Involvement of Fibroblast Growth Factor Receptor 2 Isoform Switching in Mammary Oncogenesis

Jiyoung Y. Cha,1 Que T. Lambert,2 Gary W. Reuther,2 and Channing J. Der1

1Department of Pharmacology, Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina and 2Moffitt Cancer Research Center, Tampa, Florida

Abstract

We identified the IIIb C2 epithelial cell—specific splice variant of fibroblast growth factor receptor 2 (FGFR2 IIIb C2) receptor tyrosine kinase in a screen for activated oncogenes expressed in T-47D human breast carcinoma cells. We found FGFR2 IIIb C2 expression in breast carcinoma cell lines and, additionally, expression of the mesenchymal-specific FGFR2 IIic splice variant in invasive breast carcinomas. FGFR2 IIic expression was associated with loss of epithelial markers and gain of mesenchymal markers. Although FGFR2 IIIb is expressed in epithelial cells, previous studies on FGFR2 IIIb transformation have focused on NIH 3T3 fibroblasts. Therefore, we compared the transforming activities of FGFR2 IIIb C2 in RIE-1 intestinal cells and several mammary epithelial cells. FGFR2 IIIb C2 caused growth transformation of epithelial cells but morphologic transformation of only NIH 3T3 cells. FGFR2 IIIb C2—transformed NIH 3T3, but not RIE-1, cells showed persistent activation of Ras and increased cyclin D1 protein expression. NIH 3T3 but not RIE-1 cells express keratinocyte growth factor, a ligand for FGFR2 IIib C2. Ectopic treatment with keratinocyte growth factor caused FGFR2 IIib C2—dependent morphologic transformation of RIE-1 cells, as well as cyclin D1 up-regulation, indicating that both ligand-independent and stromal cell—derived, ligand-dependent mechanisms contribute to RIE-1 cell transformation. Our results support cell context distinct mechanisms of FGFR2 IIIb C2 transformation. (Mol Cancer Res 2008;6(3):435–45)

Introduction

Fibroblast growth factors (FGF) comprise a large family of structurally related growth factors (22 human members) that mediate a variety of cellular responses that include cell proliferation, differentiation, migration, and angiogenesis (1-3). The activities of FGFs are mediated by their binding to a family of four receptor tyrosine kinases (RTK), designated FGFR1-4. FGFRs are composed of an extracellular domain that consists of two or three immunoglobulin-like domains, a single transmembrane domain, and an intracellular catalytic tyrosine kinase domain and flanking regulatory sequences (Fig. 1A).

In addition to multiple ligands and receptors, the complexity of FGF signaling is further diversified by the fact that the FGFR genes encode multiple structural variants that are generated by alternative gene splicing (1-3). Most importantly, in the case of FGFR1-3, alternative RNA splicing and exon utilization of sequences encoding the carboxyl-terminal half of the third immunoglobulin-like domain III results in the expression of either the IIIb or IIIC isoform of the FGFRs (Fig. 1A). This alternative splicing occurs in a tissue-specific manner and determines the ligand binding specificity of FGFRs. For example, FGFR2 IIIb (also called KGFR and K-sam-II) binds FGF7 (also called keratinocyte growth factor; KGF) and FGF10, but not FGF2, whereas the FGFR2 IIIC (also called Bek/K-sam-I) binds FGF2 and FGF18, but not FGF7 and FGF10. Additionally, ligand and receptor expression can be tissue restricted and expressed in a nonoverlapping tissue distribution. FGFR2 IIIb expression is restricted exclusively to normal epithelial cells, whereas FGFR2 IIIC isoform is generally expressed in normal mesenchymal cells. In contrast, the expression of the ligands for FGFR2 IIIB (KGF/FGF7 and FGF10) is restricted to mesenchymal cells, and ligands for the mesenchymal-restricted receptor are generally expressed in epithelial cells, resulting in the creation of paracrine signaling loops facilitated by epithelial and mesenchymal cell interactions during normal development. However, exon switching and expression of FGFR2 IIIC has been described in epithelial cell tumor progression. A loss of FGFR2 IIIb expression and gain of FGFR2 IIIC expression was observed in rat prostate tumor progression from androgen dependence to androgen independence (4). A similar exon switch has been described for NBT-II rat bladder carcinoma cells, where the onset of FGFR2 IIIC expression was correlated with epithelial-to-mesenchymal transition, a process associated with tumor progression and invasion (5).

Alternative splicing also results in FGFR2 variation in carboxyl-terminal sequences. To date, at least three carboxyl-terminal splice variants of FGFR2 IIIb have been identified, designated C1, C2, and C3 (ref. 6; Fig. 1B). The C2-type carboxyl-terminus is 34-amino-acid shorter than the C1-type carboxyl terminus, whereas the C3-type carboxyl terminus is 53-amino-acid shorter than C1-type carboxyl terminus. These sequence differences result in differential retention of tyrosine residues that may serve as sites of receptor autophosphorylation.
and docking sites for cytoplasmic signaling proteins. Enhanced expression of the C3 isoform was seen in gastric cancer cell lines (6). Tannheimer and colleagues (7) found that normal human mammary epithelial cells express FGFR2 IIIb C1 and C2, but C3 isoform expression is found only in the SUM-52 breast tumor cell line. Previous studies showed that FGFR2 IIIb C1 and IIIb C3 could promote growth transformation of NIH 3T3 mouse fibroblast (6) and H16N2 immortalized human mammary epithelial cells (8), with C3 exhibiting greater transforming activity. However, the transforming activity of FGFR2 IIIb C2 has not been evaluated.

