FGFR Signaling Promotes the Growth of Triple-Negative and Basal-Like Breast Cancer Cell Lines Both In Vitro and In Vivo

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Abstract

Purpose: The oncogenic drivers of triple-negative (TN) and basal-like breast cancers are largely unknown. Substantial evidence now links aberrant signaling by the fibroblast growth factor receptors (FGFR) to the development of multiple cancer types. Here, we examined the role of FGFR signaling in TN breast cancer.

Experimental Design: We examined the sensitivity of a panel of 31 breast cancer cell lines to the selective FGFR inhibitor PD173074 and investigated the potential mechanisms underlying sensitivity.

Results: TN breast cancer cell lines were more sensitive to PD173074 than comparator cell lines (P = 0.011), with 47% (7/15) of TN cell lines showing significantly reduced growth. The majority of TN cell lines showed only modest sensitivity to FGFR inhibition in two-dimensional growth but were highly sensitive in anchorage-independent conditions. PD173074 inhibited downstream mitogen-activated protein kinase and PI3K–AKT signaling and induced cell-cycle arrest and apoptosis. Basal-like breast cancer cell lines were found to express FGF2 ligand (11/21 positive) and, similarly, 62% of basal-like breast cancers expressed FGF2, as assessed by immunohistochemistry compared with 5% of nonbasal breast cancers (P < 0.0001). RNA interference targeting of FGF2 in basal-like cell lines significantly reduced growth in vitro and reduced downstream signaling, suggesting an autocrine FGF2 signaling loop. Treatment with PD173074 significantly reduced the growth of CAL51 basal-like breast cancer cell line xenografts in vivo.

Conclusions: Basal-like breast cancer cell lines, and breast cancers, express autocrine FGF2 and show sensitivity to FGFR inhibitors, identifying a potential novel therapeutic approach for these cancers.

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Introduction

Therapies that target the drivers of individual breast cancers have substantially improved the outcome of women with breast cancer, in particular endocrine therapies for luminal type cancers that express the estrogen receptor and trastuzumab for cancers with HER2 amplification (1). However, for the approximately 10% to 15% of breast cancers that are triple negative (TN), cancers that express neither the estrogen nor progesterone receptors nor have amplification of HER2, the oncogenic drivers are poorly understood (2–5). This subgroup of cancers has a poor prognosis in the adjuvant setting (6, 7) and is highly proliferative with a short time from relapse to death (8). There is substantial overlap between TN breast cancers and the basal-like subtype of breast cancer, approximately 80% of TN breast cancers are basal-like (9), and therefore the two terms describe a broadly similar group of cancers. Identifying the oncogenic drivers of TN breast cancer and basal-like breast cancer is a priority, if the outcome of women with this group of cancers is to be improved.

The oncogenic drivers and the factors that promote TN tumor growth are largely unclear with current evidence pointing to substantial heterogeneity (5, 10). Mutations of PIK3CA are found in less than 10% TN breast cancers (11), although the tumor suppressor PTEN may also be lost in a high proportion of these cancers (12), and no other high frequency kinase gene mutations have been identified (13, 14). Focal amplifications are found in the majority of TN cancers, although TN cancers often exhibit high levels of genomic instability (15, 16) and amplification of each individual genomic locus is only present in a small proportion of cancers (5). Significant progress has been made in identifying commonly activated signal transduction pathways in TN and basal-like breast cancers. Deletion...
of the phosphatase PTPN12 may set up a permissive environment for oncogenic tyrosine kinase signaling in TN cancer (17). TN cancer cell lines show high sensitivity to SRC inhibitors in vitro (18), and mitogen-activated protein kinase (MAPK) pathway activation is more prominent in these cancers than luminal type cancers in vitro (4, 19). In a subset of cancers, epidermal growth factor receptor (EGFR) has potentially been shown to be oncogenic in vitro (19), and there is recent clinical trial data supporting EGFR as a therapeutic target in a small proportion of TN cancers (20). The oncogenic drivers that activate the MAPK pathway in the remaining cancers are unknown.

