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The FGF/FGFR axis as a therapeutic target in breast cancer

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Abstract

Fibroblast growth factor receptor (FGFR) signaling is a vital component of both embryonic and postnatal mammary gland development, which has prompted researchers to investigate both its relevance to breast cancer and its potential as a therapeutic target. Deregulated FGFR signaling during breast cancer occurs through various mechanisms, including amplification of the receptor genes, aberrant ligand expression, receptor mutations and translocations. Recent experimental outcomes involving both animal models and human breast cancer cell lines have led to the initiation of multiple early clinical trials investigating the safety and efficacy of small molecule FGFR inhibitors. In this article we review both the most recent discoveries and the need for further investigation of the mechanisms through which FGF/FGFR signaling has emerged as an oncogenic driver.

Keywords

Breast cancer; fibroblast growth factor; fibroblast growth factor receptor; mammary gland; receptor tyrosine kinase; targeted therapies

Overview of FGF/FGFR in breast cancer

The FGF/FGFR axis

The fibroblast growth factor (FGF) family is comprised of 22 structurally similar ligands that mediate their effects through activation of four membrane-bound receptor tyrosine kinases [1]. Each FGF receptor (FGFR) contains three extracellular immunoglobulin (Ig)-

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like domains, an acidic box, a transmembrane domain, and a split intracellular tyrosine kinase domain [Figure 1A]. Additionally, heparin or heparin sulfate proteoglycans are required to stabilize FGF to FGFR binding, and each receptor has an extracellular heparin-binding site [2,3]. Complexity of the FGF family arises not only because each ligand can bind multiple receptors but also because FGFR1-3 undergo alternative splicing. This alternative splicing usually occurs from the differential usage of two exons both coding for the C-terminal region of the third Ig-like domain resulting in either the IIIb or IIIc isoforms [Figure 1B]. These splice variants are expressed in different tissues and have different ligand binding specificities. In general, the IIIb isoforms are expressed in epithelial cells and the IIIc isoforms are expressed in mesenchymal cells [4]. Ligand binding to an FGFR monomer induces dimerization and subsequent transphosphorylation of tyrosine residues within the kinase domains [1]. Adaptor molecules, such as FGFR substrate 2 (FRS2) or phospholipase C γ (PLC γ), bound to the activated receptor dimer can then be phosphorylated and activated, subsequently transmitting the FGFR activation signal through a variety of downstream molecules, including phosphoinositide-3 kinase (PI3K), extracellular signal-regulated kinase 1/2 (ERK1/2), various signal transducer and activator of transcription (STAT) proteins, and protein kinase C (PKC) [Figure 2]. This signaling cascade stimulates cellular processes such as proliferation, survival, migration, and angiogenesis. The FGF family has been widely studied and reviewed in normal and disease processes [1,4-7]. FGF signaling has been implicated in a variety of skin diseases as well as congenital skeletal disorders, such as Pfeiffer syndrome, in which a missense mutation in the third Ig-like domain of FGFR2 leads to autocrine activation of the receptor [8,9]. Of particular interest, FGF signaling has also been shown to be important in a number of malignancies, including prostate, endometrial, and breast cancer [6].

FGF/FGFR in normal mammary gland development

In order to understand how aberrant FGFR activation might contribute to breast cancer, it is first important to consider the functions of FGFR in normal mammary gland development. FGF signaling has been linked to many developmental processes, including formation of limb buds, stimulation of angiogenesis, and induction of branching morphogenesis in organs such as the kidneys, lungs, prostate, and mammary glands [10-15]. During embryonic mammary gland development, FGF10 and its receptor FGFR2-IIIb are essential for proper mammary placode formation as shown by analysis of mice lacking these genes [16,17].

More recent studies have focused on the contributions of FGFR to postnatal mammary gland development [18,19]. FGFR2 expression is required within the mammary epithelium during pubertal ductal morphogenesis, as Cre-mediated deletion of *Fgfr2-IIIb* within the epithelium results in reduced ductal morphogenesis and a lack of terminal end buds [18]. Interestingly, while FGFR2 is expressed in the epithelial cells, its ligand (FGF10) is highly expressed in the mammary fat pad, suggesting that FGFR2 activation is regulated via a paracrine mechanism. Furthermore, recent studies in which *Fgfr1* and *Fgfr2* were deleted in the epithelium led to inhibition of ductal outgrowth and a decrease in the repopulating cell population, suggesting a role for FGFR signaling in maintenance of the stem cell population in the mammary gland [19]. Together, these studies demonstrate the importance of the FGF/FGFR signaling axis during both embryonic and mammary gland development. The specific

mechanisms of FGFR function in the mammary epithelium, including promotion of proliferation and stem cell function, suggest potential mechanisms through which aberrant FGFR signaling might contribute to tumor formation and progression.

Alterations in the FGF/FGFR axis in breast cancer

Amplification of FGFR genes, including *FGFR1* and *FGFR2*, was initially documented in human breast cancer samples in the early 1990s [20]. A surge of studies within the last 5-10 years has both confirmed these initial observations and expanded significantly upon the mechanisms through which the FGF/FGFR axis contributes to breast cancer [21,22]. In addition to gene amplification, increased protein expression of both ligands and receptors, single nucleotide polymorphisms (SNPs) and mutations in FGFRs have been identified in human breast cancer cell lines and patient samples, suggesting that there are multiple mechanisms through which aberrant FGFR activation might occur.

