Oncogenic Gene Fusion FGFR3-TACC3 Is Regulated by Tyrosine Phosphorylation

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Abstract

Fibroblast growth factor receptors (FGFR) are critical for cellular proliferation and differentiation. Mutation and/or translocation of FGFRs lead to aberrant signaling that often results in developmental syndromes or cancer growth. As sequencing of human tumors becomes more frequent, so does the detection of FGFR translocations and fusion proteins. The research conducted in this article examines a frequently identified fusion protein between FGFR3 and transforming acidic coiled-coil containing protein 3 (TACC3), frequently identified in glioblastoma, lung cancer, bladder cancer, oral cancer, head and neck squamous cell carcinoma, gallbladder cancer, and cervical cancer. Using titanium dioxide–based phosphopeptide enrichment (TIO2)-liquid chromatography (LC)-high mass accuracy (MS/MS), it was demonstrated that the fused coiled-coil TACC3 domain results in constitutive phosphorylation of key activating FGFR3 tyrosine residues. The presence of the TACC coiled-coil domain leads to increased and altered levels of FGFR3 activation, fusion protein phosphorylation, MAPK pathway activation, nuclear localization, cellular transformation, and IL3-independent proliferation. Introduction of K508R FGFR3 kinase-dead mutation abrogates these effects, except for nuclear localization which is due solely to the TACC3 domain.

Implications: These results demonstrate that FGFR3 kinase activity is essential for the oncogenic effects of the FGFR3-TACC3 fusion protein and could serve as a therapeutic target, but that phosphorylated tyrosine residues within the TACC3-derived portion are not critical for activity.

Introduction

A subset of the Receptor Tyrosine Kinase (RTK) family is the fibroblast growth factor receptor (FGFR) family, which contains four homologous receptors: FGFR1, FGFR2, FGFR3, and FGFR4. FGFR activation results in changes in cellular proliferation and migration, antiapoptosis, angiogenesis, and wound healing. All FGFRs contain three immunoglobulin-like (Ig) domains, a transmembrane (TM) domain, and a split tyrosine kinase (TK) domain. Binding of fibroblast growth factors (FGF) and heparin sulfate proteoglycans (HSPG) to the extracellular Ig domains collectively induces FGFR activation through dimerization of receptor monomers and transautophosphorylation of kinase domain activation loop tyrosine residues. Tyrosine phosphorylation of the kinase domain initiates activation of RAS-MAPK, PI3K-AKT, and JAK/STAT pathways (1).

Point mutations in FGFRs have been linked to numerous human cancers and somatic disorders, many of which have been extensively studied. More recently, FGFR fusion proteins have been increasingly detected in various human cancers (1). Since their initial discovery in the late 1990s, the detection of these fusion proteins has steadily increased at a surprising rate. The focus of this article is a fusion protein consisting of FGFR3 fused to transforming acidic coiled-coil containing protein 3 (TACC3) that has been identified in glioblastoma, lung cancer, bladder cancer, oral cancer, head and neck squamous cell carcinoma, gallbladder cancer, and cervical cancer (1, 2). The FGFR3-TACC3 fusion protein is a consequence of a 70 kb tandem duplication at 4p16.3 (3). This causes a reversal of the two genes, as TACC3 is normally upstream of FGFR3. TACC3 is a member of the TACC family, which consists of 3 known human proteins, TACC1, TACC2, and TACC3, all of which are involved in key roles of microtubule organization during mitosis. TACC3 is believed to be essential for the stabilization of kinetochore fibers and the mitotic spindle. A particularly important domain of this family is the C-terminal coiled coil domain (named TACC domain), which is highly conserved in all family members. This domain is believed to play an important role in localization of the protein during mitosis (4). Although TACC2 has been shown to be dispensable for normal mouse development without affecting proliferation, cell-cycle progression, or centrosome numbers (5), in contrast, knockdown of TACC3 has been shown to result in G2 M phase arrest, apoptosis, abnormal spindle assembly, chromosome misalignment, and impaired microtubule assembly and nucleation (6–8).

The frequent occurrence of this fusion protein in many cancer types raises important questions as to whether activation of the FGFR3 kinase domain occurs as a result of the TACC3 fusion, and whether this domain contributes through its localization to the observed biologic effects. Using mass spectrometry to analyze the phosphorylation of key tyrosine residues as our point of...
departure, this work investigates the FGFR3-TACC3 fusion pro-tein with regard to its oncogenicity, activation of downstream signaling, altered subcellular localization, and induction of cell transformation.

Materials and Methods

DNA constructs

The TACC3 gene was purchased from Sino Biological Inc (pMDS-TACC3) and was subcloned into pcDNA3. FGFR3, FGFR3(K650E), and FGFR3(K508R) were developed as described previously (9). To construct FGFR3-TACC3 fusion gene, a unique ClaI site was introduced by PCR-based site-directed mutagenesis after residue 758 in FGFR3 and before residue 648 in TACC3. This unique site was used to subclone TACC3 3′ of FGFR3 in pcDNA3, creating a fusion breakpoint of FGFR3 exon 18 to TACC3 exon 11 with a 9 basepair linker, containing the ClaI site, and encoding the residues ASM. Fragments containing K650E or K508R mutations were subcloned into the FGFR3-TACC3 fusion gene. Single and multiple tyrosine mutations in the TACC3 region (Y798F, Y853F, Y867F, Y878F) were introduced by PCR-based site-directed mutagenesis. DNA constructs were subcloned into pLXSN vector (10) for focus, proliferation, and 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. All clones were confirmed by DNA sequencing.