We have previously suggested that amplification of the fibroblast growth factor receptor (FGFR) genes may represent a therapeutic target in breast cancer, with amplification of FGFR1 occurring in approximately 10% of breast cancers (21), predominantly of luminal subtype (22). Amplification of FGFR2 also occurs more rarely being found in only approximately 1% to 2% of breast cancers overall, although approximately 4% of TN breast cancer have FGFR2 amplification (5). These data suggest that aberrant activation of FGF signaling can play a role in breast tumorigenesis (23). In this study, we examine the prevalence of FGF signaling as a driver in breast cancer, analyzing the sensitivity of a panel of breast cancer cell lines to PD173074, a potent and selective FGFR inhibitor (24). We find that TN and basal-like breast cancers are characterized by a poor prognosis, and this work identifies a potential novel therapeutic strategy for these cancers. Potent and selective FGFR inhibitors are in early clinical development, and this study provides a rational for assessing these inhibitors, or therapies targeting FGF2 ligand, in TN and basal-like breast cancer.

**Translational Relevance**

The oncogenic drivers of triple negative (TN) and basal-like breast cancer are largely unknown. In this study, we show that multiple TN and basal-like breast cancer cell lines are sensitive to a fibroblast growth factor receptor (FGFR) inhibitor and provide evidence that the growth of basal-like breast cancer cell lines is promoted by autocrine FGF2 signaling. Basal-like breast cancers are characterized by a poor prognosis, and this work identifies a potential novel therapeutic strategy for these cancers. Potent and selective FGFR inhibitors are in early clinical development, and this study provides a rational for assessing these inhibitors, or therapies targeting FGF2 ligand, in TN and basal-like breast cancer.

**Materials and Methods**

**Cell lines, materials, and antibodies**

Cell lines were obtained from American Type Culture Collection or Asterand and maintained in phenol red–free Dulbecco’s modified Eagle’s medium or RPMI with 10% FBS (PAA gold) and 2 mmol/L-glutamine (Sigma-Aldrich). All 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) and arrayCGH profiling. PD173074 and recombinant FGF2 were from Sigma. siRNAs were from Dharmacon: FGFR2 siGenome SMARTpool (siFGFR2, M-00695-00), FGFR2 siGenome individual siRNA (siFGFR2-A-C, D-006695-02/03/04, respectively), FGFR1 siGenome SMARTpool (siFGFR1, M-003131-03), FGFR1 siGenome individual siRNA (siFGFR1-A-C D003131-09/22/23, respectively), siGenome Non-Targeting siRNA Pool#1 (siCON, D-001206-13), and PLK1 siGenome SMARTpool (siPLK1, M-003290-01). Antibodies used were phosphorylated AKT-Ser473 (4058), AKT (4691), phosphorylated ERK1/2-Thr202/Tyr204 (4370), phosphorylated RSK-Thr359/Ser363 (9344), ERK1/2 (9102; all Cell Signalling Technology), PARP-1 (sc-8007), FGF2 (sc-79) β-actin (sc-1616; all Santa-Cruz Biotechnology).

**Allocation of molecular subtype and receptor status**

Breast cancer cell lines were ascribed to be TN as described by Neve and colleagues (25), with the exception of MDA-MB-453 which is HER2 amplified (26). CAL51, CAL120, MFM223, and SUM52PE are ER, PR, and HER2 negative by Western blotting as previously described (5). S68 is ER positive by Western blotting and VP229 and JIM51 are HER2 amplified as assessed by HER2 FISH and array Comparative Genomic Hybridization (data not shown). Cell line gene expression subtype was as described by Neve and colleagues, with the exception of CAL51 and CAL120 that are of basal B subtype by using the cancer class prediction of Neve and colleagues (5).

Breast cancers in a tissue microarray (27) were classified into the molecular subtypes by using the immunohistochemical surrogate described by Nielsen and colleagues (28) and Cheang and colleagues (29). TN cancers were divided into core basal-like (positive for EGFR or CK5/6) and nonbasal TN (negative for EGFR and CK5/6).