Amplification of a region at chromosome 8p12, which contains the *FGFR1* gene, has been identified in approximately 10% of human breast cancers, with a range from 8.7% to 22.8% depending on the study, and is associated with reduced metastasis free survival [23-25]. Furthermore, analysis of defined regions of the 8p12 amplicon demonstrated that a 1 Mb region within this amplicon that contains the *FGFR1* gene is significantly associated with poor outcome [26]. Recent studies have demonstrated that amplification of *FGFR1* correlates with increased expression of protein [27]. Studies of breast cancer cell lines harboring amplification of *FGFR1* have shown that blocking FGFR activity leads to decreased growth and survival, demonstrating that *FGFR1*-amplified cells become dependent upon aberrant FGFR1 activity [27]. Among the potential mechanisms through which amplified *FGFR1* might lead to increased pathway activation are increased sensitivity of the amplified receptor to ligand or through abnormally high expression levels leading to ligand-independent activation. *FGFR2* amplification has been identified in 5-10% of human breast cancers [20], as well as in the SUM52-PE breast cancer cell line [28,29]. Subsequent work has demonstrated that *FGFR2* is a transforming oncogene in mammary epithelial cells, which is capable of conferring an invasive phenotype to the cells [30].

Amplification of FGF ligands, including *FGF3*, *FGF4* and *FGF19*, has also been observed in human breast cancer samples [31]. These ligands are located on chromosome 11q13, which is amplified in 15% of human breast cancers [32]. In addition, *FGF10* is located on chromosome 5p12, which is associated with 5-10% of breast cancers [33]. To date, it is unclear whether these ligands themselves have the capacity to drive tumor formation or if additional oncogenic changes are required. However, all of these ligands activate FGFR1 and/or FGFR2, which have both been strongly implicated in breast cancer. Interestingly, FGF3 and FGF4 bind to different isoforms (IIIb and IIIc isoforms, respectively) [33], suggesting that they may have different cell-type specific effects on the tumor cells and the cells residing in the microenvironment. In breast cancers, the 11q13 amplicon, on which *FGF3*, *FGF4* and *FGF19* are found, is frequently co-amplified with 8p12, where the *FGFR1* gene resides [24]. This co-amplification suggests the existence of a potential loop in which increased expression of both ligand and receptor could contribute to these tumors, although this possibility remains to be investigated. Finally, there is a well-established

cancer driver gene at the 11q13 amplicon, *CCND1* (cyclin D1) [32]. Whether these FGFs may be cooperating with cyclin D1 to further drive breast cancer remains to be determined.

Another mechanism by which the FGF/FGFR axis may contribute to cancer is through increased protein expression of FGF ligands. For example, in human breast cancer tissues, immunohistochemical analysis demonstrated increased expression of FGF2 in 62% of basal-like breast cancers [34]. Furthermore, triple negative breast cancer cell lines secrete FGF2 *in vitro*, suggesting that the cellular source of the increased ligand is likely the tumor cell itself [34]. In addition, FGF8 has been found to be increased in human breast cancers samples [35] and has also been found to be produced by breast cancer cells in culture [36]. Although the specific mechanisms leading to increased expression of these ligands in triple negative breast cancer cells remain unknown, recent studies demonstrated that treatment of the estrogen receptor positive (ER⁺) breast cancer cell line MCF-7 cells with estrogen leads to increased production of numerous FGF ligands, including FGF2, FGF4, FGF6, FGF7 and FGF9, providing a novel mechanistic link between aberrant FGFR activation and ER⁺ breast cancers [37]. Further studies are clearly required to understand both the mechanisms leading to increased FGF ligand expression and the specific contributions of the different FGF ligands to breast tumor formation and progression.

Interestingly, large-scale genome wide association studies have identified SNPs specifically in intron 2 of the *FGFR2* gene, which have been linked to increased breast cancer susceptibility [38,39]. Further studies have demonstrated that SNPs in *FGFR2* correlate with increased FGFR2 expression in breast tumors from patients homozygous for the risk allele [40]. While it remains unclear specifically how these SNPs contribute to breast cancer susceptibility, recent studies have explored potential mechanisms. For example, it was shown that SNPs can affect the binding affinities of specific transcription factors that regulate transcription of *FGFR2* [40]. Results from a separate study demonstrated that two of the SNPs, rs2981582 and rs2981578, correspond with increased FGFR2 expression and activation of downstream signaling pathways in stromal fibroblasts, suggesting an alternative potential mechanism through which *FGFR2* SNPs may contribute to increased breast cancer risk [41]. These observations raise the interesting possibility that activation of FGFRs in non-tumor cells may contribute to breast cancer as well.