Cell culture

HEK293, HEK293T, and NIH3T3 cells were maintained in DMEM plus 10% FBS and 1% penicillin/streptomycin in 10% CO2 at 37°C. MCF7 cells were maintained at 5% CO2 in DMEM plus 10% FBS and 1% penicillin/streptomycin in 37°C. 32D clone 3 (ATCC CRL-11346) cells were maintained in RPMI1640 medium with 10% FBS, 1% penicillin/streptomycin, and 5 ng/mL mouse IL3 in 5% CO2 at 37°C.

Antibodies and reagents

Antibodies were obtained from the following sources: FGFR3 (B-9), mSin3A (K-20), β-tubulin (H-235), STAT1 (E-23), STAT3 (C-20) from Santa Cruz Biotechnology; phosphotyrosine (4G10) from Millipore; TACC3 C-terminal (SAB4500103) from Sigma; phospho-STAT1 (Tyr701; 9171), phospho-STAT3 (Tyr705; D347), phospho-p44/42 MAPK (Erk1/2; T202/Y204; E10), p44/42 MAPK (Erk1/2; 9102) from Cell Signaling Technology; horseradish peroxidase (HRP) anti-mouse, HRP anti-rabbit, and Enhanced chemiluminescence (ECL and Prime-ECL) reagents were from GE Healthcare. MG132, aFGF, and recombinant mouse IL3 were obtained from R&D Systems. Heparin was from Sigma, Geneticin (G418) was from Gibco, and Lipofectamine 2000 Reagent was from Invitrogen.

Transfection, immunoprecipitation, and immunoblot analysis

HEK293 were plated at a density of 1 × 10⁶ cells/100-mm plate and transfected with 3 μg plasmid DNA using calcium phosphate transfection in 3% CO2 as described previously (11). Twenty to 24 hours after transfection, media were changed to DMEM with 0% FBS. Cells were starved for 20 hours before collecting and lysis. Transfected HEK293 cells were collected, washed once in PBS, and lysed in 1% NP40 Lysis Buffer [20 mmol/L Tris-HCl (pH 7.5), 137 mmol/L NaCl, 1% Nonidet P-40, 5 mmol/L EDTA, 50 mmol/L NaF, 1 mmol/L sodium orthovanadate, 1 mmol/L phenylmethylsulfonylfluoride (PMSE), and 10 μg/mL aprotinin] or RIPA buffer [50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 1% TritonX-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mmol/L NaF, 1 mmol/L sodium orthovanadate, 1 mmol/L PMSE, and 10 μg/mL aprotinin]. Bradford assay or Lowry assay was used to measure total protein concentration. Antibodies were added to lysates for overnight incubation at 4°C with rocking, followed by immunoprecipitation, as described previously (10). Samples were separated by 10% or 12.5% SDS-PAGE and transferred to Immobilon-P membranes (Millipore). Membranes were blocked in 3% milk/TBS/0.05% Tween 20 or 3% bovine serum albumin (BSA)/TBS/0.05% Tween 20 (for anti-phosphotyrosine, anti-phospho-STAT1, and anti-phospho-STAT3 blots). Immunoblotting was performed as described previously (12).

Mass spectrometry sample preparation

HEK293T cells were plated one day prior to transfection at 3.0 × 10⁶ cells per 15-cm tissue culture plate. Ten plates per sample were transfected by calcium phosphate precipitation with 9 μg of FGFR3 or FGFR3-TACC3 derivatives. Eighteen hours after transfection, cells were serum starved overnight and then treated with 10 μmol/L MG132 for 4 to 6 hours, and then washed once in 1× PBS + 1 mmol/L Na3VO4 before being lysed in RIPA. Clarified lysates were immunoprecipitated with FGFR3 antisera overnight at 4°C with rocking. Immune complexes were collected with Pierce protein A/G magnetic beads as per manufacturer's directions.

Following immunoprecipitation, proteins were digested directly on-beads using Trypsin/Lys-C mix. Briefly, samples were washed with 50 mmol/L ammonium bicarbonate, and then resuspended with 8 mol/L urea, 50 mmol/L ammonium bicarbonate, and cysteine disulfide bonds were reduced with 10 mmol/L tris(2-carboxethyl)phosphine (TCEP) at 30°C for 60 minutes followed by cysteine alkylation with 30 mmol/L iodoacetamide (IAA) in the dark at room temperature for 30 minutes. Following alkylation, urea was diluted to 1 mol/L urea using 50 mmol/L ammonium bicarbonate. The samples were finally subjected to overnight digestion with mass spectrometry grade Trypsin/Lys-C mix (Promega). Finally, peptides were collected into a new tube, and the magnetic beads were washed once with 50 mmol/L ammonium bicarbonate to increase peptide recovery. The digested samples were partially dried to approximately 50% of the total volume and desalted using a C₁₈ TopTip (PolyLC) according to the manufacturer's recommendations, and dried.

Aliquots were resuspended in 80% acetonitrile, 5% trifluoroacetic acid in 1 mol/L glycic acid and incubated with TiO₂ magnetic beads (GE) for 30 minutes at room temperature. Unbound peptides were removed from magnetic beads with two washes of 80% acetonitrile, 5% trifluoroacetic acid. Finally, phosphopeptides were eluted with 5% NH₄OH and dried.