**Cell line drug sensitivity, siRNA transfection, and FGF2-neutralizing antibody**

Cell lines were transfected with siRNA (50 nmol/L final concentration) in 96-well plates with RNAiMax (Invitrogen) or Dharmafect4 (Dharmacon) according to manufacturers instructions, and survival was assessed by Cell Titre-Glo cell viability assay (Promega) after 5 days growth. For sensitivity to PD173074, cells were plated in 96-well plates and starting 24 hours postplating were exposed for 72 hours to 1 μmol/L PD173074 or vehicle, and survival assessed by using Cell Titre-Glo. For assessment of activated caspase 3/7, cells were treated as for sensitivity to PD173074, assessed by using the Apo-ONE Homogeneous Caspase-3/7 Assay (Promega) according to manufacturer’s instructions, and the level of activated caspase 3/7 adjusted for cell number as assessed by Cell Titre-Glo in corresponding wells. For sensitivity to FGF2-neutralizing antibody, cells were plated in 96-well plates coated with polyHEMA [poly(2-hydroxyethylmethacrylate)].
and starting 24 hours postplating were exposed for 72 hours to various concentrations of control normal Goat IgG (AB-108-C; R&D Systems) or FGF2-neutralizing antibody (AB-233-NA) and survival assessed by using CellTitre-Glo.

**Anchorage-independent growth**

Unless stated otherwise, assays of anchorage independence were on plates coated with 1.2% polyHEMA (Sigma) in 95% ethanol, as previously described (30). Soft agar assays were done as previously described (22).

**Western blotting and fluorescence-activated cell sorting**

Indicated cell lines were grown on 10-cm plates, treated as indicated, and lysed in NP40 lysis buffer. Western blots were carried out with precast TA or Bis-Tris gels (Invitrogen) as previously described (22). Fluorescence-activated cell sorting (FACS) analysis was done as previously described (22).

**Xenografts**

CAL51 cells were injected into the flank of 20 nude mice (3 × 10⁶ cells per injection). After 21 days to establish tumors, half of the mice were treated with PD170374 25 mg/kg by daily intraperitoneal injection in 50 mmol/L lactate buffer pH 5.0 and half with lactate buffer. Tumors were bidimensionally measured twice weekly, and tumor volume of each cohort expressed relative to baseline of vehicle only group [tumor volume]. The growth of tumors treated with PD173074 and vehicle were compared with ANOVA.

**Quantitative reverse transcriptase PCR**

cDNA was synthesized from RNA by using Superscript III and random hexamers (Invitrogen). Quantitative PCR (qPCR) was done as previously described (22). Expression of FGF2 (Hs00241111_m1) was expressed relative to the mean of endogenous controls S18 (4310881E), MRPL19 (Hs00608519_m1), and β-actin (4310881E). Tumor samples for FGF2 mRNA expression analysis were only included if more than 50% cells in the section were tumor cells.

**FGF2 ELISA**

FGF2 ELISA was done with DUoSet DY233 (R&D Systems) according to manufacturer’s instructions with serial dilutions of recombinant FGF2 for a standard curve.

**FGF2 immunohistochemistry**

FGF2 immunohistochemistry (IHC) was done on a tissue microarray, extensive characterization of which has been reported previously (27). Antigen retrieval was with pH 6.0 citrate buffer for 30 minutes at 90°C, before FGF2 antibody (rabbit polyclonal 500-P18; PeproTech) 1:100 dilution for 60 minutes and development with dual Envision kit (Dako). FGF2 was scored by 2 observers (JSRF and NCT), blinded to the clinicopathologic data. Data were recorded separately for nuclear and cytoplasmic FGF2 expression. FGF2 expression was considered positive for cytoplasmic staining if unequivocally malignant cells expressed FGF2 and for nuclear staining with a Quick score more than 2 (31).

**Statistical analysis and analysis of gene expression data**

All statistical tests were done with GraphPad Prism version 5.0. Unless stated otherwise, P values were 2 tailed and considered significant if P < 0.05. Error bars represent SEM of 3 experiments.

