

Inhibition of Fibroblast Growth Factor 19 Reduces Tumor Growth by Modulating β -Catenin Signaling

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Abstract

Fibroblast growth factors (FGF) play important roles in development, angiogenesis, and cancer. FGF19 uniquely binds to FGF receptor 4 (FGFR4). Our previous study has shown that FGF19 transgenic tumors have an activated Wnt-pathway phenotype. Wnt signaling is implicated in initiating or promoting FGF signaling in various cell types and organs. In this study, we examined whether FGF19 or inhibition of FGF19 affects the β -catenin signaling pathway using human colon cancer cell lines (HCT116, Colo201). Our results show that FGF19 increases tyrosine phosphorylation of β -catenin and causes loss of β -catenin–E-cadherin binding. FGF19 increases p-GSK3 β and active β -catenin levels and anti-FGF19 antibody (1A6) treatment abrogates this effect of FGF19. Anti-FGF19 antibody treatment increases S33/S37/T41 phosphorylation and ubiquitination of β -catenin. Ion-trap mass spectrometric analysis confirmed that 1A6 increases phosphorylation of β -catenin in the NH₂ terminus. Using HCT116-paired β -catenin knockout cells, we show that FGF19 induces TCF/LEF reporter activity in parental (WT/ Δ 45) and in WT/– but not in mutant (–/ Δ 45) cells, and that inhibition of endogenous FGF19 reduces this reporter activity, indicating that wild-type β -catenin is accessible for modulation. FGFR4 knockdown using inducible short hairpin RNA significantly reduces the colony-forming ability *in vitro* and tumor growth *in vivo*. Although cleaved caspase-3 immunoreactivity remains unchanged, the number of ki67-positive nuclei is reduced in FGFR4 knockdown tumor xenograft tissues. Consistent with the reduced β -catenin activation, Taqman analyses show that FGF19/FGFR4 inhibition reduced β -catenin target gene (*cyclin D1*, *CD44*, *c-jun*, *Cox-2*, *UPAR*) expression. These findings highlight that FGF19/FGFR4 cross-talk with β -catenin and that pathway intervention reduces tumor growth. [Cancer Res 2008; 68(13):5086–95]

Introduction

The fibroblast growth factor (FGF) family of signaling molecules plays important roles in development, angiogenesis, and cancer (1). FGF19 binds uniquely to FGFR4 (2). Our previous studies showed that ectopic expression of FGF19 in transgenic mice results in development of hepatocellular carcinomas by 10 months of age and that liver tumors of these transgenic mice have neoplastic cells that show nuclear localization of β -catenin indicative of activation (3). Interestingly, recombination hotspots associated with carcino-

genesis include *Wnt* or *FGF* gene clusters. More recent studies have shown that Wnt and FGF signaling cross-talk during a variety of cellular processes, including human colorectal carcinogenesis, and that coactivation of Wnt and FGF signaling pathways in tumors leads to more malignant phenotypes (4).

β -Catenin is a dual-function protein that plays a key role in maintaining cell-cell adhesion and as a downstream effector of the Wnt-signaling cascade. Association of E-cadherin with β -catenin and α -catenin is crucial for stable cell-cell adhesion and binding of β -catenin to these partners has been shown to be regulated by tyrosine phosphorylation of β -catenin (5, 6). The stability of β -catenin in the cytoplasm is regulated by a protein complex composed of adenomatous polyposis coli (APC), axin, conductin, and glycogen-synthase 3 β (GSK-3 β), which targets β -catenin for ubiquitination and proteasomal degradation. Activation of Wnt signaling leads to inactivation of GSK-3 β , resulting in accumulation of cytoplasmic β -catenin, which then enters the nucleus and interacts with TCF/LEF transcription factors that activate transcription of genes containing TCF/LEF binding sites (7–9). Phosphorylation of GSK3 α at serine 21 or GSK3 β at serine 9 upon stimulation with growth factors such as insulin has been shown to result in inhibition of GSK-3 kinase activity in mammalian cells (10–12). Deregulation of Wnt signaling has been implicated as an important event in the initiation and/or progression of several malignancies, including colon cancer; hepatocellular carcinoma; melanoma; and ovarian, endometrial, and prostate cancers (7–9, 13). The *APC* gene is mutated in up to 80% of sporadic colon cancers and loss of APC has been shown to induce irregular stabilization and accumulation of β -catenin (14, 15).