LC-MS/MS and proteomics analysis

Samples were analyzed by LC-MS/MS using a 0.180 × 20 mm C₁₈ trap Symmetry column (Waters) connected to an analytical C₁₈ BEH130 PicoChip column (0.075 × 100 mm, 1.7 μm particles; NewObjective) mounted on a nanoACQUITY Ultra Performance Liquid Chromatography system (Waters), directly coupled to an Orbitrap Velos Pro mass spectrometer (Thermo Fisher Scientific). The peptides were separated with a 90-minute
null linear gradient of 2%–35% solvent B (acetonitrile) at a flow rate of 400 nl/minute. The mass spectrometer was operated in positive data-dependent acquisition mode. MS1 spectra were measured with a resolution of 60,000, an AGC target of 10⁶, and a mass range from 350 to 1,400 m/z. Up to 5 MS2 spectra per duty cycle were triggered, and each precursor was fragmented twice by collision-induced dissociation (CID) and electron transfer dissociation (ETD), and acquired in the ion trap with an AGC target of 10⁴, an isolation window of 2.0 m/z, and a normalized collision energy of 35. Dynamic exclusion was set to 5 seconds to allow multiple fragmentation of phosphopeptides.

All mass spectra from were analyzed with MaxQuant software version 1.3. Briefly, MS/MS spectra were searched against the cRAP protein sequence database (http://www.thegpm.org/crap/) indexed with the mutant FGFR3 or FGFR3-TACC3 sequences described here. Precursor mass tolerance was set to 20 ppm and 4.5 ppm for the first search where initial mass recalibration was completed and for the main search, respectively. Product ions were searched with a mass tolerance 0.5 Da. The maximum precursor ion charge state used for searching was 7. Carbamidomethylation of cysteines was searched as a fixed modification, while phosphorylation of serines, threonines, and tyrosines, and oxidation of methionines was searched as variable modifications. Enzyme was set to trypsin in specific mode and a maximum of two missed cleavages was allowed for searching. The target-decoy-based false discovery rate (FDR) filter for spectrum and protein identification was set to 1%. Modification site decoy fraction was also set to 1% and minimum score and delta score for modified peptides were set to 40 and 6, respectively, to assure high confidence phosphopeptide identification. All phosphorylation sites reported here were identified with a localization site of at least 0.75. Second peptide mode of MaxQuant software was also enabled. Spectra presented were produced in MaxQuant and manually annotated using Canvas Draw. Peak assignments were confirmed using software provided by ICSF ProteinProspector (http://prospector.ucsf.edu/prospector/).

Focus assay
Focus assays were performed using NIH3T3 cells plated at a density of 2 × 10⁵ cells/60-mm plates in DMEM with 10% FBS 24 hours before transfection. Cells were transfected by Lipofectamine 2000 Reagent with 10 μg plasmid DNA. Between 22 and 24 hours after transfection, cells were re-fed with DMEM 10% FBS. Cells were split 1:12 onto duplicate 100-mm plates between 22 and 24 hours later. Foci were scored at 12 to 14 days, fixed in methanol, stained with Giemsa stain, and photographed. Efficiency of transfection was determined by Geneticin (G418, 0.5 mg/mL)-resistant colonies plated on duplicate plates at a dilution of 1:240.

IL3-independent growth in 32D cells
A total of 1 × 10⁶ exponentially growing 32D cells were electroporated (1,500 V, 10 ms, 3 pulse) by the Neon Transfection System ( Invitrogen ) using 30 μg of FGFR3, FGFR3-TACC3 or PR/neu* derivatives in pLXSN in triplicate. Twenty-four hours after transfection, cells were selected with 1.5 mg/mL Geneticin (G418) sulfate for 10 days to generate stable cell lines. For IL3-independent proliferation assays, 2 × 10⁵ cells/well were seeded in 12-well plates in the absence of IL3 or 6-well plates in the presence of IL3. The media also contained 1 nmol/L aFGF and 30 μg/mL heparin (14). Cell numbers were determined in triplicate, with a hemocytometer and Trypan blue exclusion on days 2, 4, 6, and 7. Media were added to cultures when cell numbers reached approximately 1 × 10⁶ cells/mL during the assays to maintain at viable concentrations. For metabolic assays, MTT assays were performed. A stock solution of 5 mg/mL in PBS of MTT (Sigma) was added at a ratio of 1:10 to the cultures. After incubation at 37°C, 5% CO2 for approximately 4 hours, equal volume of 0.04 mol/L HCl in isopropanol was added and mixed well and incubated again for at least 30 minutes (15). Cultures were transferred to microfuge tubes, spun for 30 seconds at room temperature, and supernatant absorbance was measured at 570 nm. A total of 5 × 10⁴ cells per well were plated in triplicate in 24-well plates in the presence or absence of IL3 and 1 nmol/L aFGF and 30 μg/mL heparin and assayed 3 days later. The cell viability at day 7 was measured using cultures from the proliferation assay. In triplicate, 0.5 ml of the cultures were transferred to 24-well plates and treated with the MTT reagent.