FGFRs are activated by ligand-induced dimerization, causing stimulation of their intrinsic tyrosine kinase activity, tyrosine autophophorylation, and recruitment of signaling proteins to specific phosphorylated tyrosine residues in their cytoplasmic carboxyl termini (2). FGFRs activate the FGFR substrate 2 (FRS2) docking proteins. FGFR phosphorylation of FRS2 creates phosphorylated tyrosine docking sites for the growth factor receptor binding protein 2 (Grb2) adaptor protein. Recruitment of Grb2 in complex with the SOS Ras-specific guanine nucleotide exchange factor causes activation of Ras and the extracellular signal-regulated kinase (ERK) mitogen-activated protein kinase cascade. Ras can also activate the phosphatidylinositol 3-kinase–AKT serine/threonine kinase pathway in some cell types. FRS2-dependent FGFR signaling can also activate the phosphatidylinositol 3-kinase–AKT pathway through Grb2 and the Grb2-associated docking protein Gab1. Because the majority of studies evaluating FGFR signaling are based on transient stimulation with FGFs that can activate multiple FGFRs (e.g., FGF1 binds and stimulates all four FGFR1-4 isoforms), it is not clear whether the identified FGFR signaling activities described above are common for all four FGFRs. Furthermore, the majority of studies have focused on FGFR1 and there has been little effort made in studying the signaling activities that mediate FGFR2 IIIb oncogenesis.

The accumulated evidences indicate that cell type–specific differences exist in cellular transformation. For example, transforming growth factor-β inhibits the growth of epithelial cells, whereas it induces oncogenic transformation of fibroblasts (9-11). Furthermore, we and others have previously found that although activating Ras or Raf transforms fibroblasts, only Ras but not Raf transforms epithelial cells, suggesting that distinct mechanisms exist for Ras transformation in epithelial cells and fibroblasts (12, 13). The majority of studies have evaluated the transforming potential of FGFR2 IIIb in NIH 3T3 mouse fibroblasts (6, 14, 15). NIH 3T3 cells express KGF and a KGF monoclonal antibody blocked FGFR2 IIIb C1 transformation of NIH 3T3 cells. Because fibroblasts and not epithelial cells express FGFR2 IIIb ligands, the biological analyses of FGFR2 IIIb in fibroblasts are complicated by an autocrine growth loop not present in epithelial cells. Because FGFR2 IIIb is an epithelial cell–specific splice variant, an evaluation of FGFR2 IIIb signaling and biological activity in an epithelial cell model system may provide a more physiologically relevant assessment of the role and mechanism of aberrant FGFR2 IIIb function in human epithelial cell–derived carcinomas. Therefore, in the present study, we evaluated the transforming activities and signaling mechanisms of FGFR2 IIIb in epithelial cells. Because we identified the C2 isoform of FGFR2 IIIb in a screen for activated oncogenes, and determined that C2 expression was characteristic of the majority of breast carcinoma cells, we focused our analyses on this isoform. We found that although FGFR2 IIIb C2 caused growth transformation of NIH 3T3 fibroblasts and RIE-1 epithelial cells, FGFR2 IIIb C2 transformation of RIE-1 cells was mediated primarily by a ligand-independent mechanism that did not involve activation of Ras and Ras-mediated
Aberrant Expression of the Mesenchymal FGFR2 IIIc Isoform in Invasive Breast Cancer Cell Lines

The SUM-52 breast cancer cell line expresses nine distinct FGFR2 IIIb isoforms that include the C1, C2, and C3 variants (7). However, aside from this cell line, a systematic analysis of established breast carcinoma cell lines for FGFR2 IIIb isoform expression has not been done. Therefore, we did reverse transcription-PCR analyses to determine transcription of FGFR2 IIIb carboxyl-terminal isoforms in the immortalized, nontransformed MCF-10A human breast epithelial cell line and a panel of breast carcinoma cell lines. We found that MCF-10A cells expressed predominantly C1, and very weakly, the C2 and C3 isoforms (Fig. 2A). In contrast, the majority of tumor cell lines (six of nine) showed significant expression of all three isoforms.