Another potential mechanism of aberrant FGFR signaling is the presence of activating somatic mutations in FGFRs. Mutations in FGFR1 leading to constitutive tyrosine kinase activity are found in Type 1 Pfeiffer syndrome and other bone disorders. These mutations can occur in either the ligand binding domain or the tyrosine kinase domain. Somatic mutations have also been found in some lung cancers [42,43] and *FGFR1* translocations have been identified in 8p11 myeloproliferative syndrome [44]. Furthermore, recent studies identified *FGFR* translocations in a variety of solid tumors, including lung cancer, bladder cancer, oral cancer, head and neck cancer, thyroid cancer and glioblastoma [45]. These gene fusion products all retained the *FGFR* tyrosine kinase domains, suggesting that the resulting products are active and remain targets of FGFR inhibitors. The authors demonstrated that these translocation events contribute to cancer cell proliferation in an FGFR dependent manner, suggesting that patients with cancers that harbor these translocations may be candidates for FGFR targeted therapies [45]. FGFR mutations have also been identified in

human breast cancers. For example, FGFR2 mutations were identified in a kinome screen of metastatic breast cancer [46]. Furthermore, recent studies have identified somatic mutations in FGFR2 in breast cancer cell lines that confer constitutively activated signaling [47]. Current analysis of the Catalog Of Somatic Mutations In Cancer (COSMIC) database identifies limited numbers of mutations in FGFRs in breast cancer samples. Specifically, mutations are found in FGFR1 in 2 out of 1031 samples (S125L, K566R), in FGFR2 in 1 out of 637 samples (R203C) and in FGFR4 in 1 out of 550 samples (V550E) [Figure 3A]. A SNP has also been identified in the transmembrane domain of FGFR4 (G388R), however the functional consequences of this polymorphism remain to be described. Interestingly, analysis of the COSMIC database also reveals a possible fusion between FGFR1 and ZNF703 in a sequenced breast cancer sample (<http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/>), the latter of which has been suggested to be an oncogene for luminal B breast cancers [48] [Figure 3B]. However, the functional relevance and frequency of this translocation in the breast cancer patient population remains to be determined. Although somatic activating mutations are unlikely to be a common source of aberrant FGFR activity in breast cancers, they may still represent a potential targetable pathway in a small percentage of breast cancer patients and warrant further investigation.

Association of aberrant FGFR activity with breast cancer subtypes

In the clinic, breast cancers are typically characterized by the presence or absence of the hormone receptors, ER and progesterone receptor (PR), and the human epidermal growth factor receptor 2 (HER2). Tumors that are found to be HER2⁺ are treated with therapies that specifically target HER2, such as the monoclonal antibody trastuzumab [49]. Tumors that express ER are treated with the ER-specific antagonists such as tamoxifen [50]. Additionally, aromatase inhibitors, which indirectly inhibit ER function by blocking the production of the ER ligand estrogen, are used to treat patients with ER⁺ and ER⁺/PR⁺ tumors [50,51]. However, the subset of breast cancers that are ER⁻/PR⁻/HER2⁻ (known as triple negative) does not have any currently available and widely used targeted therapies [52]. Instead, women with triple negative-designated tumors have only systemic chemotherapy and surgery as treatment options. However, not all patients respond well to therapies when given based solely on the presence or absence of the hormone receptors and HER2, and the complexity and heterogeneity of breast cancer is not completely described with these markers alone. Since the pioneering study by Perou and colleagues in 2000, much research has been done using high-throughput microarray-based global gene expression profiling and has resulted in the identification of several molecular intrinsic subtypes of breast cancer [53-58]. Currently, there are six distinct breast cancer intrinsic subtypes based on molecular profiling: luminal A, luminal B, normal breast-like, HER2-enriched, basal-like and claudin-low. Each subtype can be distinguished from the others based on gene cluster expression patterns and it has been proposed that each subtype arises from distinct progenitors.

Aberrant activation of the FGF/FGFR axis has been implicated in many of the breast cancer subtypes, including the luminal B, HER2-enriched and basal-like subtypes [27,34,59,60]. Recent studies found that amplification of *FGFR1* is frequently found in ER⁺ luminal B tumors and that tumors overexpressing FGFR1 exhibited increased proliferation and

decreased distant metastasis-free survival [27]. Furthermore, ER⁺ breast cancer cell lines harboring *FGFR1* amplification rely upon active FGFR signaling for anchorage independent growth [27]. Finally, these studies demonstrated that *FGFR1* amplification conferred resistance to endocrine-based therapies [27]. In another study, FGFR3 expression was found to be upregulated in tamoxifen-resistant breast cancers [61]. Further studies of breast cancer cells *in vitro* demonstrated that activated FGFR3 could promote resistance to tamoxifen through downstream activation of PLC γ 1 [61]. Together, these studies demonstrate a potential link between FGFR overexpression and hormone-responsive breast cancers and suggest that targeting FGFR activity may be a rational therapeutic approach for breast cancers that are resistant to endocrine-based therapies.

Comprehensive profiling of the different subtypes of breast cancers has demonstrated that HER2⁺ tumors have higher levels of expression of various receptor tyrosine kinases, including FGFR4 [54]. Furthermore, experimental studies using a lapatinib-resistant HER2⁺ breast cancer cell line demonstrated that amplification and overexpression of FGFR2 and targeting FGFR signaling using a receptor tyrosine kinase inhibitor PD173074 led to increased apoptosis of the cells [59]. Studies using mouse models of mammary tumors have demonstrated that combination therapies targeting both FGFR activation and HER2 activity are more effective than either therapy alone [60]. Together, these studies suggest that activated FGFR signaling may contribute to HER2-driven tumor formation and resistance to therapy, and that combinatorial targeting of both pathways may have clinical relevance.