Fractionation
MCF7 cells were plated at a density of 1.5 × 10⁶ cells/100-mm plates 24 hours before transfection. Immediately prior to transfection, media was changed to DMEM 0% FBS with no antibiotic. Cells were transfected with 8μg of plasmid DNA using Lipofectamine 2000 Reagent. Twenty-three hours after transfection, cells were collected in PBS and 1 mmol/L EDTA for fractionation as described previously (16). Total protein concentrations of separated fractions were analyzed by Bradford assay, separated by 10% SDS-PAGE, and transferred to Immobilon-P membrane for Western Blot analysis.

Results
Constitutive phosphorylation of FGFR3-TACC3 fusion protein
In the FGFR3-TACC3 fusion protein, tyrosine kinase domain dimerization and autophosphorylation may be elevated by the presence of the TACC3 coiled-coil domain, which could be crucial to cancer progression. To investigate changes in phosphorylation and biologic activity, various FGFR3-TACC3 derivatives were constructed. All fusion constructs contain the breakpoint between exon 18 of FGFR3 to exon 11 of TACC3 as shown in Fig. 1A, chosen so due to the high occurrence of this particular fusion breakpoint (3, 17). This fusion is predicted to contain the extracellular, transmembrane, and intracellular kinase domains of FGFR3 fused 5’ to the coiled-coil domain of TACC3 (18). Constitutively activated FGFR3 clones were produced by the K650E mutation. This mutation, originally identified as the cause of the lethal skeletal disorder Thanatophoric Dysplasia type II (TDII), results in profound FGFR3 kinase activation (1). The kinase activity of FGFR3 was abrogated by the mutation K508R, known as the “kinase-dead” mutant (Fig. 1A).

To examine the phosphorylation of each fusion construct compared with FGFR3 WT, FGFR3(K650E) and FGFR3(K508R) were expressed in HEK293 cells, collected, and immunoprecipitated with an N-terminal FGFR3 antibody (Fig. 1B, top). HEK293 cells were chosen as they have been used previously for studies of protein phosphorylation, downstream signaling, and protein–protein interactions (11, 12, 19). An increase in tyrosine phosphorylation was seen in FGFR3-TACC3 compared with FGFR3 WT (lanes 2 and 6). No phosphorylation signal could be detected for the kinase-dead FGFR3 with or without the fused TACC3 (Fig. 1B). These results show that tyrosine phosphorylation was increased...
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LC-MS/MS analysis identifies elevated and novel phosphorylation sites

The strong increase in tyrosine phosphorylation seen by immunoblotting leads to the question of whether TACC3 leads to a constitutively phosphorylated FGFR3 kinase and whether additional or novel phosphorylation sites exist on the fusion protein. Following immunoprecipitation with the FGFR3 N-terminal antibody and on-bead tryptic digestion, titanium dioxide–based phosphopeptide enrichment (TiO2)-liquid chromatography (LC)-high mass accuracy MS/MS was used with samples from HEK293T cells expressing FGFR3 or FGFR3-TACC3 derivatives. HEK293T cells provide a successful model for phosphorylation, signal transduction, and protein–protein interactions, and thus are useful for mass spectrometry studies (11, 19). Significant results are presented in Fig. 2A and B. As potential negative controls, duplicate samples of kinase-dead derivatives FGFR3 (K508R) and FGFR3(K508R)-TACC3 were analyzed by mass spectrometry, and no phosphorylated tyrosine residues were detected (data not shown). All tyrosine phosphorylation sites detected on the fusion proteins are indicated in Fig. 2C.

Figure 2A presents the spectral intensity of each phosphorylation site normalized against internal constitutive phosphorylation sites pS424 and pS444, which were found to be phosphorylated in all samples, even kinase-dead samples, as background cellular phosphorylation with no known function. Figure 2B presents another view of the same data, presenting the intensity of each phosphotyrosine site as a percentage of the total phosphotyrosine signal in that sample. In the FGFR3 WT samples, a very low level of phosphorylation was observed on the activation loop residues, Y647 and Y648 (Fig. 2A, top), with each site accounting for 50% of the phosphotyrosine in that sample (Fig. 2B, top). By comparing nonfused FGFR3 to FGFR3-TACC3, the effect of the coiled-coil domain on receptor phosphorylation and activation can be seen. The addition of the TACC3 domain leads to a modest increase in the overall phosphotyrosine (Fig. 2A, second panel), but surprisingly leads to more promiscuous phosphorylation as additional sites such as Y577, Y599, Y607, and Y798 are now observed (Fig. 2B, second panel).

Introduction of the activating mutation K650E into the FGFR3 background leads to an overall increase in tyrosine phosphorylation (Fig. 2A, third panel); furthermore, many additional phosphorylation sites are now observed, such as Y107, Y226, Y278, Y305, Y599, Y607, and Y724, in addition to the activation loop residues Y647 and Y648 (Fig. 2B, third panel). Finally, the introduction of the activating K650E mutation into the FGFR3-TACC3 background, creating FGFR3 (K650E)-TACC3, leads to massive activation of tyrosine phosphorylation (Fig. 2A and B, fourth panel), including all sites observed in the preceding samples, but with pronounced phosphorylation of the site Y798 in the TACC3 domain.

Representative spectra of phosphorylated peptides are shown in Fig. 3. A commonly identified FGFR3 WT peptide containing double phosphorylation of Y647 and Y648 in the activation loop is shown in panel A. In FGFR3-TACC3 fusion protein constructs, this double phosphorylated peptide becomes less frequent, with detection of peptides containing single Y647 phosphorylation becoming more common (Fig. 2, Fig. 3B and C). Also shown are spectra containing primary phosphorylation sites Y577, Y798, and Y867 in FGFR3-TACC3 and FGFR3(K650E)-TACC3 (Fig. 3D–F).

