristen Olewine Milke Breast Cancer Research Fund.

NIH Funding: This work was supported by the National Institutes of Health grants R01CA183702 (PI: R. J. Weigel) and T32CA148062 (PI: R. J. Weigel). JPD, AWL, VTW and PMS were Surgical Oncology Fellows supported by the NIH grant T32CA148062.

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Figure 1. TFAP2C Regulates EGFR in Luminal Breast Cancer and Enhances Cell Viability

A. Expression of EGFR RNA and protein is shown for MCF-7, ZR-75-1 and T-47D luminal breast cancer lines with knockdown of TFAP2C (C) compared to non-targeting (NT) siRNA at 96 hours after transfection. **B.** ChIP-seq data from Woodfield et al.(6), reported in GEO Series accession number GSE21234. **C.** Stable MCF-7 cell clones established with shRNAs to TFAP2C (sKD-C) or non-targeting shRNA (sKD-NT) were evaluated for expression of TFAP2C and EGFR RNA (top) and protein (bottom); * $p < 0.05$. **D.** Relative viability of sKD-C cells compared to sKD-NT; $p < 0.05$. **E.** Relative viability of MCF-7 cells following transient knockdown of TFAP2C (C) compared to non-targeting (NT) siRNA. Expression of TFAP2C and relative viability with knockdown of TFAP2C (C) compared to non-targeting (NT) siRNA transfection in ZR-75-1 (**F**) and T-47D (**G**).

Figure 2. TFAP2C Regulates Tumor Growth and Proliferative Index

A. Volume of tumor xenografts comparing sKD-C and sKD-NT cells, N=8 mice per group.

B. Average tumor volume compared at day 15 from data in A; * p=0.006. **C.**

Immunohistochemistry for Ki-67 and CC3 of tumor xenografts from sKD-NT and sKD-C cells as indicated with quantitative differences shown in graphic form at the right; *p<0.05, NS: not significant.

Figure 3. EGFR Regulates Tumor Growth and Progression

A. Tumor-free survival of mice inoculated with MCF-7 cells transfected with siRNA to EGFR (siEGFR) vs. non-targeting (NT); N=7 mice. **B.** Tumor free survival at day 5 post-inoculation (time when all siNT had tumors) showing significant difference between NT and EGFR siRNA transfected cells. **C.** Average tumor volume for all animals inoculated with NT or EGFR siRNA transfected cells. **D.** Average tumor volume at 12 days post-inoculation for experiment shown in C; * $p = 0.002$.

Figure 4. Additive Effects of RET and EGFR in ERK Activation and Cell Viability

In all top panels, western blots show expression of TFAP2C, RET, EGFR, ERK, p-ERK and GAPDH after transfection with siRNA to NT, RET, EGFR or both EGFR and RET (R+E) in MCF-7 (A), ZR-75-1 (B) and T-47D (C). The relative level of p-ERK compared to NT with knockdown of RET, EGFR and R+E for MCF-7 cells: 66%, 7%, 4%; for ZR-75-1: 61%, 45%, 15%; for T-47D: 57%, 40%, 36%. Bottom panels show parallel assessment of cell viability for all three cell lines with knockdown of RET and EGFR or both RTKs as indicated; * $p < 0.01$.

Figure 5. Role of RET and EGFR in Response to Vandetanib

A. Relative viability of MCF-7 cells after knockdown of RET, EGFR or both RTKs (R+E) without treatment (Vehicle) (From figure 4A, bottom panel) or with vandetanib treatment (VAN) for 24 hours prior to harvest. **B.** Tumor volume of sKD-NT (black) and sKD-C (blue) xenografts with vandetanib treatment or without vandetanib treatment (from figure 2A). **C.** Tumor volume (mm³) at day 15 for experiment shown in B; * $p < 0.05$, NS: non significant. **D.** Immunohistochemistry of xenografts stained for Ki-67 for experiment shown in C. Quantitative results of relative Ki-67 for xenografts from sKD-NT (black) and sKD-C (blue) xenografts; * $p < 0.05$.

Figure 6. RET and EGFR Expression in Breast Cancer Samples and Response to TKI therapy
A. H&E and immunohistochemistry of three luminal A primary human breast cancers stained for expression of RET and EGFR show expression of both RTKs. **B.** Western blots for total ERK, p-ERK and GAPDH demonstrate the change in p-ERK expression of nine breast tumor tissue after treatment with vehicle control (CTL), vandetanib (VAN), or PD153035 (PD). **C.** Plot of relative *RET* and *EGFR* expression in nine primary breast cancers relative to MCF-7. Color of circle indicates relative response to treatment with vandetanib (yellow) or PD (blue). Grey indicates no response to RTK; X and Y-axes are logarithmic. **D.** Table summarizes relative decrease in p-ERK compared to control treatment based on densitometer analysis of western blots in B; NC: no change.