Breast cancer subtype profiling has also demonstrated that *FGFR1* and *FGFR2* are amplified in basal-like cancers [54]. Several basal-like breast cancer cell lines were found to be sensitive to inhibition of FGFR signaling using PD173074 [34]. Specifically, treatment of these cell lines led to decreased activation of ERK and Akt signaling and increased cell cycle arrest and apoptosis, demonstrating the dependence of these cells on FGFR activity [34]. One of the mechanisms of FGFR activation in these cells appears to be increased production of FGF2 ligand by the cancer cells, and further studies revealed that up to 62% of human basal-like breast cancers expressed FGF2 by immunohistochemistry [34]. Thus, FGFR activation may be involved in a subset of basal-like breast cancers as well.

While these recent studies have suggested that aberrant activation of the FGF/FGFR axis contributes to various subtypes of human breast cancers, further studies will be required to fully understand the actual percentage of FGFR-responsive and dependent breast cancers. As described in more detail below, the development of reagents that can effectively detect activated FGFRs and FGF ligand expression in human breast cancer tissues will lead to a more thorough understanding of the numbers of breast cancer patients with FGFR-driven tumors.

Experimental studies shed light on FGFR function in tumor formation and progression

Numerous studies using both *in vitro* and *in vivo* models have demonstrated a wide range of functions for FGFR signaling in breast tumor formation and progression. It has been well-established that FGFR stimulation in breast cancer cells results in proliferation and survival via activation of specific signaling pathways such as ERK and Akt [1]. More recent studies have focused on identifying novel mechanisms through which FGF/FGFR activation

regulates breast cancer cells. For example, recent studies of ER⁺ breast cancer cells demonstrated that FGF9 can cooperate with estrogen to induce expression of the transcription factor TBX3, leading to expansion of the cancer stem cell population [37]. In other studies, microarray analysis was performed on ER⁻ breast cancer cells treated with FGF8b to identify novel genes involved in mediating FGF-driven tumorigenesis [62]. Results from these studies demonstrated that FGF8b regulates the expression levels of a number of genes involved in proliferation and survival, including *BTG2*, *CCND1*, *CCNB1* (cyclin B), *PLK1*, survivin and aurora kinase A [62]. Using gene profiling approaches, it has also been shown that FGFR1 activation leads to increased expression of epidermal growth factor (EGF) family members, which then act on the tumor cells via ErbB family member activation to promote proliferation and migration, demonstrating a functional link between FGFR and ErbB activity in tumor cells [63,64]. Together, these studies demonstrate that FGFR activation leads to regulation of numerous different signaling pathways and transcriptional target genes. Identification of novel pathways that mediate FGFR-induced effects on breast cancer cells will ultimately lead to more rational design of therapeutic strategies targeting downstream FGFR activities.

While aberrant activation of the different receptors, including FGFR1, FGFR2 and FGFR3, has been implicated in breast cancers as described above, the abilities of these receptors to function through either redundant or different mechanisms to promote breast cancer are not well understood. Comparison of inducible versions of FGFR1 and FGFR2 demonstrated differences in activation of downstream signaling pathways and receptor regulation [65]. Specifically, activation of inducible FGFR1 led to stronger and more stable activation of ERK than FGFR2. Furthermore, activation of inducible FGFR2 led to rapid receptor downregulation in a Cbl-dependent manner, which was not observed following inducible FGFR1 activation [65]. Recent studies focusing on identifying the different effects of FGFR1, 2 and 3 knock-down on mammary tumor growth were performed in which each receptor was knocked down in breast cancer cells using short hairpin RNA (shRNA) strategies [66]. Decreased expression of FGFR1 led to smaller tumors with less vasculature, demonstrating a critical role for FGFR1 in promoting these tumors [66]. Interestingly, decreased FGFR2 expression led to an increase in tumor size and vasculature compared with the control along with a significant increase in expression of FGFR1. These results suggest that decreased FGFR2 expression leads to a compensatory increase in expression of FGFR1 and subsequent tumor formation [66]. Further studies of the differences between these receptors in promoting breast cancer are clearly required to determine the most effective methods for targeting specific FGFRs while avoiding compensation by the other receptors.

In addition to canonical activation of the transmembrane FGFR and subsequent receptor activation, recent studies have also demonstrated that FGFR1 can be cleaved by granzyme B [67]. Once cleaved, the intracellular portion of FGFR1 translocates to the nucleus where it contributes to the transcriptional regulation of genes that promote migration and invasion [67]. Interestingly, nuclear FGFR1 expression was also identified in human breast cancer samples, suggesting a novel mechanism for FGFR1-induced breast cancer [67]. Other recent studies of nuclear FGFRs have revealed the presence of FGFR2 in the nucleus of steroid-hormone responsive cells, where it interacts with other transcription factors, such as PR and

STAT5, at progesterone response elements leading to regulation of expression of important oncogenes such as *MYC* [68]. Together, these studies demonstrate that cellular localization of FGFRs is an important consideration of their functions and further analysis of non-membrane bound forms of FGFRs may reveal novel mechanisms of action in promoting breast cancer.