There are four tyrosine residues in the TACC3 portion of the FGFR3-TACC3 fusion protein: Y798, Y853, Y867, and Y878, corresponding to residues Y684, Y739, Y753, and Y764 in TACC3 WT. In FGFR3-TACC3, it was previously unknown whether these tyrosine residues were also phosphorylated, possibly by the fused kinase domain, and whether they play a role in cancer development. Through MS/MS analysis, significant phosphorylation was observed at Y798 and Y867 in FGFR3-TACC3, with trace amounts detected at Y853 (Fig. 2C). Phosphorylation was not detected at Y878, although this may be due to the small predicted tryptic peptide containing this residue. Increasing receptor activation by
The K650E mutation led to an increase in intensity levels of TACC3 tyrosine phosphorylation (Fig. 2A, second and fourth panels).

Of the phosphorylation sites detected in the TACC3 portion of the fusion protein, Y798 and Y853 have been previously identified as phosphorylation sites in TACC3 WT. The function of these sites is unclear and these residues are not conserved in the TACC family (20, 21). However, Y867 is a conserved tyrosine residue in the TACC family and our data identify it as a novel phosphorylation site for the FGFR3(K650E)-TACC3 fusion protein.
As mentioned above, mass spectrometry of HEK293T cells expressing FGFR3(K508R)-TACC3 (kinase dead mutation) revealed no tyrosine phosphorylated peptides within the FGFR3 or TACC3 domains. This indicates that an active FGFR3 kinase domain is required for tyrosine phosphorylation of the fusion proteins and the TACC domain is most likely phosphorylated by the FGFR3 kinase domain, not another tyrosine kinase.

Cell transforming ability of FGFR3-TACC3 by focus assay

To examine the transforming activity of FGFR3-TACC3 and subsequent mutants, focus-forming assays with NIH3T3 cells were performed. NIH3T3 cell transformation assays represent one of the original assays for the identification and characterization of oncogenes, having been used for analysis of activated RAS, MUC4, AKT, and many other oncogenes (22–25). This assay relies on the oncogenic transformation of transfected cells to overcome the contact-inhibited monolayer of NIH3T3 cells and establish a focus of proliferating cells. FGFR3-TACC3 and FGFR3(K650E)-TACC3 produced extremely high foci formation and cell transformation compared with FGFR3 WT or FGFR3(K650E) (Fig. 4). Expression of PR/neu*, a focus assay positive control, displayed less transformation than FGFR3-TACC3, which also consistently produced much larger foci. PR/neu* is a platelet-derived growth factor receptor, beta (PDGFR-β) with a Neu receptor transmembrane domain with the activating V664E mutation (p185*neo*; ref. 26). Despite the previously demonstrated elevated activation of PR/neu*, its transforming ability was dwarfed by the foci formation seen by FGFR3(K650E)-TACC3. As a result, samples were normalized to FGFR3(K650E)-TACC3 (Fig. 4). Expression of FGFR3(K508R)-TACC3 (kinase-dead mutation) and TACC WT in NIH3T3 cells did not produce significant foci formation, indicating that an active FGFR3 kinase domain is essential for cell transforming ability of FGFR3-TACC3.

Within the coiled-coil domain in FGFR3-TACC3, there are four tyrosine residues. Three of these residues were shown to be phosphorylated by MS/MS analysis, as discussed above. To assess the importance of these FGFR3-TACC3 phosphorylation sites, all four TACC3 tyrosine residues were mutated to phenylalanine (Y798F, Y853F, Y867F, Y878F) with and without the activating FGFR3 K650E mutation by site-directed mutagenesis and analyzed for focus forming ability. NIH3T3 cells expressing the fusion constructs with all four tyrosine mutations (Fig. 4) displayed high levels of focus formation when compared with FGFR3-TACC3 and FGFR3(K650E)-TACC3 with no additional phosphorylation was strongly elevated by FGFR3-TACC3 and FGFR3(K650E)-TACC3, FGFR3-TACC3(4xYF), and PR/neu* and their fusion counterparts displayed a clear difference in localization (Fig. 6A). All three fusions, FGFR3-TACC3, FGFR3(K650E)-TACC3, and FGFR3(K508R)-TACC3 (nuclear fraction, lanes 5, 6, and 7) displayed strong nuclear localization. The nonfused tyrosine kinase domains (lanes 2, 3, and 4) were present mainly in the cytoplasmic fraction, which also includes the cell membrane fraction. Perinuclear localization of FGFR3 (K650E) has been demonstrated previously (37), but fusion of FGFR3(K650E) to TACC3 dramatically increased nuclear localization. These results indicate the presence of the TACC3 coiled-coil domain is responsible for nuclear localization of the FGFR3 kinase, regardless of receptor activation. Immunoblotting for nuclear localizing mSin3A and cytoplasmic β-tubulin confirmed separation of nuclear and cytoplasmic fractions (Fig. 6A).