**Results**

**Sensitivity to PD173074 in breast cancer cell lines**

We examined the sensitivity of a panel of 31 breast cancer cell lines to the FGFR selective inhibitor PD173074 (24, 32), treating cell lines plated in 96-well plates for 72 hours with 1 μmol/L PD173074. Eight breast cancer cell lines showed a significant reduction in growth after 72 hours treatment (P < 0.01 Student’s t test). We noted that 7 of these cell lines were of TN phenotype (Fig. 1A) and that TN cell lines were more sensitive to PD173074 than other cell lines (P = 0.011, Mann–Whitney U Test; Fig. 1A). The sensitivity of the majority of cell lines to PD173074 was only modest (Fig. 1A). We have previously shown that one of the sensitive cell lines, CAL120, was substantially more sensitive to PD173074 growing in anchorage-independent conditions (22). Therefore, we assessed the sensitivity of the moderately sensitive cell lines in anchorage-independent conditions. Substantially increased sensitivity to PD173074 under these conditions was shown for TN cell lines (Fig. 1B), with no sensitivity seen in control cell lines that were ER and/or HER2 positive (Fig. 1C). Confirming these results, the growth of CAL51 cells in soft agar was blocked by PD173074, with no effect of PD173074 on the growth of control T47D ER-positive cell line (Fig. 1D and Supplementary Fig. S1).

We have previously identified FGFR gene amplification in 3 of the sensitive TN cell lines: FGFR2 amplifications in SUM52 and MDM223 (5) and FGFR1 amplification in CAL120 (22). MDA-MB-453, a HER2 amplified non-TN cell line that was sensitive to PD173074, has been shown to have an activating FGFR4 mutation (26). The data shown above suggested a more pervasive sensitivity to PD173074 in TN breast cancer, and we, therefore, examined the factors underlying the sensitivity of the remaining TN breast cancer cell lines.

**Cellular consequences of FGFR inhibition**

We examined downstream signaling in the TN breast cancer cell lines CAL51, Hs578T, and BT549 after exposure to PD173074 (Fig. 2A). We have previously shown that MAPK signaling is inhibited by PD173074 in the CAL120 cell line (22), and we confirmed that phosphorylation of RSK and ERK1/2 was substantially decreased by
PD173074 in all cell lines, with a decrease in AKT phosphorylation in Hs578T under anchorage-independent conditions (Fig. 2A). We note that CAL51 has previously been shown to have an activating mutation in PIK3CA (c.1624G>A, p.E542K), and BT549 has a homozygous mutation of PTEN (c.821delG, p.V275fs*1) that results in a truncated nonfunctional protein (www.sanger.ac.uk/cosmic), which might explain the lack of modulation of AKT in these cell lines. Mirroring the increase in sensitivity seen with PD173074 in anchorage-independent conditions (Fig. 1B), there was an increase in the FGFR dependence of downstream signaling in anchorage-independent conditions. To confirm inhibition of FGFR signaling by PD173074, we showed that FGFR-Tyr653/654 autophosphorylation and phosphorylation of FRS2-Tyr196, a key FGFR adapter protein required for MAPK pathway activation (33), was decreased by PD173074 in CAL120 and CAL51 cells (Supplementary Fig. S2).

We examined the consequences of FGFR inhibition. CAL51 and BT549 cells exhibited G1 cycle arrest in response to PD173074 (Fig. 2B) and Hs578T arrested at 4N (Fig. 2B). There was a substantial increase in subG1 cells in CAL120 and CAL51 in response to PD173074, suggesting the induction of apoptosis, but only a minor increase in Hs578T. To confirm that the increase in subG1 represented apoptosis, we measured PARP cleavage in response to PD173074, with evidence of increased PARP cleavage in CAL120 and Hs578T cell lines (Fig. 2C). In addition, CAL120 and Hs578T showed increased levels of activated caspase 3/7 on exposure to PD173074 (Fig. 2D). We could therefore confirm apoptosis in CAL120 and Hs578T but not in CAL51.

Therefore, multiple basal-like TN breast cancer cell lines are sensitive to FGFR inhibitor PD173074 with a decrease in downstream signaling, and this is associated with induction of both cell-cycle arrest and apoptosis.
Basal-like breast cancer cell lines express FGF2 ligand

We investigated the potential mechanisms underlying sensitivity to PD173074, in the cell lines in which the mechanism of sensitivity was unknown (MDA-MB-157, CAL51, BT549, and Hs578T). As well as being TN, we noted that all these cell lines were basal-like in phenotype and, in particular, the basal B cell line subtype as described by Neve and colleagues (25). Basal B like cell lines are reported to be the cell line subtype most enriched in “stem cell-like” gene expression patterns, as well as having high levels of mesenchymal markers (25). Assessing PD173074 sensitivity data according to cell line subtype, basal B cell lines were more sensitive to PD173074 compared with cell lines of other subtypes (P = 0.0328 Mann–Whitney U test, Fig. 1A).