Analysis of mouse models has also demonstrated important roles for the FGF/FGFR axis in mammary tumor formation and progression. Expression of an inducible FGFR1 in mammary epithelial cells of transgenic mice using the mouse mammary tumor virus (MMTV) promoter was shown to lead to the formation of early stage epithelial lesions, demonstrating a driving role for FGFR1 activity in tumorigenesis [69]. Crossing these mice with the MMTV-Wnt1 mice led to a dramatic reduction in tumor latency, demonstrating the ability of FGFR1 to interact with other oncogenes to promote tumor formation [70]. In a separate study, *Fgf2* knock-out mice were crossed the MMTV-PyMT transgenic mice, which form rapid and aggressive tumors [71]. Interestingly, loss of *Fgf2* led to increased tumor latency and decreased tumor size, demonstrating that FGFR activation acts to promote tumor formation in this model. Finally, orthotopic transplant models using the well-studied 4T1 cells have demonstrated that growth and metastasis of 4T1 tumors can be inhibited using inhibitors of FGFR activity [72].

While the discussion thus far has centered on autocrine activation of FGFR signaling either by increased expression of FGFRs or increased tumor cell production of FGF ligands, it is important to consider that stromal-derived FGFs may be capable of inducing FGFR activation in breast cancer cells. FGFs can be produced by a variety of cell types located in the stromal environment, including fibroblasts, endothelial cells and immune cells [73], raising the possibility that these cell types may contribute to aberrant FGFR activity in breast cancer cells in a paracrine manner. For example, recent studies demonstrated that FGF2 is highly expressed by carcinoma-associated fibroblasts in the C4-hormone independent mammary tumor transplant model [74]. Furthermore, inhibition of FGFR in this model using PD173074 decreased tumor growth, demonstrating the contribution of stromal-derived FGFs to tumor formation [74]. These studies highlight the potentially complex nature of the mechanisms involved in driving FGF-dependent tumor formation and progression that are only beginning to be uncovered.

In addition to the autocrine effects of FGFR activation on epithelial and tumor cell functions, FGFR activation in mammary epithelial cells and tumor cells can also contribute to profound changes within the stroma. Activation of an inducible FGFR1 in mammary epithelial cells in transgenic mice led to a rapid induction of angiogenesis in the mammary gland [69]. Furthermore, analysis of the same mouse model demonstrated that FGFR1 activation also induced a rapid inflammatory response characterized by increased levels of proinflammatory cytokines and macrophage recruitment [64]. Mechanistic studies found that activation of FGFR signaling in mammary epithelial cells led to the induction of soluble factors such as cytokines, chemokines and growth factors that can affect the surrounding stromal environment [64,75,76]. In another study, expression of a dominant negative FGFR2 construct in mouse mammary carcinoma cells resulted in decreased tumor growth and metastasis of 66c14 cells [77]. Further analysis of these tumors revealed that this decrease

was accompanied by a decrease in lymphangiogenesis through suppression of vascular endothelial growth factor C (VEGF-C) production [77]. Together, these studies suggest that effects of FGFR activation in tumor cells on the surrounding microenvironment may also be an important component of FGF-driven breast cancers.

Therapeutic targeting of FGF/FGFR activity

The recent development of agents that target FGFR activity signifies a growing interest in targeting this pathway in the clinical setting. While pharmacological inhibition of FGFR activity has been used extensively in pre-clinical studies, new classes of receptor tyrosine kinase inhibitors have been developed that either selectively target FGFR or target FGFR in addition to other receptor tyrosine kinases [5]. As discussed below, a number of these agents are currently being examined in clinical trials for breast and other cancers [Table 1]. While this discussion will focus primarily on approaches to specifically target breast cancers, it is important to note that there are a number of other FGFR-targeted approaches being examined at both the pre-clinical and clinical stages in a variety of cancers [5].

Of the selective FGFR tyrosine kinase inhibitors, NVP-BGJ398 and AZD4547 are currently being tested in clinical trials. NVP-BGJ398 was developed as an orally bioavailable selective pan-FGFR inhibitor with potent anti-FGFR activity in the nanomolar range [78]. Further studies using this small molecule inhibitor demonstrated that genetic alterations in FGFR could be used as biomarkers to predict sensitivity to NVP-BGJ398 [79]. Specifically in breast cancer, *FGFR1* amplification in cell lines correlated with increased responsiveness to NVP-BGJ398 [79], suggesting that patients with breast cancers that harbor *FGFR1* amplification may benefit from this therapeutic agent. A phase I dose escalation trial is currently recruiting patients to determine the maximum tolerated dose and safety profile of NVP-BGJ398 in patients with advanced solid tumors with amplification of *FGFR1* and *FGFR2* or mutation of *FGFR3* (NCT01004224).

AZD4547 was also recently developed as a selective FGFR tyrosine kinase inhibitor [80]. AZD4547 exhibits efficacy against FGFR1, FGFR2 and FGFR3, with weaker activity against FGFR4 and 120-fold increased sensitivity over VEGF receptor (VEGFR) [80]. Pre-clinical studies demonstrated effective FGFR inhibition in a panel of cancer cell lines, including the FGFR2-expressing breast cancer cell line SUM52-PE [80]. Several trials are currently recruiting patients to examine safety and efficacy of AZD4547, three of which specifically include breast cancer patients. NCT01202591 is a Phase IIa clinical trial in which patients with ER⁺ breast cancer with either *FGFR1* polysomy or gene amplification that have progressed following endocrine-based therapy are being recruited to test the safety and efficacy of AZD4547 with fulvestrant compared with fulvestrant alone. NCT01791985 is a Phase IIa study in which ER⁺ patients whose tumors have progressed despite previous treatment with anastrozole or letrozole are being recruited. Once dosage of AZD4547 is established, patients will receive either exemestane alone or AZD4547 with letrozole or anastrozole. Finally, a third trial is recruiting patients with breast, gastric, esophageal and squamous cell lung carcinomas with amplified *FGFR1* or *FGFR2* and progression following chemotherapy. Safety and tolerability will be monitored and response to AZD4547 will be assessed by analyzing ERK phosphorylation and tumor size (NCT01795768). While clearly

still in early phases of testing, important information regarding selective targetability of FGFR activity in breast cancer patients will likely be obtained from these trials.