Downstream signaling activation by FGFR3-TACC3

It has been shown previously that FGFR3 WT and FGFR3 (K650E) activate the STAT pathway and MAPK pathway, but it is not clear how this activation compares with FGFR3-TACC3 or FGFR3(K650E)-TACC3 fusions. HEK293 cells, previously used in FGFR signal transduction studies (12, 16), expressing these fusions and their nonfused counterparts were analyzed for STAT1 and STAT3 activation. FGFR3 expression resulted in undetectable or slight phosphorylation of STAT1 and STAT3, whereas the activated mutant FGFR3(K650E) resulted in significant phosphorylation of both STAT1 and STAT3 (Fig. 6B, lanes 2 and 3). In contrast, the addition of the TACC3 domain in either FGFR3-TACC3 or FGFR3(K650E)-TACC3 did not lead to a significant change (Fig. 6B, lanes 6 and 7). However, MAPK phosphorylation was strongly elevated by FGFR3-TACC3 and FGFR3(14) select fusion proteins were examined in an alternate IL3-dependent cell line, 32D. Both are murine-derived cell lines dependent on IL3 for viability and growth and have been extensively used as a model system for studying transforming properties of oncogenes (27–31). The choice of the 32D cells over Ba/F3 cells was due to their more established origin (32–34) and availability. FGFR3 WT, FGFR3-TACC3, FGFR3(K650E), FGFR3(K650E)-TACC3, FGFR3-TACC3(4xFY), and PR/neu* were electroporated into the 32D cell line and selected as described in Materials and Methods. As seen in Fig. 5A, in the absence of IL3, all the clones expressed were able to stimulate IL3-independent growth indicating their transforming potential. Interestingly, the FGFR3-TACC3(4xFY) clone had the highest proliferation even without the activating K650E mutation. This could support the suggestion of the TACC3 tyrosine residues as being inhibitory. In addition, even in the presence of IL3 (Fig. 5B), the expression of some of the clones enhanced the proliferation of the 32D cells compared with nonexpressing cells. The MTT metabolic assays performed on days 3 and 7 shown in Fig. 5C support the cell population assay results. All transfected constructs display cell viability, whereas 32D control cells do not, indicating that FGFR3-TACC3 and other constructs promote cell proliferation.

FGFR3-TACC3 displays nuclear localization

The presence of the TACC3 domain, derived from a nuclear localizing protein (4), raises the question whether the fusion protein FGFR3-TACC3 relocalizes to the nucleus as a result of this domain. MCF7 cells have been previously used for fractionation studies with high success (16, 35, 36). Indeed, fractionation of MCF7 cells expressing FGFR3 WT, FGFR3(K650E), FGFR3(K508R), and their fusion counterparts displayed a clear difference in localization (Fig. 6A). All three fusions, FGFR3-TACC3, FGFR3(K650E)-TACC3, and FGFR3(K508R)-TACC3 (nuclear fraction, lanes 5, 6, and 7) displayed strong nuclear localization. The nonfused tyrosine kinase domains (lanes 2, 3, and 4) were present mainly in the cytoplasmic fraction, which also includes the cell membrane fraction. Perinuclear localization of FGFR3 (K650E) has been demonstrated previously (37), but fusion of FGFR3(K650E) to TACC3 dramatically increased nuclear localization. These results indicate the presence of the TACC3 coiled-coil domain is responsible for nuclear localization of the FGFR3 kinase, regardless of receptor activation. Immunoblotting for nuclear localizing mSin3A and cytoplasmic β-tubulin confirmed separation of nuclear and cytoplasmic fractions (Fig. 6A).

FGFR3-TACC3 promotes IL3 independent cell growth

Because of the role of activated FGFR3 mutants in multiple myeloma and previous studies showing their oncogenic and proliferative potential in the IL3-dependent cell line, Ba/F3 (48), select fusion proteins were examined in an alternate IL3-dependent cell line, 32D. Both are murine-derived cell lines dependent on IL3 for viability and growth and have been extensively used as a model system for studying transforming properties of oncogenes (27–31). The choice of the 32D cells over Ba/F3 cells was due to their more established origin (32–34) and availability. FGFR3 WT, FGFR3-TACC3, FGFR3(K650E), FGFR3(K650E)-TACC3, FGFR3-TACC3(4xFY), and PR/neu* were electroporated into the 32D cell line and selected as described in Materials and Methods. As seen in Fig. 5A, in the absence of IL3, all the clones expressed were able to stimulate IL3-independent growth indicating their transforming potential. Interestingly, the FGFR3-TACC3(4xFY) clone had the highest proliferation even without the activating K650E mutation. This could support the suggestion of the TACC3 tyrosine residues as being inhibitory. In addition, even in the presence of IL3 (Fig. 5B), the expression of some of the clones enhanced the proliferation of the 32D cells compared with nonexpressing cells. The MTT metabolic assays performed on days 3 and 7 shown in Fig. 5C support the cell population assay results. All transfected constructs display cell viability, whereas 32D control cells do not, indicating that FGFR3-TACC3 and other constructs promote cell proliferation.
Figure 3.
Representative spectra of selected peptides. MS/MS spectra are presented for several key phosphorylation sites. In the fragmentation patterns, spectral peak evidence was observed for all indicated fragments and, furthermore, fragmentation evidence is present for all phosphorylation sites shown here. As noted, due to space constraints, all identified fragment ions are not individually labeled on the spectra. Peptides presented are as follows: A, from a sample of FGFR3 WT, an ETD (electron transfer dissociation) spectrum of the doubly phosphorylated peptide DVHNLDpYpYKK including pY647 and pY648 within the activation loop. B, from a sample of FGFR3-TACC3, a CID (Collision Induced Dissociation) spectrum of the phosphorylated peptide DVHNLDpYpYKK including pY647 within the activation loop. In this spectrum, the following fragment ions were identified but not labeled: y6, m/z 909.41; z8, 1144.49. C, from a sample of FGFR3(K650E)-TACC3, an ETD spectrum of the phosphorylated peptide DVHNLDpYpYKETTNGRLPVK including pY647 within the activation loop. In this spectrum, the z3 fragment ion with m/z 372.22 was identified but not labeled on the spectrum. (Continued on the following page.)
(K650E)-TACC3 (Fig. 6C, lanes 6 and 7), compared with non-fused FGFR3 WT and FGFR3(K650E) (lanes 2 and 3), indicating that FGFR3-TACC3 induces MAPK pathway activation. The kinase-dead FGFR3(K508R)-TACC3 did not display this activation (lane 8), indicating that FGFR3 kinase activity in the fusion protein is essential to downstream signaling activation.