We first examined the presence of amplifications by microarray-based comparative genomic hybridization (aCGH) and showed that none of these cell lines exhibited amplification of either the FGF receptors or FGF ligands (Supplementary Table S1). We next examined the correlation between sensitivity to PD173074 and whole genome gene expression in the panel of 31 cell lines, as assessed by Illumina human WG6 gene expression microarrays, examining for components of FGFR signaling pathways (Supplementary Table S2). We noted that FGF2 mRNA expression correlated significantly, but modestly, with sensitivity to PD173074 (r = −0.43 P = 0.024 Spearman’s correlation coefficient; Supplementary Fig. S3).

To extend this observation, we assessed FGF2 mRNA expression by quantitative PCR in the panel of breast cancer cell lines. The majority of cell lines had undetectable FGF2 mRNA. Basal-like breast cancer cell lines expressed substantially greater FGF2 mRNA levels than luminal cell lines (P = 0.0001 Mann–Whitney U Test, Fig. 3A). Within the
basal-like cell lines, the basal B cell lines (25) showed substantially higher levels of FGF2 than basal A cell lines (Fig. 3A). We confirmed FGF2 protein expression in these cell lines by Western blotting and that sensitivity to PD173074 correlated with FGF2 protein expression (Fig. 3B and C). Basal B breast cancer cell lines were also observed to condition media with FGF2 (Fig. 3D) and had substantially elevated FGF2 protein in cellular lysates assessed by FGF2 ELISA (Supplementary Fig. S3).

Basal-like breast cancers express FGF2 ligand
To assess FGF2 expression in breast cancers, we examined the expression of FGF2 by IHC in a well characterized breast cancer tissue microarray (27). FGF2 IHC was validated as illustrated in Supplementary Figure S4. FGF2 was expressed in the cytoplasm of 12% (24/199) breast cancers (Fig. 4A), frequently in a vesicular distribution, as well as nuclear staining without cytoplasmic staining in a further 10% (19/199). FGF2 staining was heterogenous in the majority of tumors (Fig. 4A). FGF2 cytoplasmic staining was only weakly associated with nuclear staining [presence of nuclear staining, 29% (7/24) cytoplasmic positive tumors vs. 11% (19/171) cytoplasmic negative tumors, \( P = 0.019 \) Fisher’s exact test]. To confirm the validity of the FGF2 IHC staining, we assessed FGF2 mRNA expression by qPCR in a randomly selected sample of FGF2 IHC–positive and FGF2 IHC–negative cancers. This comparison was complicated by the substantial FGF2 expression seen in stromal cells such as fibroblasts and normal breast elements (Supplementary Fig. S4), with stromal mRNA likely contaminating qPCR analysis in some tumors. Despite these limitations, both FGF2 cytoplasmic and FGF2 nuclear IHC staining was associated with increased FGF2 mRNA expression (Fig. 4B).

We showed above that basal-like breast cancer cell lines condition media with FGF2 (Fig. 3D) implying secretion of FGF2. We therefore initially scored cytoplasmic FGF2 IHC expression, as this was likely to represent FGF2 stored in...
Cytoplasmic FGF2 expression was strongly associated with core basal-like breast cancers, TN cancers positive for EGFR or CK5/6 (28, 29), with 62% (binomial 95% CI: 41–80) of core basal-like breast cancers expressing FGF2 compared with 5% (95% CI: 2.0–8.9) of nonbasal-like breast cancers \( (P < 0.0001 \text{ Fisher's exact test, Table 1 and Fig. 4C}) \). Within TN breast cancers, FGF2 expression was associated specifically with cancers of a core basal-like phenotype (TN: core basal-like 62% vs. nonbasal 0% \( P = 0.0015 \) Fisher’s exact test). Number in each group luminal \( n = 138 \), HER2 \( n = 26 \), core basal-like \( n = 26 \), TN nonbasal \( n = 9 \). D, Kaplan–Meier curves of overall survival for breast cancers with cytoplasmic FGF2 expression \( (n = 24) \) compared with cancers without cytoplasmic FGF2 expression \( (n = 175; P = 0.035 \text{ log-rank test, } P = 0.007 \text{ Gehan–Breslow–Wilcoxon test}) \).