To date, there has been limited analysis of the expression of the mesenchymal-specific FGFR2 IIIc in breast cancer cells. Whereas exon switching and preferential expression of FGFR2 IIIc has been associated with tumor progression in two rat carcinoma models (4, 5), analyses of breast cancer tissue found that exon switching to favor IIIc expression was associated with patients with advanced clinical staging (16). Therefore, we used exon III-specific primers together with restriction enzyme analyses (17) to detect the expression of FGFR2 IIIb and IIIc in breast carcinoma cell lines (Fig. 2B). Because exon IIIb contains a unique AvaI site, whereas exon IIIc contains two HinII sites, digestion with these two enzymes allows detection of these two isoforms. We found that FGFR2 IIIb expression was characteristic of untransformed MCF-10A and six of six noninvasive (BT-474, ZR-75-1, MCF-7, T-47D, MDA-MB-468, and SK-BR-3) breast cell lines, whereas FGFR2 IIIc was detected in all three invasive breast carcinoma cell lines (Hs578T, BT-549, and MDA-MB-231). Previously, we verified that the Hs578T, BT-549, and MDA-MB-231, but not the untransformed MCF-10A or other carcinoma cell lines, showed invasive activity in vitro by using the Matrigel invasion and other assays (18). We also found that expression of FGFR2 IIIc was correlated with an epithelial-to-mesenchymal transition in eight of nine cell lines, as indicated by the loss of epithelial (E-cadherin) and gain of mesenchymal (N-cadherin and vimentin) protein expression (Fig. 2B). Thus, breast cancer progression may involve two distinct alternative splicing events, with expression of FGFR2 IIIb/C3 isoforms associated with the progress from normal breast epithelia to noninvasive breast cancer, and the conversion to the mesenchymal FGFR2 IIIc isoform in invasive breast cancer cells.

Previous studies have determined that FGFR2 IIIb C3 exhibits greater transforming activity than FGFR2 IIIb C1

Results

Identification of the FGFR2 IIIb C2 Splice Variant as a Transforming Protein from T-47D Breast Carcinoma Cells

To identify novel oncopgenes involved in breast epithelial cell growth transformation, we generated a retrovirus-based cDNA expression library from mRNA expressed in T-47D human breast carcinoma cells. The cDNA expression library was introduced into Rat-1 fibroblasts and RIE-1 epithelial cells. One 2.3-kb cDNA sequence was identified independently as a sequence that induced focus formation in both our Rat-1 and RIE-1 screenings. This sequence encodes for FGFR2 IIIb C2, the epithelial cell–specific splice variant of FGFR2 (Fig. 1). Because the isolated sequence encodes for the wild-type protein, overexpression of FGFR2 IIIb C2 alone is sufficient for FGFR2 IIIb C2 to cause transformation.

FGFR2 deregulation in breast cancer progression. Taken together, our results suggest cell context distinct mechanisms by which FGFR2 IIIb causes growth transformation and that isoform switching might be a mechanism for FGFR2 deregulation in breast cancer progression.

To date, there has been limited analysis of the expression of the mesenchymal-specific FGFR2 IIIc in breast cancer cells. Whereas exon switching and preferential expression of FGFR2 IIIc has been associated with tumor progression in two rat carcinoma models (4, 5), analyses of breast cancer tissue found that exon switching to favor IIIc expression was associated with patients with advanced clinical staging (16). Therefore, we used exon III–specific primers together with restriction enzyme analyses (17) to detect the expression of FGFR2 IIIb and IIIc in breast carcinoma cell lines (Fig. 2B). Because exon IIIb contains a unique AvaI site, whereas exon IIIc contains two HinII sites, digestion with these two enzymes allows detection of these two isoforms. We found that FGFR2 IIIb expression was characteristic of untransformed MCF-10A and six of six noninvasive (BT-474, ZR-75-1, MCF-7, T-47D, MDA-MB-468, and SK-BR-3) breast cell lines, whereas FGFR2 IIIc was detected in all three invasive breast carcinoma cell lines (Hs578T, BT-549, and MDA-MB-231). Previously, we verified that the Hs578T, BT-549, and MDA-MB-231, but not the untransformed MCF-10A or other carcinoma cell lines, showed invasive activity in vitro by using the Matrigel invasion and other assays (18). We also found that expression of FGFR2 IIIc was correlated with an epithelial-to-mesenchymal transition in eight of nine cell lines, as indicated by the loss of epithelial (E-cadherin) and gain of mesenchymal (N-cadherin and vimentin) protein expression (Fig. 2B). Thus, breast cancer progression may involve two distinct alternative splicing events, with expression of FGFR2 IIIb/C3 isoforms associated with the progress from normal breast epithelia to noninvasive breast cancer, and the conversion to the mesenchymal FGFR2 IIIc isoform in invasive breast cancer cells.

Previous studies have determined that FGFR2 IIIb C3 exhibits greater transforming activity than FGFR2 IIIb C1
expressing C2 exhibited a 3-, 4-, 16-, 2-, or 8-fold higher transforming potency when compared with the human lines used. This trend is similar to our previous observations with Ras-transforming activity, supporting an oncogene function for FGFR2 in T-47D breast carcinoma growth. Generally, we found that FGFR2 IIIb C2–expressing NIH 3T3 cells caused morphologic transformation of epithelial cells. Mass populations of the indicated cell lines stably infected with the empty pBabe-puro vector or encoding the indicated FGFR2 isoforms were suspended in 0.4% soft agar and the number of proliferating colonies was quantitated after 2 to 4 wk. Representative fields of soft agar colonies.