There are a number of tyrosine kinase inhibitors that bind and inhibit multiple kinases in addition to FGFR and are thus termed non-selective tyrosine kinase inhibitors. An example of a non-selective tyrosine kinase inhibitor being tested in clinical trials is TKI258 (dovitinib). TKI258 inhibits a broad panel of receptors including VEGFR, FGFR and platelet-derived growth factor receptor (PDGFR) and has demonstrated good efficacy in inhibiting tumor growth in pre-clinical models of mammary tumor formation [60,72]. TKI258 is currently being evaluated in numerous clinical trials for various cancers. Specifically related to breast cancer, a completed study evaluated the safety and efficacy of TKI258 in HER2⁺ metastatic breast cancer (NCT00958971). In addition, a more recent Phase II study is currently recruiting patients to test TKI258 in HER2⁺ inflammatory breast cancers (NCT01262027).

A number of other non-selective receptor tyrosine kinase inhibitors that show efficacy against FGFRs have been recently developed and examined in pre-clinical studies. For example, recent studies demonstrated that a novel inhibitor, E-3810, inhibits VEGFR1, VEGFR2, VEGFR3, FGFR1 and colony stimulating factor 1 receptor (CSF1R) with inhibition of FGFR2 at higher concentrations [81]. Studies using an MDA-MB-231 xenograft model demonstrated that treatment with E-3810 alone led to tumor stabilization [81]. Furthermore, combination treatment with paclitaxel led to tumor regression and was well-tolerated [81]. In another recent study, AP24534 (ponatinib), which was initially identified as a potent inhibitor of BCR-ABL, was also found to inhibit growth of breast cancer cell lines *in vitro* [82]. Furthermore, AP24534 inhibited phosphorylation of FGFR in breast cancer cell lines that harbor amplifications of *FGFR1* and *FGFR2*, suggesting specific inhibition of FGFR activation [82]. Although further studies are required to determine the utility of these types of inhibitors in FGFR-driven patient tumors, the results from these experimental studies suggest that these types of non-selective approaches certainly warrant further investigation.

Although many of the efforts to target FGFR activation have focused on developing inhibitors of FGFR kinase activity, several other approaches of inhibiting the FGF/FGFR axis have also been investigated. The use of antibody-based therapy in breast cancer has been successful for targeting other receptors, such as HER2 [49]. Therefore, the development of antibodies that bind and inhibit specific FGFR isoforms seems to be a rational approach to targeting the FGFR pathway. Recent studies describe the development of an antibody, GP369, which specifically recognizes the FGFR2-IIIb isoform [83]. The GP369 antibody was effective in blocking proliferation of *FGFR2*-amplified cell lines, including the SUM52-PE breast cancer cells *in vitro* [83]. Furthermore, GP369 induced tumor stasis of MFM-223 breast cancer xenografts *in vivo* [83]. These studies provide strong rationale for further studies aimed at targeting specific FGFR isoforms. In an earlier study, however, a single chain antibody to the FGFR1-IIIc isoform was found to be anorexigenic when administered to mice [84]. Whether this approach might be more effective using a different isoform that is not as widely expressed as FGFR1-IIIc remains to be determined.

Another potential approach for targeting the FGF/FGFR axis is the development of strategies that specifically target the ligands. In a recent study, long pentraxin-3 (PTX3), a soluble pattern recognition receptor, was shown to be capable of binding and inhibiting specific FGF ligands, including FGF2 and FGF8b [85-87], both of which have been implicated in breast cancer as described above. Expression of PTX3 in hormonally-responsive mouse mammary tumor cells led to decreased proliferation *in vitro* as well as decreased angiogenesis and tumor growth *in vivo* [86]. Recently, a ligand trap (FP-1039) consisting of the ligand-binding domain of FGFR1 fused to an Ig-F_c domain has shown antitumor activity in pre-clinical models [88]. This effect was most evident against tumors with an upregulation of FGF/FGFR signaling axis. A Phase I clinical trial (NCT00687505) was recently completed in patients with advanced solid tumors and while not specific to breast cancer, these results suggest that targeting FGF ligands represents a feasible therapeutic approach.