Figure 4. FGF2 expression is associated with the core basal-like phenotype in breast cancers. A, assessment of FGF2 expression by IHC, with a cancer negative for FGF2 expression (left), with FGF2 cytoplasmic expression (middle), and FGF2 nuclear expression (right). B, validation of FGF2 IHC staining, with assessment of FGF2 expression by quantitative RT-PCR on mRNA extracted from a random selection of tumors positive and negative for FGF2 expression \( (n = 28) \). FGF2 mRNA was expressed at higher levels in FGF2 IHC-positive cancers \( (P < 0.0001 \text{ Kruskal–Wallis one way ANOVA}) \), and at higher levels in cancers with FGF2 cytoplasmic expression \( (n = 6, P = 0.018) \) and FGF2 nuclear expression \( (n = 3, P = 0.013 \text{ Mann–Whitney } U \text{ test}) \) compared with FGF2 negative cancers \( (n = 15) \). C, Cytoplasmic FGF2 expression according to tumor subtype, defined using the IHC criteria of Nielsen and colleagues (28), with the percentage of tumors in each subtype positive for cytoplasmic FGF2 expression \( (P < 0.0001 \text{ comparison of all subtypes with } \chi^2 \text{ test and } P = 0.0015 \text{ comparison of core basal-like and nonbasal TN cancers with Fisher’s exact test}) \). Number in each group luminal \( n = 138 \), HER2 \( n = 26 \), core basal-like \( n = 26 \), TN nonbasal \( n = 9 \). D, Kaplan–Meier curves of overall survival for breast cancers with cytoplasmic FGF2 expression \( (n = 24) \) compared with cancers without cytoplasmic FGF2 expression \( (n = 175; P = 0.035 \text{ log-rank test, } P = 0.007 \text{ Gehan–Breslow–Wilcoxon test}) \).

secretory granules. Cytoplasmic FGF2 expression was strongly associated with core basal-like breast cancers, TN cancers positive for EGFR or CK5/6 (28, 29), with 62% (binomial 95% CI: 41–80) of core basal-like breast cancers expressing FGF2 compared with 5% (95% CI: 2.0–8.9) of nonbasal–like breast cancers \( P < 0.0001 \text{ Fisher's exact test, Table 1 and Fig. 4C}) \). Within TN breast cancers, FGF2 expression was associated specifically with cancers of a core basal-like phenotype (TN: core basal-like 62% vs. nonbasal 0% \( P = 0.0015 \text{ Fisher's exact test) \). FGF2 expression was associated with an adverse prognosis because of the relatively early relapse of FGF2-expressing cancers (Fig. 4D). However, it is unclear whether this simply reflects an association with the basal-like subtype, a subtype that itself has a poor prognosis, or whether FGF2 has a causal role in the poor prognosis.

To extend these observations, we interrogated the publicly available TransBIG dataset (34). In this dataset, basal-like breast cancers, as defined by Parker and colleagues (35), had higher FGF2 expression compared with other breast cancer subtypes (Supplementary Fig. S5). Within the basal-like subset of cancers, high FGF2 expression was associated with poor outcome \( (HR = 2.924, 95\% \text{ CI: 1.019–8.393, } P = 0.046 \text{ log-rank test, Supplementary Fig. S5}) \). However, only 41 basal-like cancers were included in this analysis and therefore the results should be interpreted with appropriate caution.

Nuclear FGF2 IHC staining, in the absence of cytoplasmic staining, was not associated with any tumor phenotype (Supplementary Table S3), suggesting a distinct biological role for nuclear FGF2. Interestingly, the myoepithelial cells of normal breast ducts were observed to strongly express FGF2 in both nuclear and cytoplasmic compartments (Supplementary Fig. S4).

FGF2 promotes the growth of basal-like cell lines

To examine whether FGF2 potentially acted in an autocrine fashion, we silenced FGF2 expression by using siRNA in CAL120, CAL51, and Hs578T cells which caused a significant reduction in cell line growth with both siFGF2
SMARTpool and individual siRNAs (Fig. 5A). In addition, we noted that silencing FRS2, the key adapter protein required for MAPK pathway activation by FGFRs (33), reduced the survival of all 3 basal-like cell lines and to a greater extent than control cell lines (Supplementary Fig. S6). Silencing of FGFR1 in CAL120 cells decreased survival (Fig. 5B), suggesting an FGF2–FGFR1 autocrine loop in this FGFR1-amplified cell line (22).