The transforming activity of the IIIb C2 isoform has not been evaluated. Therefore, we focused our analyses on the biological activity of the FGFR2 IIIb C2 isoform isolated in our transformation screen. Our identification of FGFR2 IIIb C2 as a transforming protein in T-47D breast carcinoma cells suggests that wild-type FGFR2 IIIb C2 will function as an oncogene and promote breast epithelial cell growth transformation. To address this possibility, we ectopically expressed FGFR2 IIIb C2 in untransformed NMuMG mouse mammary epithelial cells; in spontaneously immortalized, untransformed MCF-10A; and in telomerase-immortalized HMEC human breast epithelial cell lines. All three cell lines have been shown previously to be sensitive to activated Ras-mediated growth transformation (19-22). Whereas empty vector–infected cells did not grow in soft agar, we found that FGFR2 IIIb C2–expressing NMuMG, MCF-10A, and HMEC cells showed the ability to form colonies when suspended in soft agar (Figs. 3 and 4A), supporting an oncogene function for FGFR2 in T-47D breast carcinoma growth. Generally, we found that FGFR2 IIIb C2 transforming potency was much greater in rodent cell lines when compared with the human lines used. This trend is similar to our previous observations with Ras-transforming activity, where human cells showed greater resistance to growth transformation.

We also compared the transforming activity of FGFR2 IIIb C2 with that of the longer IIIb C1 isoform. We found that MCF-10A, HMEC, NMuMG, Rat-1, and RIE-1 cells stably over-expressing C2 exhibited a 3-, 4-, 16-, 2-, or 8-fold higher frequency of colony formation, respectively, in soft agar when compared with C1-expressing cells (Fig. 4A). These results indicate that the carboxyl-terminal truncation enhanced FGFR2 IIIb C2–transforming activity.

Finally, because we found FGFR2 IIIc expression in some breast carcinoma cells, we also evaluated FGFR2 IIIc C1 transforming activity. We found that the IIIc C1 isoform caused very weak focus-forming activity, whereas the IIIb C1 isoform caused potent focus-forming activity (10-fold higher than IIIc C1) in NIH 3T3 fibroblasts (Fig. 4B). We also found that the IIIb C1 isoform induced a 2.5-fold greater frequency of soft agar colony formation than that caused by the IIIc C1 isoform in Rat-1 fibroblasts (Fig. 4C). We speculate that KGF secreted by fibroblasts may contribute to the greater transforming potency of the IIIb C1 isoform, when compared with the IIIc C1 isoform, in NIH 3T3 and Rat-1 fibroblasts. In contrast, the IIIb C1 showed the same soft agar colony-forming activity as the IIIc C1 in RIE-1 cells (Fig. 4C).

Distinct Transforming Activities of FGFR2 IIIb C2 in NIH 3T3 Fibroblasts and RIE-1 Epithelial Cells

Because previous studies evaluating FGFR2 IIIb function in NIH 3T3 mouse fibroblasts are complicated by fibroblast production of FGFR2 IIIb ligand, we focused our analyses using the RIE-1 cell line, a well-characterized rodent epithelial cell model system for the analyses of oncogene transformation. Because previous studies used NIH 3T3 cells, we did parallel analyses in NIH 3T3 cells to identify possible cell type differences in FGFR2 IIIb C2 transforming activity.

As expected from the identification of FGFR2 IIIb C2 in both our screens with Rat-1 and RIE-1 cells, we found that FGFR2 IIIb C2 caused potent formation of foci of transformed cells in NIH 3T3, RIE-1, and Rat-1 cells (Fig. 5A and B). Formation of foci of transformed cells is indicative of a loss of density-dependent growth inhibition and/or morphologic transformation. Therefore, we determined if FGFR2 IIIb C2 can cause these cellular parameters of oncogenesis.

For transformation analyses, we established mass populations of NIH 3T3 and RIE-1 cells stably infected with the pBabe-puro empty vector or encoding FGFR2 IIIb C2. Additionally, because FGFR2 IIIb signaling can involve activation of Ras, we used activated H-Ras(61L) as a positive control for these analyses. First, we determined whether FGFR2 IIIb C2 caused morphologic transformation of NIH 3T3 and RIE-1 cells. To our surprise, although FGFR2 IIIb C2 caused focus formation in both NIH 3T3 fibroblasts and RIE-1 cells (Fig. 5B), only NIH 3T3 fibroblasts but not RIE-1 or other epithelial cells were morphologically transformed by FGFR2 IIIb C2 (Fig. 5C). FGFR2 IIIb C2–expressing NIH 3T3 cells showed a highly retractile, elongated, and spindle-shaped cell morphology that was essentially identical to that of Ras-transformed NIH 3T3 cells. In contrast, the morphology of FGFR2-expressing RIE-1 cells was indistinguishable from that of control vector–infected cells (Fig. 5C). Similar results were seen with two mammary epithelial cell lines (NMuMG and MCF-10A), where activated Ras, but not FGFR2 IIIb C2, caused morphologic transformation.