FGFR4 is overexpressed in several types of human tumors, including colon, liver, breast, neuroendocrine, and pancreatic carcinoma (16–19). In addition, a single nucleotide polymorphism changing the sense codon 388 from glycine to arginine in the transmembrane domain of the *FGFR4* gene has been associated with a poor prognosis for positive node breast cancer, high-grade soft-tissue sarcoma, colon carcinoma, and head and neck squamous cell carcinoma (20–22). However, the role of FGF19 or the effect of FGF19 inhibition in cancer remains poorly understood. In the present study, using human colon cancer (HCT116, Colo201) cells, we show that FGF19 increases GSK-3 β phosphorylation (S9) and active β -catenin, and induces β -catenin/TCF4-regulated transcriptional activity. Inhibition of FGF19 with a blocking antibody (1A6) increases S33/S37/T41 phosphorylation and ubiquitination of β -catenin and decreases β -catenin target gene expression. Using paired HCT116 β -catenin knockout cells, we show that FGF19 exerts its effect through modulation of wild-type (WT) β -catenin. Moreover, inducible short hairpin RNA (shRNA)-mediated FGFR4 knockdown show reduced β -catenin pathway signaling and decreased tumor growth *in vivo* and clonal growth *in vitro*. These findings show that FGF19-FGFR4 cross-talk with β -catenin signaling and that inactivation of either FGF19 or FGFR4 could help reduce tumorigenesis.

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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Materials and Methods

HCT116 and Colo201 cell lines (American Type Culture Collection) were routinely maintained at 37°C and 5% CO₂ in RPMI 1640 containing 10% fetal bovine serum (FBS) and 4 mmol/L L-glutamine. HCT116 cells harboring WT/ Δ 45 (parental) and their derivatives expressing only WT/– or mutant (–/ Δ 45) β -catenin were obtained from the Johns Hopkins University (Baltimore, MD; ref. 23) and routinely maintained in McCoy's 5A containing 10% FBS. Serum-starved cells were incubated with either vehicle or FGF19 (50–250 ng/mL, 10 min). In separate experiments, cells were treated with either control antibody (gp120) or FGF19 antibody (1A6, 20 μ g/mL) for the indicated time points. To further evaluate the effects on β -catenin activation, cells were pretreated with a proteasome inhibitor, MG132 (Biomol) at 1 μ mol/L concentration for 4 h followed by 1A6 treatment for 24 h to evaluate ubiquitination and phosphorylation of S33/S37, S45, and T41 on β -catenin. After incubation, cells were washed in cold PBS and lysed for either protein or RNA analyses.

Immunoprecipitation and Western blot analysis. Cells were lysed in modified radioimmunoprecipitation assay buffer [RIPA; 50 mmol/L Tris-Cl (pH 7.5), 150 mmol/L NaCl, 1% IGEPAL, 1 mmol/L EDTA, 0.25% sodium deoxycholate, 1 mmol/L NaF, 1 mmol/L Na₂VO₄, and protease inhibitors cocktail (Sigma-Aldrich, St. Louis, MO)] and clarified by centrifugation. Protein concentrations of the lysates were determined using the BCA protein assay kit (Pierce). Equal amounts of proteins were incubated with specific antibody immobilized onto protein A-Sepharose and washed, and immunocomplexes were eluted in 2 \times Laemmli buffer. Proteins were resolved on SDS-PAGE, transferred to nitrocellulose membrane, and incubated with specific primary and secondary antibodies. The antibodies used for immunoprecipitation and immunoblotting were β -catenin monoclonal antibodies (mAb) from BD Transduction; active β -catenin directed against NH₂-terminally dephosphorylated β -catenin, FRS2, phosphotyrosine (4G10), and E-cadherin mAb from UpState Biotech; phospho- β -catenin (S33/S37 and S45/T41 specific), phospho-GSK3 β (S9), and GSK-3 β antibodies from Cell Signaling; and ubiquitin mAb from EMD Biosciences. Mouse monoclonal FGF19 (1A6), FGFR4 (1G7), and control (gp120) antibodies were developed in house (24). Where indicated, the membranes were stripped (Pierce) and reprobed with another antibody. Quantitative analyses of tyrosine and Ser/Thr phosphorylation of β -catenin were done by determining the ratio between relative density of the total protein and the phosphorylation band using the data from three separate experiments.