We previously noted that PD173074 reduced both MAPK pathway and AKT phosphorylation in Hs578T cells, and we therefore examined the effect of silencing FGF2 on downstream signaling in this cell line. Silencing of FGF2 reduced both ERK1/2 phosphorylation and AKT phosphorylation in Hs578T (Fig. 5C). A similar but more modest drop in ERK1/2 phosphorylation was observed on silencing FGF2 in CAL120 cells that may reflect the partial knockdown of FGF2 achieved (Supplementary Fig. S6). To extend these observations further, we examined the effect of an FGF2-neutralizing antibody on the growth of Hs578T cells. The FGF2-neutralizing antibody reduced the growth of Hs578T cells compared with cells treated with normal goat IgG (Fig. 5D). There was no effect of the neutralizing antibody on control ER-positive cell lines (MCF7, T47D, and ZR75.1, data not shown).

**FGFR inhibitors decrease growth of CAL51 xenografts in vivo**

To investigate whether FGFR signaling presented a potential therapeutic target, we established xenografts with the basal-like breast cancer cell line CAL51 cells in nude mice and treated established tumors with PD173074 (Fig. 5E). We were unable to establish xenografts reliably from Hs578T cells. PD173074 substantially reduced the growth of CAL51 xenografts compared with xenografts treated with vehicle alone (Fig. 5E).

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<th><strong>Table 1.</strong> Tumor features associated with cytoplasmic FGF2 expression assessed by IHC in an invasive breast cancer tissue microarray</th>
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Abbreviations: IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; CK5/6, cytokeratin 5/6; CK14, cytokeratin 14, any basal-marker (positive for any of EGFR, CK5/6 or CK14).

<sup>a</sup>Mann–Whitney U Test or<sup>b</sup>c<sup>2</sup> test.

NOTE: Statistical analysis was with Fisher’s exact test, unless indicated. Tumor subtype as defined by Nielsen and colleagues (ref. 28; HER2–HER2 amplified, Luminal–HER2 negative/ER positive, core basal-like–TN expressing CK5/6 or EGFR, TN nonbasal–TN with no expression of CK5/6 and EGFR).

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**FGFR inhibitors decrease growth of CAL51 xenografts in vivo**

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Discussion

TN breast cancers have a poor prognosis and the factors that drive the proliferation of these breast cancers have been largely unclear. Here, we provide evidence that FGFR signaling may promote the growth of a proportion of TN breast cancers. Multiple TN cell lines show sensitivity to FGFR inhibitor PD173074, with evidence that MAPK pathway signaling in particular is under control of FGFR signaling. We further showed that TN breast cancer cell line CAL51 xenografts are sensitive to PD173074 in vivo.

The underlying mechanism of sensitivity to PD173074 seems to be heterogeneous in TN breast cancer cell lines, although autocrine FGF2 ligand expression seems to be the predominant mechanism (Supplementary Table S4). Cytoplasmic FGF2 expression is found specifically in core basal-like TN cancers, and not in TN cancers that are not basal-like (Fig. 4C), and we provide data that suggests FGF2 acts in an autocrine fashion as both siRNA targeting FGF2 and an FGF2-neutralizing antibody reduce the growth of basal-like cell lines. These data suggest that it is specifically basal-like cancers, as opposed to TN per se, whose growth is promoted by autocrine FGF2 signaling, further adding to the evidence that TN cancers are a heterogenous group of cancers.