As discussed above, experimental studies have suggested that inhibiting FGFR activity may enhance tumor responses to other established drugs, such as endocrine-based therapies and ErbB-targeted therapies [27,60]. Furthermore, FGFR activation also promotes resistance to chemotherapeutic-based treatments (unpublished observations). Therefore, it is possible that combination therapies using FGFR inhibitors along with standard treatments may lead to better responses in patients with high levels of FGFR activity. In addition, as further studies continue to reveal the mechanisms that drive breast cancer, more potential opportunities for developing effective combination therapies involving FGFR inhibition can be considered. For example, due to the high prevalence of mutations in *PIK3CA* in breast cancer [54], combining FGFR inhibitors with inhibitors of the PI3K/Akt pathway seems to be a logical combination. In fact, combined inhibition of FGFR2 and mammalian target of rapamycin (mTOR) was found to be an effective therapy in endometrial models [89]. Further studies are required to determine whether this combination might also be effective for breast cancer, although the recent interest in using mTOR inhibitors in breast cancer makes this an attractive possibility [90]. In addition, recent studies of renal cancer have suggested that FGF can regulate endothelial cell proliferation and tubule formation even in the presence of the VEGFR inhibitor sunitinib, and that blocking FGF2 can enhance the anti-VEGFR effects [91]. While these studies have yet to be performed in the context of breast cancer, these studies demonstrate the feasibility of targeting FGFR in combination with other targeted therapies and warrant further investigation into the complex interactions through which FGFR mediates in pro-tumorigenic effects in breast cancer.

Expert commentary

Over the past several years, there has been a dramatic increase in the interest of targeting FGFR activity in a number of cancers, including breast, lung and gastrointestinal cancers [5]. Numerous studies in cell culture and animal models have suggested that aberrant FGFR signaling can be a driving factor during tumor formation and pre-clinical studies have demonstrated the feasibility of targeting FGFR activation as a novel anti-tumor strategy. Finally, recent clinical studies have begun to explore the feasibility of targeting FGFR in patients, bringing to fruition many years of basic and pre-clinical research studying aberrant

FGFR activation in breast cancer. However, there are still several questions that remain to be addressed regarding the effective targeting of FGFR signaling in breast cancer.

First and foremost, it will be critical to identify the specific patient population that will receive FGFR-targeted therapies. An initial challenge will be to develop methods for identifying patients that will respond to FGFR-driven therapies. Currently, amplification of receptors can be identified using fluorescence in situ hybridization. Sequencing approaches can be used to identify receptor mutations and translocations, although as discussed previously these are not likely to be a driving source of altered FGFR signaling in the majority of breast cancers. However, it is possible that other patients have activated FGFR signaling in their breast cancers due to alterations that are not associated with amplification, mutation or translocation. One possibility would be an increase in gene or protein expression of FGFRs due to altered transcriptional or translational regulation. Interestingly, recent studies have found that microRNAs can directly regulate expression of FGFRs [92] or expression of factors that modulate FGFR activation [93]. Whether these mechanisms of FGFR regulation occurs in the breast cancer setting remain to be investigated. Another possibility for altered receptors activity that has been implicated in prostate cancer is switching of the expression of FGFR isoforms in the tumor cell from the epithelial to mesenchymal receptor isoforms [13]. This switching would allow the cells to respond to a different repertoire of ligands and may affect tumor cell behaviors. Whether this switching occurs in breast cancers in a similar manner to that observed in prostate cancers is not well understood, but it is tempting to speculate that isoform switching could play a central role in epithelial-mesenchymal transition. What hampers many of these studies is the lack of suitable reagents available for studying the relative levels of ligands, receptor isoforms and activated receptors in breast cancer samples. Specifically, the development of antibodies suitable for immunohistochemical detection of FGF ligands and the specific isoforms of the FGFRs, both unmodified and phosphorylated, would aid in identifying specific patient populations with aberrant FGFR activation. Furthermore, the generation of inhibitors that are specific for the different receptor isoforms would provide important information addressing the ability of isoform targeting to effectively inhibit tumor growth with less side effects than general FGFR inhibitors. Overall, the generation of these reagents would greatly enhance our understanding of the precise patient population that might benefit from FGFR targeted therapies.

As the newly developed FGFR-selective targeted therapies are being evaluated for safety and efficacy in current clinical trials, it will be important to continue to understand the mechanisms of FGFR action in promoting breast cancer formation, progression, and response to therapy. For example, understanding the mechanisms that lead to aberrant regulation of the FGF/FGFR axis, such as the specific pathways that promote ligand and receptor overexpression, could lead to identification of novel therapeutic approaches upstream of FGFR activation. Furthermore, obtaining a better understanding of the tumor cell-stromal interactions driven by FGFR activity is necessary for developing combinatorial therapies that target both the tumor cell and its pro-tumorigenic microenvironment. Of interest, conditional activation of FGFR1 in prostate epithelial cells of transgenic mice leads to an induction of angiogenesis that is initially reversible but after a defined period time becomes irreversible [94]. These results suggest that FGFR activation in developing tumors

potentially lead to pro-tumorigenic alterations in the microenvironment that may not be reversible solely by targeting FGFR activity, thus requiring a combination therapy approach. Together, the development of more specific targeted therapies along with a more thorough understanding of the mechanisms of FGFR action in promoting breast cancers will ultimately lead to the design of effective therapeutic strategies that incorporate FGFR inhibition for a defined subset of patients.