We next evaluated the growth rate and saturation density of NIH 3T3 fibroblasts and RIE-1 cells stably expressing FGFR2

![FIGURE 3. FGFR2 IIIb and IIIc isoforms cause anchorage-independent growth transformation of epithelial cells. Mass populations of the indicated cell lines stably infected with the empty pBabe-puro vector or encoding the indicated FGFR2 isoforms were suspended in 0.4% soft agar and the number of proliferating colonies was quantitated after 2 to 4 wk. Representative fields of soft agar colonies.](Image 76x423 to 257x690)
IIIb C2. When the cultures were at subconfluent densities (between day 0 and 6 postplating; Fig. 6), NIH 3T3, but not RIE-1, cells expressing FGFR2 IIIb C2 displayed an increased growth rate when compared with their empty vector counterparts. However, at confluent densities (after the vector control cells reached at saturation densities; after day 6), both NIH 3T3 and RIE-1 cells expressing FGFR2 IIIb C2 showed increased growth rates when compared with their empty vector–infected counterparts (Fig. 6). In contrast, both NIH 3T3 and RIE-1 cells expressing Ras showed increased growth rates at both subconfluent and confluent densities.

FGFR2 IIIb C2 Causes Activation of Ras, ERK, and AKT and Up-Regulation of Cyclin D1 Protein Levels in NIH 3T3 but not in RIE-1 Cells

FGF stimulation activates multiple downstream signaling networks, including the Ras and ERK mitogen-activated protein kinase cascade (2). Surprisingly, little has been reported regarding whether FGFR2 IIIb is an activator of Ras and Ras-mediated signaling pathways. Furthermore, most of the studies done for FGFR2 IIIb signaling have focused on transient activation of FGFR2 IIIb (23-27) rather than sustained activation of FGFR2 IIIb in transformed cells. Therefore, we focused on characterizing the consequences of sustained activation of FGFR2 IIIb in stably transformed cells.

We determined whether FGFR2 IIIb C2 caused sustained activation of Ras. To measure Ras activity, we did a pull-down assay using a glutathione S-transferase fusion protein containing the GTP-dependent Ras-binding domain from the Ras effector, the Raf-1 serine/threonine kinase (GST-Ras-RBD). As expected, the H-Ras–transformed NIH 3T3 and RIE-1 cells showed greatly elevated levels of ectopically overexpressed H-Ras-GTP. Surprisingly, we found that FGFR2 IIIb C2–transformed NIH 3T3 but not RIE-1 cells showed an elevated level of endogenous activated GTP-bound Ras (Fig. 7A).

Next, we determined whether FGFR2 IIIb C2 caused sustained activation of downstream effector pathways of Ras. The two best-characterized effector pathways of Ras important...
for growth transformation are the Raf-MEK-ERK mitogen-activated protein kinase and the phosphatidylinositol 3-kinase–AKT pathways. For these analyses, we used immunoblot analyses with phosphospecific antibody that recognizes the phosphorylated and activated forms of ERK1 and ERK2, or AKT. As we have shown previously (28), activated Ras causes sustained ERK activation in NIH 3T3 and RIE-1 cells, and sustained AKT activation in NIH 3T3 but not RIE-1 cells (Fig. 7B). Expression of FGFR2 IIIb C2 also led to sustained activation of ERK1/2 and AKT in NIH 3T3 fibroblasts, although the degree of activation was less than that seen in Ras-transformed NIH 3T3 cells. In contrast, we found no increase in activated ERK or AKT levels in FGFR2-transformed RIE-1 cells (Fig. 7B). Expression of FGFR2 IIIb C2 also led to sustained activation of ERK1/2 and AKT in NIH 3T3 fibroblasts, although the degree of activation was less than that seen in Ras-transformed NIH 3T3 cells. In contrast, we found no increase in activated ERK or AKT levels in FGFR2-transformed RIE-1 cells (Fig. 7B). Finally, we evaluated the consequences of FGFR2 IIIb C2 transformation on altered cell cycle regulation. Both the Raf-MEK-ERK and phosphatidylinositol 3-kinase–AKT pathways have been shown to contribute to Ras-mediated up-regulation of cyclin D1, a key positive regulator of cell cycle progression through G1 (29). Therefore, we examined whether FGFR2 IIIb C2 increased the steady-state levels of cyclin D1 protein. Consistent with the ERK and AKT activity levels, we found that cyclin D1 protein levels are up-regulated in FGFR2 IIIb C2-transformed NIH 3T3 cells but not in RIE-1 cells (Fig. 7B). These results suggest that FGFR2 IIIb C2 causes sustained activation of Ras and Ras-mediated effector signaling in NIH 3T3 but not RIE-1 cells.

KGF Stimulates Morphologic Transformation and Increases Cyclin D1 Protein Levels in FGFR2 IIIb C2-Transformed RIE-1 Cells

Because fibroblasts, but not epithelial cells, express the ligand (KGF/FGF7) for FGFR2 IIIb, it is expected that FGFR2 IIIb C2 will be stimulated by an autocrine mechanism in fibroblast but not epithelial cells. This may account for the absence of Ras activation that we observed in FGFR2-transformed RIE-1 cells (Fig. 7A). Thus, we postulated that exogenous ligand stimulation of FGFR2 IIIb C2–expressing RIE-1 cells should mimic the biological and signaling
consequences of FGFR2 IIIb C2 seen in NIH 3T3 cells. KGF stimulation of control untransformed cells showed no evidence for KGF induction of apoptosis (data not shown). We found that sustained KGF stimulation (for 2 days) caused morphologic transformation of FGFR2 IIIb C2–expressing but not control vector cells (Fig. 8A), indicating that the morphologic transformation seen with FGFR2-transformed NIH 3T3 cells (Fig. 5C) is dependent on ligand stimulation of FGFR2 IIIb C2.