Liquid chromatography-mass spectrometry/mass spectrometry. Indirect quantification of NH₂-terminal β -catenin phosphorylation levels was performed using linear ion-trap mass spectrometry (MS). β -Catenin was immunoprecipitated from cells pretreated with MG132 followed by control (gp120) or 1A6 antibody and separated using SDS-PAGE. Gels were Coomassie stained and the β -catenin bands were cut out and reduced in 10 mmol/L DTT and cysteines were alkylated with 50 mmol/L iodoacetamide before tryptic digestion. The peptides were digested in trypsin (10 ng/ μ L) in 50 mmol/L sodium bicarbonate (pH 8.0) and peptide mixtures (3 mL) were loaded onto a 0.25 \times 30 mm trapping cartridge packed with Vydac 214MS low-TFA C4 beads. This cartridge was placed in-line with a 0.1 \times 100 mm resolving column packed with Vydac 218MS C18 beads. The resolving column was constructed using a "picofrit" (New Objective) fused silica capillary pulled to a 15-mm metal-coated tip, which formed a microelectrospray emitter. Peptides were eluted with 1-h gradients of acetonitrile containing 0.1% formic acid at a rate of 0.3 mL/min. Data-dependent tandem MS was performed using a linear ion-trap instrument (LTQ; Finnigan). The Sequest database searching program was used to generate cross correlation scores for each CID spectrum. Proteins matched by only a single peptide were confirmed by manual interpretation of the collision-induced dissociation spectra. Phosphorylated peptides were manually confirmed. Peak areas were then integrated to determine relative abundance of peptides.

ShRNA studies. The pHUSH inducible vector system comprising a shRNA expression shuttle plasmid and a viral vector backbone containing a TetR-IRES-Puro cassette was used. FGFR4 knockdown vectors were constructed by designing custom siRNA sequences converting them into

shRNA and testing their efficacy in transient cotransfection experiment in 293T cells. Selected shRNA sequences (shFGFR4 forward: GATCCCGAACCATTGGAGGCATTATCAAGAGAAATGCCTCCAATGCGTTCTTTTTTGAAA; reverse: AGCTTT TCCAAAAAGAACCGCATTGGAGGCAATTTCTCTTGATAATGCCTCCAATGCGGTTCCGGG; hEGFP (shCTRL) forward: GATCCCGCAGCAGCAGTCTTCAAGTTCAAGA-GACTTGAAGAAGTCTGCTGCTTTTTTGAAA; reverse: AGCTTTTC-CAAAAAGCAGCAGCAGTCTTCAAGTCTCTTGAAGTTGAA-GAAGTCTGCTGCGGG) were cloned into pShuttle-H1 and then H1-shRNA cassette was transferred into pHUSH-GW by a Gateway (Invitrogen) recombination reaction. All constructs were verified by sequencing.

Generation of inducible-shRNA cell clones. HCT116 cells were maintained in a medium containing tetracycline-free FBS and were transfected using Lipofectamine 2000 plus (Invitrogen). As the puromycin resistance gene encoded in the vector is under the control of a constitutive β -actin promoter, 5 μ g/mL puromycin were used to select transfected cells expressing shRNA. Stable clones were isolated, treated with 1 μ g/mL doxycycline (BD Clontech) for 7 d, and functional FGFR4 knockdown was assessed by Western blotting and fluorescence-activated cell sorting (FACS) analysis.