Five-year view

This is a pivotal time in the field of FGFR and breast cancer. Basic research and pre-clinical studies have yielded numerous insights into the mechanisms involved in FGFR-driven breast cancer. A number of FGFR-focused clinical trials are in early stages of recruitment and testing and the results will bring critical information regarding the safety and efficacy of selective FGFR inhibitors. The results from these studies will also provide information regarding possible side effects and therapeutic resistance, which will require further refining of therapeutic approaches. Furthermore, the identification of patients that will respond to FGFR-targeted therapies, whether through amplification of receptor and/or ligand, activating receptor mutations or translocations or general overexpression of receptor and/or ligand protein is clearly needed. Continuing to combine bench research with translational and clinical work is crucial for defining the next steps in FGFR targeting. The next 5 years will likely see an increase in the identification of mechanisms through which the FGF/FGFR axis is regulated and through which activation of this axis contributes to breast cancer through complex autocrine and paracrine mechanisms. In addition, it is expected that FGFR selective inhibitors will continue to be developed and tested in both pre-clinical studies and clinical trials. Finally, as clinical studies move towards combinatorial therapeutic approaches, it seems possible that FGFR targeting will be considered in the context of combination therapies, or that the non-selective receptor tyrosine kinases may gain favor due to their inherent combinatorial effects. It is anticipated that effectively targeting this pathway will ultimately lead to improved therapeutic options for breast cancer patients of all subtypes.

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Key issues

- FGFR signaling is crucial to the embryonic and postnatal development of the mammary gland.
- A number of possible mechanisms could contribute to aberrant FGFR signaling including receptor amplification, overexpression of receptor and/or ligand protein, SNPs, mutations and receptor translocations.
- Experimental evidence suggests that FGFR signaling contributes to both tumor initiation and tumor maintenance in mouse model studies.
- Both FGFR-selective and non-selective tyrosine kinase inhibitors are in early clinical testing after demonstrating effects in pre-clinical trials both as stand alone therapy and in combination with other inhibitors and/or chemotherapy.
- Above all, with the advancement of clinical trials utilizing FGFR inhibitors, better reagents for assessing FGFR activation and inhibition are required in order to most effectively target patient populations and continue basic scientific research on the complex roles of the FGF/FGFR axis in breast cancer.

Figure 1. Schematic overview of the protein domain structure of FGFR

A) Protein domain structure of FGFR family members. Receptors contain an extracellular ligand-binding domain, a transmembrane domain (TM), and two intracellular tyrosine kinase domains (TKD). The extracellular portion of the receptor is comprised of three immunoglobulin (Ig)-like domains and an acidic box. B) Schematic depicting alternative splicing of FGFR isoforms. Differential splicing of exon 8 or 9 leads to different Ig III domains (Ig IIIb / Ig IIIc).

Figure 2. FGFR structure and downstream signaling

FGFR signaling occurs as a result of a fibroblast growth factor (FGF) binding to a monomeric FGF receptor (FGFR), leading to receptor dimerization and stabilization of the dimer by HSPGs. Dimerization is followed by receptor transautophosphorylation and activation of its kinase domain. The scaffolding proteins FRS2 and GRB2 are then recruited to the receptor's intracellular domain and act to initiate the downstream signaling cascade leading to the activation of RAS GTPase and PLCγ (among others). Together, these act to initiate ERK and AKT signaling, resulting in the cell's increased survival and proliferation, as well as increased migration and invasion potential. HSPGs, heparin sulfate proteoglycans; FRS2, fibroblast receptor substrate-2; GRB2, growth factor receptor-bound-2; PI3K, phosphoinositide 3-kinase; PIP2, phosphatidylinositol 4,5-bisphosphate; IP3, inositol 1,4,5-trisphosphate; DAG, diacylglycerol; PKC, protein kinase C; PLCγ = phospholipase C-γ; STAT, signal transducer and activator of transcription.

Figure 3. Map of known FGFR mutations linked with breast cancer

A) Mutations in parentheses indicate that the mutation has not been experimentally shown to be an activating mutation. *: FGFR1, **: FGFR2, ^: FGFR4. B) Diagram of FGFR1-ZNF703 fusion protein identified in breast cancer samples in the COSMIC database. Exons 1-13 of FGFR1 are fused with exon 2 of ZNF703 (ZNF703 ex2), resulting in a truncation of the FGFR1 TKD2 (TKD2) and fusion with ZNF703.

Table 1

Current state of FGF/EGFR-targeted therapies in breast cancer

Drug Name	Manufacturer	Target(s)	IC ₅₀ (nM)	Development Stage
NVP-BGJ398	Novartis	FGFR1-3 FGFR4 VEGFR2	9.9 - 13.9 391.5 1019.0	Phase I - NCT01004224 (Recruiting)
AZD4547	AstraZeneca	FGFR1-3 VEGFR2 FGFR4	0.2 - 2.5 24.0 165.0	Phase IIa - NCT01202591 (Recruiting) Phase IIa - NCT01791985 (Recruiting) Phase II - NCT01795768 (Recruiting)
TKI258 (dovitinib)	Novartis	FLT3 c-KIT FGFR1-3 VEGFR1-3 PDGFRa/b	2.0 8.0 - 9.0 10.0 - 13.0 27.0 - 210.0	Phase II - NCT00958971 (Completed) Phase II - NCT01262027 (Recruiting)
E-3810	Ethical Oncology Science	CSF-1R VEGFR1-3 FGFR1-2	5.0 7.0 - 25.0 17.5 - 82.0	Phase I - NCT01283945 (Recruiting)
FP-1039 (FGFR1:Fc)	Five Prime Therapeutics	FGF Ligands	-	Phase I - NCT00687505 (Completed)