The transformed morphology of KGF-stimulated FGFR2-transformed RIE-1 cells (Fig. 8A) was similar to that seen with Ras-transformed RIE-1 cells (Fig. 5C), with cells exhibiting a refractile, elongated, and fibroblastic morphology. This suggested that KGF stimulation may cause sustained Ras activation in FGFR2 IIIb C2–expressing RIE-1 cells. First, we assessed whether sustained KGF stimulation led to constitutive activation of ERK and AKT in FGFR2 IIIb C2–expressing RIE-1 cells. Surprisingly, KGF stimulation did not increase the steady-state level of ERK phosphorylation levels in FGFR2 IIIb C2–expressing RIE-1 cells (Fig. 8B). As expected, because activated H-Ras(61L) did not increase AKT phosphorylation in RIE-1 cells, whereas H-Ras(61L) increased AKT phosphorylation in NIH 3T3 fibroblasts (Fig. 7B), no increase in AKT activity was seen in FGFR2 IIIb C2–expressing RIE-1 cells. However, we found that sustained KGF increased cyclin D1 protein expression levels in these cells. Cyclin D1 forms a complex with cyclin-dependent kinases CDK4/6 and phosphorylates and inactivates the retinoblastoma tumor suppressor protein (Rb). Thus, we further assessed if the increased cyclin D1 protein levels in FGFR2 IIIb C2–expressing RIE-1 cells stimulated with KGF corresponded to hyperphosphorylation and inactivation of Rb. Immunoblot analyses with phospho-specific Rb antibody that specifically recognizes the phosphorylated and inactive form of Rb revealed that KGF stimulation caused Rb hyperphosphorylation in FGFR2 IIIb C2–expressing RIE-1 cells (Fig. 8B). Finally, KGF stimulation stimulated a 5-fold enhancement in the ability of FGFR2 IIIb C2–expressing RIE-1 cells, but not control vector cells, to form colonies in soft agar (Fig. 8C and D). These results indicate that a KGF-mediated autocrine growth loop can further enhance the ability of FGFR2 IIIb C2 to cause growth transformation of RIE-1 cells.

**Discussion**

We identified FGFR2 IIIb C2 as a transforming gene expressed in human breast carcinoma cells. Although FGFR2 IIIb is expressed exclusively in normal epithelial cells, the majority of studies evaluating FGFR2 IIIb transformation have been done in fibroblasts (6, 14, 15). Because fibroblasts express FGFR2 IIIb ligands, the analyses of FGFR2 IIIb transformation in fibroblasts are complicated by an autocrine mechanism. Because cell type–specific differences exist in mechanisms of cellular transformation and oncogenesis, we have compared FGFR2 IIIb C2 transforming and signaling activities in NIH 3T3 fibroblasts and RIE-1 epithelial cells. Our observations suggest that FGFR2 IIIb–mediated transformation of epithelial cells may involve ligand-independent signaling that is independent of Ras activation.

Previous studies found low expression of the C1 isoform of FGFR2 IIIb in normal mammary epithelial cells, with greatly elevated C1, as well as C2 and C3, expression in SUM-52 human breast cancer cells (7). We extended these observations and found enhanced FGFR2 IIIb C1, C2, and C3 expression in seven of nine breast carcinoma cell lines evaluated, when compared with the levels seen in immortalized MCF-10A human breast epithelial cell line. Interestingly, the two cell lines that lacked FGFR2 IIIb expression harbor mutationally activated Ras. FGFR2 IIIb C3 exhibited higher transforming activity when expressed in NIH 3T3 fibroblasts (6) or in H16N2 immortalized human mammary epithelial cells (8). We have determined that FGFR2 IIIb C2 also displays greater transforming potency than FGFR2 IIIb C1. Hence, increased expression of C2 and C3 may contribute to the aberrant growth of breast carcinoma cells. Furthermore, we identified expression of the mesenchymal-specific FGFR2 IIIc isoform in
invasive breast carcinomas. A similar switch from FGFR2 IIIb to IIIc has been observed in a rat prostate tumor progression model, where IIIc expression promoted progression to malignant tumors that were independent of the stroma (4). Our cell line observations are consistent with a previous study that evaluated IIIb and IIIc transcript expression in 77 human breast cancer tissues, 12 nonmalignant breast biopsies, and 29 cell lines. They found a shift to IIIc expression in more advanced tumor stages (16). We also found that expression of FGFR2 IIIc correlated with an epithelial-to-mesenchymal transition, as indicated by the loss of epithelial (E-cadherin) and gain of mesenchymal (N-cadherin and vimentin) protein expression. Because FGFR expression has been shown to promote epithelial-to-mesenchymal transition (5), perhaps the enhanced expression of FGFR2 IIIc in invasive breast carcinomas may promote epithelial-to-mesenchymal transition and increased tumor cell invasion. Expression of FGFR2 IIIc may also render breast cancer cells independent of stromal cell-derived ligands.