Reporter assays. TCF/LEF reporter assays were done following the previously described procedure (25). HCT116 parental cells harboring WT/ Δ 45 and their derivatives expressing only wild-type (WT/–) or mutant (–/ Δ 45) β -catenin were obtained from the Johns Hopkins University (23). HEK293 cells stably expressing FGFR4 (HEK293-hFGFR4) or empty vector (HEK293-EV; control; ref. 26) were gifts from Dr. Desnoyers' laboratory. Cells were seeded in 24-well plates at 1.5×10^5 per well and the following day were transiently transfected with pTOPglow (containing TCF binding sites), or pFOPglow (mutant motif), pCAN LEM1, and pCMV-Renilla luciferase (Promega) using Lipofectamine 2000 reagent according to the manufacturer's instructions (Invitrogen). Twenty-four hours after transfection, cells were either treated with 1A6 (10–50 μ g/mL) or serum starved overnight and then treated with FGF19 (100–500 ng/mL) for 6 h. Luciferase activity was measured using the dual luciferase assay system (Promega). The relative reporter activity was determined by calculating the mean TOP/FOP ratios normalized to Renilla luciferase activity. Values are presented as means \pm SE from three separate experiments performed in duplicate.

Cell proliferation assay. The effect of FGFR4 knockdown on HCT116 cell proliferation was studied using shCTRL and shFGFR4 stable cell clones. In brief, shCTRL and shFGFR4 stable cells were pretreated with or without doxycycline (1 μ g/mL, 5 d), plated in 96-well culture plates, and continued with or without doxycycline treatment during the assay. Cellular proliferative activity was measured by quantification of 5'-bromo-2'-deoxyuridine (BrdUrd) incorporation into the genomic DNA during cell growth using a colorimetric BrdUrd ELISA assay kit following the manufacturer's (Roche) instructions. Absorbance was analyzed at dual wavelengths of 450 to 540 nm. Values are presented as means \pm SE of two individual experiments performed in replicate of 8.

Clonal growth assay. The effect of FGFR4 knockdown on cell clonogenicity was assessed following a previously described protocol (27). In brief, shCTRL and shFGFR4 stable cell clones (100 cells per well) were seeded in 96-well culture plates in the presence and absence of doxycycline (1 μ g/mL) and incubated for 10 d until cells in control wells have formed sufficiently large clones. Colonies were stained (0.5% crystal violet) and the relative density of stained colonies in a fixed area per well was measured using Adobe Photoshop software. Values are presented as means \pm SE ($n = 10$).

Xenograft studies. All animal protocols were approved by an Institutional Animal Care and Use Committee. Six- to eight-week-old female athymic nu/nu mice (Charles River Laboratories) were inoculated in the right flank with either 5×10^6 shCTRL- or human shFGFR4-containing cell clones resuspended in 100 μ L PBS. When tumors reached a mean volume of 100 to 150 mm³, the mice with similarly sized tumors were grouped into treatment cohorts. Mice received 5% sucrose only or 5% sucrose plus 1 mg/mL doxycycline for control and knockdown cohorts, respectively. All water bottles were changed thrice a week. Tumors were measured with calipers and mice were weighed twice a week. Mice whose