Discussion

We extensively analyzed the FGFR3-TACC3 fusion protein by tyrosine residue phosphorylation changes and the impacts on oncogenic potential. We demonstrate that introduction of a C-terminal TACC3 coiled-coil domain results in constitutive activation and phosphorylation of key residues in FGFR3. Clearly, the presence of the TACC domain leads to an increase of total kinase activity, as well as increased promiscuity of phosphorylation sites, as shown by the additional phosphorylation sites detected by LC-MS/MS (Fig. 2). Activation by this coiled coil domain has a more severe impact on cell transformation and downstream signaling than the activating K650E mutation alone. The oncogenic potential of the fusion protein was demonstrated by focus formation assay (Fig. 4), IL3-independent proliferation assay (Fig. 5), and metabolic activity assay (Fig. 5). The absence of biologic activity and the lack of tyrosine phosphorylation of the fusion protein shown by FGFR3(K508R)-TACC3 kinase-dead mutant indicates that kinase activity is required for gain of

Figure 4.
Transformation of NIH3T3 cells by FGFR3 and FGFR3-TACC3 derivatives. Representative plates from a focus assay are shown, with transfected constructs indicated. Number of foci were scored, normalized by transfection efficiency, and quantitated relative to FGFR3(K650E)-TACC3 ± SEM. PR/neu’ is a positive control. Assays were performed a minimum of three times per DNA construct.

(Continued) D, from a sample of FGFR3-TACC3, an ETD spectrum of the phosphorylated peptide RPPGLDpY577SFDTCKPPEEQLTFK including pY577. In this spectrum, the following fragment ions were identified but not labeled: y3, m/z 395.23; y4, 508.31; y7, 894.46. Note that the Cys in this peptide was carbamidomethylated. E, from the FGFR3-TACC3 sample, a CID spectrum of the phosphorylated peptide IMDRFEEVpYQAMEEVGK including py798 within the TACC3 domain. In this spectrum, the following fragment ions were identified but not labeled: y2-NH3, m/z 258.14; y3-NH3, 357.21; y7, 1403.63; y13-H2O, 1643.7; a2, 217.14; b11, 1490.64; b12, 1561.68; b13, 1692.72. F, from the FGFR3(K650E)-TACC3 sample, a CID spectrum of the phosphorylated peptide KCVEDpY867AR including py967 within the TACC3 domain. In this spectrum, the following fragment ions were identified but not labeled: y1, m/z 175.12; y5-H2O, 699.29; y5-NH3, 700.27; y62++, 423.67; y8-H2O, 1087.43; b2-NH3, 272.11; b5-NH3, 571.17; b4-NH3, 500.22; b6-NH3, 858.27. Note that the Cys in this peptide was carbamidomethylated.
function and cancer progression, but not required for nuclear localization of the fusion protein, as shown by cellular fractionation (Fig. 6).

The K650E mutation in FGFR3, originally identified as the cause of the neonatal lethal skeletal malformation syndrome Thanatophoric Dysplasia type II (38), has been identified frequently as a somatic mutation in many different human cancers such as bladder cancer and multiple myeloma (1). This mutation has been exploited in many studies to examine signaling pathways utilized by FGFR3 (14, 39–43). However, the K650E mutation has not yet been identified together with the FGFR3-TACC3 oncogenic gene fusion in human cancer, and thus represents an artificial construct not yet found in nature. Nonetheless, our use of this construct here allowed us to compare different routes of activation of FGFR3.

Examining the nonfused FGFR3 proteins, the analysis by LC-MS/MS indicates key FGFR3 residues are being phosphorylated, primarily residues Y647 and Y648 as part of the YYKK activation.
loop motif essential to FGFR kinase activity (43). Activation due to the K650E mutation reveals an increase in total phosphorylation as well as phosphorylation at additional sites, including Y724 (Fig. 2). Residue Y724 has been shown to be critical for activation of downstream signaling pathways, such as MAPK, STAT, and PI3K, as well as cell transformation (40). In the fusion protein FGFR3-TACC3, Y647 was the major site of phosphorylation within the activation loop, and phosphorylation of additional sites such as Y577, Y599, Y607, and Y798 was also observed. Introduction of the K650E mutation into the fusion protein FGFR3(K650E)-TACC3 resulted in increased phosphorylation of all the sites seen in the FGFR3-(K650E) as well as the sites seen in the FGFR3-TACC3 sample, with the additional appearance of phosphorylation at Y867 in the TACC3 domain.