Although FGFR2 IIIc expression induced focus formation and soft agar growth of both NIH 3T3 fibroblasts and RIE-1 epithelial cells, only NIH 3T3 fibroblasts were morphologically transformed by FGFR2 IIIb C2. However, when we treated FGFR2-expressing RIE-1 cells with exogenous KGF, cells became morphologically transformed. These data indicate that FGFR2 IIIb–induced morphologic transformation is dependent on stromal cell–derived KGF. In addition, we found that FGFR2 IIIb C2 caused up-regulation of cyclin D1 protein levels in NIH 3T3 fibroblasts but not RIE-1 cells. However, KGF stimulation increased cyclin D1 protein levels, leading to inactivation of the Rb tumor suppressor. Consistent with these observations, KGF stimulation also enhanced the anchorage-independent growth potential of RIE-1 cells expressing FGFR2 IIIb C2. Together, these observations indicate that FGFR2 IIIb can induce growth transformation of RIE-1 epithelial cells in a ligand-independent manner, but that paracrine stimulation by stromal cell–derived KGF will also contribute to FGFR2 IIIb C2–mediated transformation of RIE-1 epithelial cells. It has become clear that fibroblasts can regulate adjacent epithelia by secretion of growth factors and direct epithelial–mesenchymal interactions. In particular, several lines of evidences indicate that fibroblasts within the tumor stroma, the so-called carcinoma-associated fibroblasts, possess distinct biological properties from normal fibroblasts and play a role as a key modifier of cancer initiation and progression (30–33). Several oncogenic signals, including transforming growth factor-β, hepatocyte growth factor, and stromal cell–derived factor-1, were shown to be up-regulated in carcinoma-associated fibroblasts compared with normal fibroblasts (34). It is possible that KGF might be also up-regulated in carcinoma-associated fibroblasts and aberrantly activate FGFR2 IIIb in adjacent epithelial cells and contribute to cancer development.

Because KGF-stimulated FGFR2 IIIb C2–transformed cells showed characteristics that resembled Ras-transformed RIE-1 cells, the enhanced transformation caused by KGF is likely to involve activation of Ras. However, unlike mutant Ras-transformed RIE-1 cells, KGF stimulation did not cause activation of ERK in FGFR2 IIIb C2–transformed RIE-1 cells. This difference may reflect a qualitative or quantitative difference in effector utilization by mutationally activated Ras when compared with upstream activation of endogenous wild-type Ras. The signaling mechanisms for ligand-independent growth transformation are not clear, but the absence of Ras activation in FGFR2 IIIb C2–transformed RIE-1 cells suggests that it does not involve Ras-mediated signaling. Other FGFR2 IIIb C2 signaling activities that may facilitate growth transformation include activation of PLCγ and stimulation of second messenger production.

In summary, our studies provide further validation of a positive role for the tumor-associated expression of the IIIb C2 splice variant of FGFR 2 in breast cancer growth. Additionally, our studies support both ligand-independent and stromal cell–derived ligand-dependent mechanisms by which FGFR2 IIIb may promote oncogenesis in epithelial cells. Our future studies will focus on elucidation of the ligand-independent, Ras-independent mechanisms of FGFR2 IIIb transformation.

Materials and Methods

Cell Culture and Plasmid Expression Vectors

NIH 3T3, RIE-1, Rat-1, MCF-10A, NMuMG, and human breast carcinoma cell lines were maintained as we have...
described previously (12, 18). Telomerase-immortalized human mammary epithelial cells (HMEC; provided by R. Weinberg, Massachusetts Institute of Technology, Cambridge, MA) were maintained in MEGM growth medium (Clonetics). The cDNA sequence encoding FGFR2 IIIb C2 was subcloned into the 5’ blunt end and 3’ SalI site of the pBabe-puro retroviral vector. The pCTV3 and pBabe-puro H-Ras(61L) expression vectors were described previously (35).

Expression Library Screening

mRNA was purified from 2.8 × 10⁷ T-47D cells and double stranded cDNA was synthesized and used to generate the cDNA library in the pCTV1B retroviral vector as described (36, 37). The T-47D cDNA plasmid library was then converted into infectious retrovirus by transfection of 293T cells. Rat-1 fibroblasts (5 × 10⁶) and RIE-1 cells (7 × 10⁵) were plated in 10-cm dishes (10 each) the day before infection with retrovirus expressing the T-47D cDNA expression library. Genomic DNA was isolated from transformed foci and cDNAs were recovered by PCR as described (37).

FGFR2 Isoform Expression Analyses

To evaluate the expression of FGFR2 C1, C2, and C3 splicing variants in human breast cells, total cellular RNA was isolated from each cell line and reverse transcribed. The resulting cDNAs were amplified by PCR for 35 cycles. The primer sequences used for amplification of FGFR2 C1, C2, and C3 cDNA fragments were described previously (6). Our analysis of exon IIB and IIIc expression was done as described previously (17), with PCR amplification of the cDNA sequences for 50 cycles.