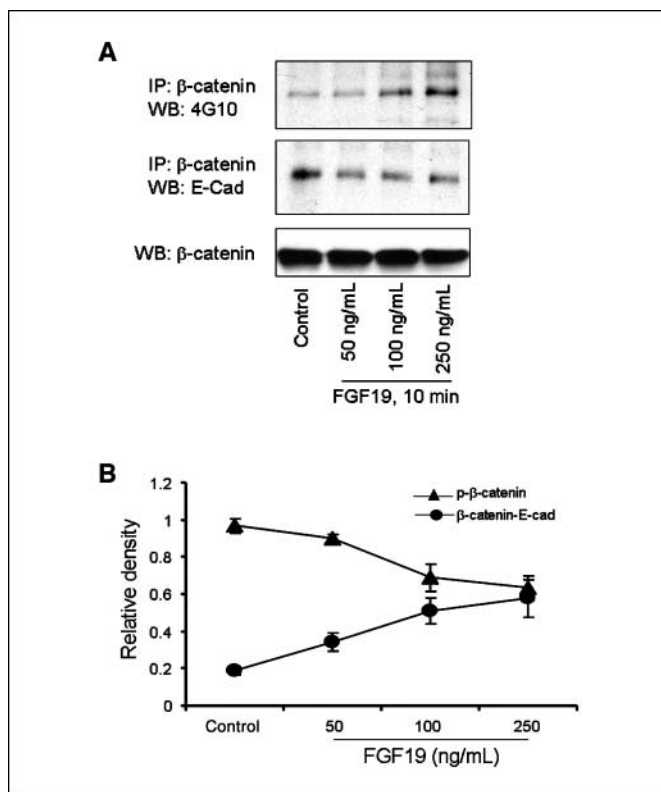


Figure 1. FGF19 induces tyrosine phosphorylation of β -catenin and causes loss of E-cadherin (*E-Cad*) binding to β -catenin in colon cancer (HCT116) cells. Serum-starved cells were treated with either vehicle (*control*) or FGF19 at 50 to 250 ng/mL for 10 min. *A*, a representative blot showing tyrosine phosphorylation of β -catenin as determined by immunoprecipitation (*IP*) and immunoblotting (*IB*). The same blot was stripped and reprobed for E-cadherin and subsequently reprobed for total β -catenin. *B*, quantitative analysis of β -catenin phosphorylation and β -catenin bound to E-cadherin as determined by calculating the ratio between the phosphorylated β -catenin or E-cadherin and total β -catenin levels from three separate experiments. Columns, mean; bars, SE.

tumors reached 2,000 mm³ were euthanized. Eight mice were used for each treatment group and results are presented as mean tumor volume \pm SE.

Immunohistochemistry. Formalin-fixed paraffin-embedded tissue specimens from shCTRL and shFGFR4 tumors treated with or without doxycycline were collected and a routine H&E slide was evaluated. Antigen retrieval was done using DAKO Target Retrieval Kit (DAKO) and immunohistochemical staining was performed using anti-ki-67 (clone MB-1, DAKO) and anti-cleaved caspase -3 (CC3; Cell Signaling) antibodies and DAKO ABC kit. Tissue sections were counterstained with hematoxylin and digital analysis for quantification of the proliferation index (i.e., the percent of cells undergoing division/total nuclei) and positive immunoreactivity for CC3 was performed. Images used for Ki-67 and CC3 analyses were acquired using an Ariol SL-50 slide scanning platform (Applied Imaging; San Jose) and analyzed using Ariol software. Values are presented as means \pm SE ($n = 6$).

β -Catenin target gene expression analyses. Total RNA was isolated using the Qiagen RNA isolation kit and DNase treated (Qiagen) following the manufacturer's protocol. RNA concentration was determined using ND-1000 spectrophotometer. Real-time quantitative PCR was performed to determine the relative abundance of β -catenin target gene mRNAs. Human-specific primers and fluorogenic probes for cyclin D1, CD44, c-jun, UPAR, Cox-2, and RPL19 (Supplementary Table S1) were designed using Primer Express 1.1 (Applied Biosystems). Analyses of data were performed using Sequence Detector 1.6.3 (Applied Biosystems) and results for genes of interest were normalized to RPL19.

Statistical analysis. Student's two-tailed *t* test was used to compare data between two groups. One-way ANOVA and Dunnett's test were used to compare data between three or more groups. $P < 0.05$ was considered statistically significant.

Results

FGF19 induces tyrosine phosphorylation of β -catenin and causes loss of E-cadherin binding to β -catenin in HCT116 cells.

Because FGFs are suggested to collaborate with Wnt during carcinogenesis (4), we sought to determine whether FGF19 affects Wnt signaling using colon cancer cells (HCT116) that endogenously express both FGF19 and FGFR4. Treatment of colon cancer cells (HCT116) with FGF19 (50–250 ng/mL) showed a significant increase in tyrosine phosphorylation of β -catenin as early as 10 minutes (Fig. 1*A*) when compared with vehicle-treated controls. Because β -catenin binding to cadherins to form stable cell-cell adhesions has been shown to be regulated by tyrosine phosphorylation of β -catenin, we next evaluated E-cadherin levels in cells treated with FGF19 by stripping and reprobing the tyrosine phosphorylation blot using E-cadherin antibody. The results showed a substantial loss of E-cadherin binding to β -catenin and this was inversely proportional to the tyrosine phosphorylation levels (Fig. 1*B*). Similar results were obtained when E-cadherin was immunoprecipitated and immunoblot analysis was performed using β -catenin antibody (data not shown).

FGF19 increases p-GSK-3 β and activates β -catenin, and treatment with 1A6 antibody reduces these effects of FGF19 in colon cancer cells.