Also deleted as a result of the FGFR3-TACC3 fusion breakpoint are the Aurora-A phosphorylation sites on TACC3. Aurora-A has been shown to phosphorylate TACC3 WT at S552 and S558 which is required for the localization of a TACC3-chTOK-chlathrin complex to mitotic spindle microtubules and spindle poles (7, 18, 45). Localization of TACC3 to kinetochoore in complex with chTOK and clathrin is believed to assist with stabilization and formation of the mitotic spindle (45). However, previous studies have found the FGFR3-TACC3 fusion protein localized only to the mitotic spindle poles during mitosis, and relocated during late stage mitosis to the midbody. A mechanism for this change in recruitment and the role of FGFR3-TACC3 during interphase remains unclear (46).

Although not analyzed in regards to cell cycle, we demonstrate a strong indication of nuclear localization for the fusion protein. In addition, localization of FGFR3-TACC3 to the nucleus is not dependent on kinase activity as shown by K508R mutation, indicating that this localization is solely due to the fused TACC domain. As the Aurora A phosphorylation sites are no longer present in the fusion protein, there must be another nuclear recruitment mechanism occurring. This delocalized kinase could be interacting with novel proteins that lead to cancer progression.

The detection of phosphorylated TACC3-derived residues in FGFR3-TACC3 (Y798, Y853 and Y867 corresponding to Y684, Y740 and Y752 in TACC3) was confirmed by immunoblotting of lysates from HEK293 cells expressing FGFR3 or FGFR3-TACC3 derivatives (Fig. 2B and C). The presence of these phosphorylated residues in the fusion protein suggests a role for TACC3 in the regulation of FGFR3 kinase activity.

Figure 6. Localization and signaling of FGFR3-TACC3 fusions. A, fractionation of MCF7 cells expressing FGFR3 or FGFR3-TACC3 derivatives. Cells were separated into cytoplasmic (left) and nuclear (right) fractions. Immunoblotting with FGFR3 antibody shows nuclear localization of FGFR3-TACC3 fusions (top). Immunoblotting for mSin3A and β-Tubulin confirm fractionation (second and third panels). B, lysates of HEK293 cells expressing FGFR3 or FGFR3-TACC3 derivatives were immunoblotted for phospho-STAT1 (Y701; top), STAT1 (second panel), phospho-STAT3 (Y705; third panel), STAT3 (fourth panel), and FGFR3 (bottom). C, HEK293 cell lysates expressing FGFR3 or FGFR3-TACC3 derivatives were immunoblotted for phospho-MAPK (T202/Y204; top), MAPK (second panel), and FGFR3 (bottom).
Y739, and Y753 in native TACC3) could indicate the ability of a highly activated kinase to self-phosphorylate the TACC domain and potentially lead to increased downstream signaling. Our results, in which mutation to phenylalanine of all four tyrosine residues within the TACC3 domain leads to increased focus formation and IL3-independent cell proliferation, leads to the conclusion that phosphorylation at these sites, while it does occur, fails to contribute significantly to the oncogenic potential of the FGFR3-TACC3 fusion oncogene.

Recently, it has been shown that chTOG (colonic and hepatic tumor overexpressed gene), a centrosomal localizing protein, recruits TACC3 to microtubule plus-ends during interphase. This localization is dependent on chTOG, not TACC3, and is independent of Aurora A phosphorylation (45). TACC3 residues 672–688 contain the binding site of ch-TOG and are present in the FGFR3-TACC3 fusion protein (at residues 786–802). Within this region, mutation Y798, which we found to be highly phosphorylated in the FGFR3-TACC3 and FGFR3{K650E}–TACC3 fusion proteins. In the K650E background, when the phosphorylation site is removed by the mutation Y798F, we did not observe a significant change in biologic activity, suggesting that the specific phosphorylation of Y798 occurs adventitiously and is extrinsic to the biological properties of the FGFR3-TACC3 fusion.

Introduction of Tyr-to-Phe mutations at all of the retained TACC3-derived residues Y798, Y853, Y867, or Y878 resulted in changes in biologic activity that were statistically insignificant, again indicating that the phosphorylation we observed at these sites is biologically inconsequential. An inhibitory phosphorylation site has been shown to occur in FGFR3 WT at Y770 which, upon phosphorylation, inhibits cell transformation (40). Interestingly, the residue Y770 has been removed from the FGFR3-TACC3 fusion, but the significance of this change was not explored here.

We have presented overwhelming evidence of the significant oncogenic potential of the FGFR3-TACC3 fusion protein. The presence of the TACC coiled-coil domain leads to increased and altered levels of FGFR3 activation, fusion protein phosphorylation, downstream signaling, cellular transformation, proliferation, and viability. The existence of FGFR3-TACC3 fusions in human cancers creates additional challenges and opportunities for identifying effective treatment strategies. For instance, a recent study showed that the HSP90 inhibitor ganetespib exerted pleiotropic effects on mitogenic and survival pathways in cells expressing oncogenic gene rearrangements of FGFR3, and provided an alternative therapeutic approach in addition to the pan-FGFR tyrosine kinase inhibitor BGJ398 (47). Further study of novel pathways activated by the FGFR3-TACC3 fusion protein, and a deeper understanding of the molecular details exploited by FGFR3-TACC3 to achieve its biologic potency, can be expected to lead to novel therapeutic paradigms.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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