Transformation Assays

For primary focus formation assays, NIH 3T3 fibroblasts were plated at 2 × 10⁵ cells per 60-mm dish. The following day, cells were transiently transfected with the pBabe-puro empty vector or encoding H-Ras(61L) or FGFR2 IIIb C2. For secondary focus formation assays, RIE-1, Rat-1, and NIH 3T3 cells were stably infected with the pBabe-puro empty vector or encoding H-Ras(61L) or FGFR2 IIIb C2. After infection, cells were selected in growth medium supplemented with puromycin

![Figure 8](https://example.com/figure8.png)

**FIGURE 8.** KGF stimulation induces morphologic transformation and increases cyclin D1 protein levels in FGFR2 IIIb C2–transformed, but not control, RIE-1 cells. A. RIE-1 cells stably infected with either the empty vector or encoding FGFR2 IIIb C2 were maintained in growth medium supplemented with 0.1% bovine serum albumin (Vehicle) or 50 ng/mL KGF and changes in cell morphology were evaluated after 2 d. B. RIE-1 cells stably expressing the indicated proteins were treated with either vehicle or 50 ng/mL KGF. After 48 h, cell lysates were prepared and analyzed by immunoblot analyses for activated ERK and AKT. Cyclin D1 protein expression was determined by immunoblotting with cyclin D1 antibody and Rb inactivation was determined by immunoblotting with a phospho specific Rb (Ser780) antibody. C. KGF stimulation enhances FGFR2 IIIb C2 induction of RIE-1 anchorage-independent growth. Mass populations of the indicated cell lines stably infected with the empty pBabe-puro vector or encoding FGFR2 IIIb C2 were suspended in 0.4% soft agar supplemented with complete growth medium alone (Vehicle) or supplemented with 50 ng/mL KGF. The appearance of proliferating colonies was visualized after 2 wk. D. Quantitation of data shown in C.
(2 µg/mL). Multiple drug-resistant colonies were pooled and replated into 60-mm dishes and maintained in growth medium for 3 wk before the appearance of foci of transformed cells was photographed. To determine the growth rate on plastic and saturation density, mass populations of NIH 3T3 and RIE-1 cells stably expressing FGFR2 IIIb C2 were plated at 5 × 10^4 cells per 60-mm dishes. The cells were then trypsinized and duplicate dishes were counted every day for 15 d. Parallel cultures of NIH 3T3 and RIE-1 cells stably infected with the empty pBabe-puro vector, or encoding activated H-Ras(61L), were included for negative and positive controls, respectively. To determine the anchorage-independent growth, mass populations of the indicated cell lines stably expressing either empty vector or the indicated FGFR2 isoform were suspended in 0.4% bacto-agar in growth medium at 5 × 10^4 cells per 60-mm dish. The single-cell suspensions were layered on top of 0.6% bacto-agar in growth medium. After 14 to 21 d, colonies were stained with 2 mg/mL 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (tetrazolium salt) and the average number of colonies on duplicate dishes was calculated.

**Signaling Analyses**

To determine the level of Ras activation, we used a glutathione S-transferase fusion protein containing the Ras-binding domain of Raf-1 as we have described previously (38, 39). To determine ERK and AKT activation, cells were lysed and then analyzed for active ERK and AKT by immunoblot analyses with antibodies to total ERK (Santa Cruz Biotechnology) and AKT (Cell Signaling Technology), respectively. The membranes were stripped and reprobed with antibodies for total ERK (Santa Cruz Biotechnology) and AKT (Cell Signaling Technology), respectively. To determine cyclin D1 protein expression levels, immunoblot analyses were done with anti-cyclin D1 antibody (Santa Cruz Biotechnology). To determine Rb phosphorylation and inactivation, immunoblot analyses were done with antibody specific for the phosphor-determination Rb phosphorylation and inactivation, immunoblot with anti-cyclin D1 antibody (Santa Cruz Biotechnology). To determine ERK and AKT activation, cells were lysed and then analyzed for active ERK and AKT by immunoblot analyses with antibodies for total ERK (Santa Cruz Biotechnology) and AKT (Cell Signaling Technology), respectively. The membranes were stripped and reprobed with antibodies for total ERK (Santa Cruz Biotechnology) and AKT (Cell Signaling Technology), respectively. To determine cyclin D1 protein expression levels, immunoblot analyses were done with anti-cyclin D1 antibody (Santa Cruz Biotechnology). To determine Rb phosphorylation and inactivation, immunoblot analyses were done with antibody specific for the phosphor-determination Rb phosphorylation and inactivation, immunoblot with anti-cyclin D1 antibody (Santa Cruz Biotechnology). To determine ERK and AKT activation, cells were lysed and then analyzed for active ERK and AKT by immunoblot analyses with antibodies to total ERK (Santa Cruz Biotechnology) and AKT (Cell Signaling Technology), respectively. The membranes were stripped and reprobed with antibodies for total ERK (Santa Cruz Biotechnology) and AKT (Cell Signaling Technology), respectively.

**Acknowledgments**

We thank Misha Rand for preparation of figures.

**References**


Involvement of Fibroblast Growth Factor Receptor 2 Isoform Switching in Mammary Oncogenesis


Updated version
Access the most recent version of this article at:
http://mcr.aacrjournals.org/content/6/3/435

Cited articles
This article cites 39 articles, 18 of which you can access for free at:
http://mcr.aacrjournals.org/content/6/3/435.full.html#ref-list-1

Citing articles
This article has been cited by 7 HighWire-hosted articles. Access the articles at:
/content/6/3/435.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.