In cancer cell lines, FGF2 expression was found at highest levels in cancer cell lines of basal B cell line subtype (Fig. 3A). Recent data has suggested that the basal B cell line subtype shows high similarity with...
the claudin-low breast tumor subtype (36). Basal B breast cancer cell lines and claudin-low tumors are characterized by high levels of mesenchymal markers, low or no expression of E-cadherin and claudins at the mRNA level, and "stem cell-like" features, in addition to expression of basal markers and the TN phenotype (25, 36). It is now generally held that claudin-low tumors are best seen as one end of the spectrum of basal-like cancers, rather than a distinct entity, as these features are also present, but to a lesser extent, in molecular subtype basal-like tumors. The nontumorigenic breast epithelial cell lines MCF10A and MCF12A expressed FGF2 (Fig. 3B), which also express a basal B transcriptional program (25), and also myoepithelial cells from normal breast ducts express FGF2 by IHC (Supplementary Fig. S4). This suggests that FGF2 expression may be a manifestation of the basal/myoepithelial phenotype. With FGF2 expression observed at the highest level in cell lines with a high expression of "stem cell-like" features, and the evidence that in vitro FGF2 supports the growth of embryonic stem cells (37), this raises the possibility that FGF2 autocrine signaling could be a lineage-specific trophic factor associated with a stem/propagating cell type.

Our data on FGF2 expression does initially seem to contrast previous observations measuring FGF2 by ELISA in tumor lysates, in which FGF2 expression in whole tumor lysates was associated with ER-positive breast cancer and low histologic grade (38). Potentially, FGF2 in whole tumor lysates represents increased expression of FGF2 by tumor associated fibroblasts and increased release of FGF2 via extracellular matrix proteolysis, as opposed to tumor-specific cytoplasmic FGF2 expression which we have shown to be specific to core basal-like breast cancer. It is also likely that high stromal element expression complicates the assessment of FGF2 mRNA by gene expression arrays. Although normal breast duct myoepithelial cells expressed both cytoplasmic and nuclear FGF2, the majority of basal-like cancers expressed only cytoplasmic FGF2. FGF2 protein is expressed as multiple different isoforms reflecting translation from differing noncanonical, translation start sites (39), with the lower molecular weight isoform being secreted through a nongolgi/endoplasmic reticulum–mediated process (40). Potentially, this observation suggests that expression of the nuclear FGF2 isoforms may be suppressed during basal-like tumor development.

It will be interesting in future research to identify the FGF receptor that mediates the FGF2 autocrine signaling. We show that FGFR1 likely mediates the autocrine signaling in the CAL120 cell line (Fig. 5), although CAL120 is the only basal-like cell line that harbors high level FGFR1 amplification and the FGF2–FGFR1 autocrine loop could be specific to this cell line. We note that FGFR2 mRNA expression is higher in TN and basal-like breast cancers, compared with other breast cancers, in external datasets (Supplementary Fig. S7). Recently, it has also been suggested that an FGF9–FGFR3 paracrine loop may promote breast cancer stem cell expansion, suggesting a potential role for FGFR3 (41).

What might be the significance of our data for the treatment of TN and basal-like breast cancer? Alternative tyrosine kinase receptors are expressed as part of the basal-like phenotype. EGFR is expressed as part of the basal-like phenotype, and clinical trials have examined the efficacy of the EGFR targeting antibody cetuximab with evidence of modest levels of activity (20). In one phase II randomized trial, the response rates increased from 10.3% to 20% with the addition of cetuximab to cisplatin (20). Overexpression of the HGF receptor c-MET drives the development of mammary cancer in mouse models, including cancers of a basal-like phenotype, and c-MET is expressed in basal-like breast cancer (42). It will therefore be interesting to establish the relationship between FGF2 autocrine signaling and these other pathways. For example, does FGF signaling and EGFR/c-MET occur mutually exclusively, or is there cooperation between signaling pathways? In endothelial cells, there is evidence of cross-talk between FGF and VEGFR signaling (43), as well as between FGFR4 and HER2 in the FGFR4 mutant and HER2-amplified MDA-MB-453 breast cancer cell line (44). This raises the possibility that FGF signaling could mediate resistance to drugs targeting alternative receptor tyrosine kinases, and it will be interesting to examine combination approaches in vitro.

Here, we show that PD173074, as a potent FGF inhibitor, has activity in vitro on basal-like breast cancer cell lines that express autocrine FGF2, as well as single agent activity against CAL51 basal-like cell line xenografts, suggesting that FGF signaling may present a therapeutic target in basal-like breast cancer. A number of potent inhibitors of the FGF receptors are in early phase clinical trials (33) and the data presented here support the investigation of these agents in TN and basal-like breast cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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FGFR Dependency of Triple-Negative Breast Cancer

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