Previous studies have established that Wnt-regulated β -catenin degradation is essential for carcinogenesis (7) and that Wnt signals are transmitted through NH₂-terminally dephosphorylated β -catenin (28). GSK-3 β plays a critical role in this process (29). Treatment of HCT116 cells with FGF19 increased p-GSK-3 β (S9) and active β -catenin levels in a dose-dependent manner (Fig. 2*A*, top left). Using our recently developed anti-FGF19 monoclonal antibody, 1A6, that blocks FGF19 binding to FGFR4 and neutralizes FGF19 tumorigenic effects *in vivo* (24), we determined the effect of FGF19 inhibition on β -catenin signaling in HCT116 cells. Treatment of these cells with 1A6 antibody reduced p-GSK-3 β (S9) levels and significantly decreased active β -catenin levels as early as 6 hours and sustained low levels up to 24 hours when compared with control antibody-treated cells (71.8 \pm 1.5% decrease, $P < 0.001$; Fig. 2*A*, top right and bottom). Similarly, treatment of Colo201 cells showed a dose-dependent increase in p-GSK-3 β (S9) and active β -catenin levels in response to FGF19 treatment (Supplementary Fig. S1*A*) and FGF19 inhibition with 1A6 antibody reduced p-GSK-3 β (S9) and active β -catenin levels as early as 3 hours and sustained the reduction up to 6 hours significantly (Supplementary Fig. S1*B*). Treatment of additional colon cancer cell lines (HT29 and SW620) with 1A6 antibody also showed a substantial decrease in active β -catenin levels, indicating that it is not a cell type-specific response (Supplementary Fig. S1*C*).

Inhibition of FGF19 using 1A6 antibody induces S33/S37/S45 and T41 phosphorylation.

Because FGF19 inhibition reduced active β -catenin levels in HCT116 cells, we next evaluated whether 1A6 treatment increases NH₂-terminal Ser-Thr phosphorylation and targets β -catenin for ubiquitination and proteasomic degradation. HCT116 cells pretreated with a proteasome inhibitor (MG132, 1 μ mol/L) for 4 hours followed by treatment with 1A6 antibody showed a significant increase in S33/S37 and S45/T41 phosphorylation (Fig. 2*B*) when compared with proteasome

inhibitor plus control antibody-treated cells. Quantification of S33/S37 phosphorylation as determined by calculating the ratio between the total β -catenin protein and phosphorylated protein levels showed a $123.4 \pm 7\%$ increase in 1A6 treated cells versus control. Similarly, Ser⁴⁵/T41 phosphorylation was increased by $166.8 \pm 11\%$ in 1A6-treated cells versus control (both $P < 0.05$). This effect of 1A6 was coupled with increased β -catenin ubiquitination (Fig. 2C) versus control. Colo201 cells pretreated with MG132 (1 $\mu\text{mol/L}$, 4 hours) followed by treatment with anti-FGF19 antibody also showed a substantial increase in S33/S37 phosphorylation (Supplementary Fig. S2) when compared with control antibody-treated cells.

Liquid chromatography-MS/MS analysis confirms increased NH₂-terminal S33/S37/S45 and T41 phosphorylation of β -catenin in 1A6 antibody-treated cells. To further confirm our data showing increased Ser-Thr phosphorylations in the NH₂

terminus of β -catenin upon 1A6 treatment, we compared signal intensities of nonphosphorylated β -catenin peptide in cells pretreated with a proteasome inhibitor followed by 1A6 or control antibody using linear ion-trap MS. Because the phosphorylated peptide is not detectable due to multiple phosphorylation sites and its stoichiometric ratio to other nonphosphorylated peptides, indirect quantification method was used to detect nonphosphorylated (control) versus phosphorylated (1A6) peptides. Under these conditions, nonphosphorylated peptide would give higher intensity and phosphorylated peptide would give lower intensity. The data were normalized to other nonrelated peptides (containing all four phosphorylation sites) that showed no difference in signal intensities from the treated and untreated samples. The β -catenin peptide isolated from 1A6-treated cells showed lower signal intensity when compared with β -catenin peptide isolated from control antibody-treated cells (Fig. 2D), clearly indicating an

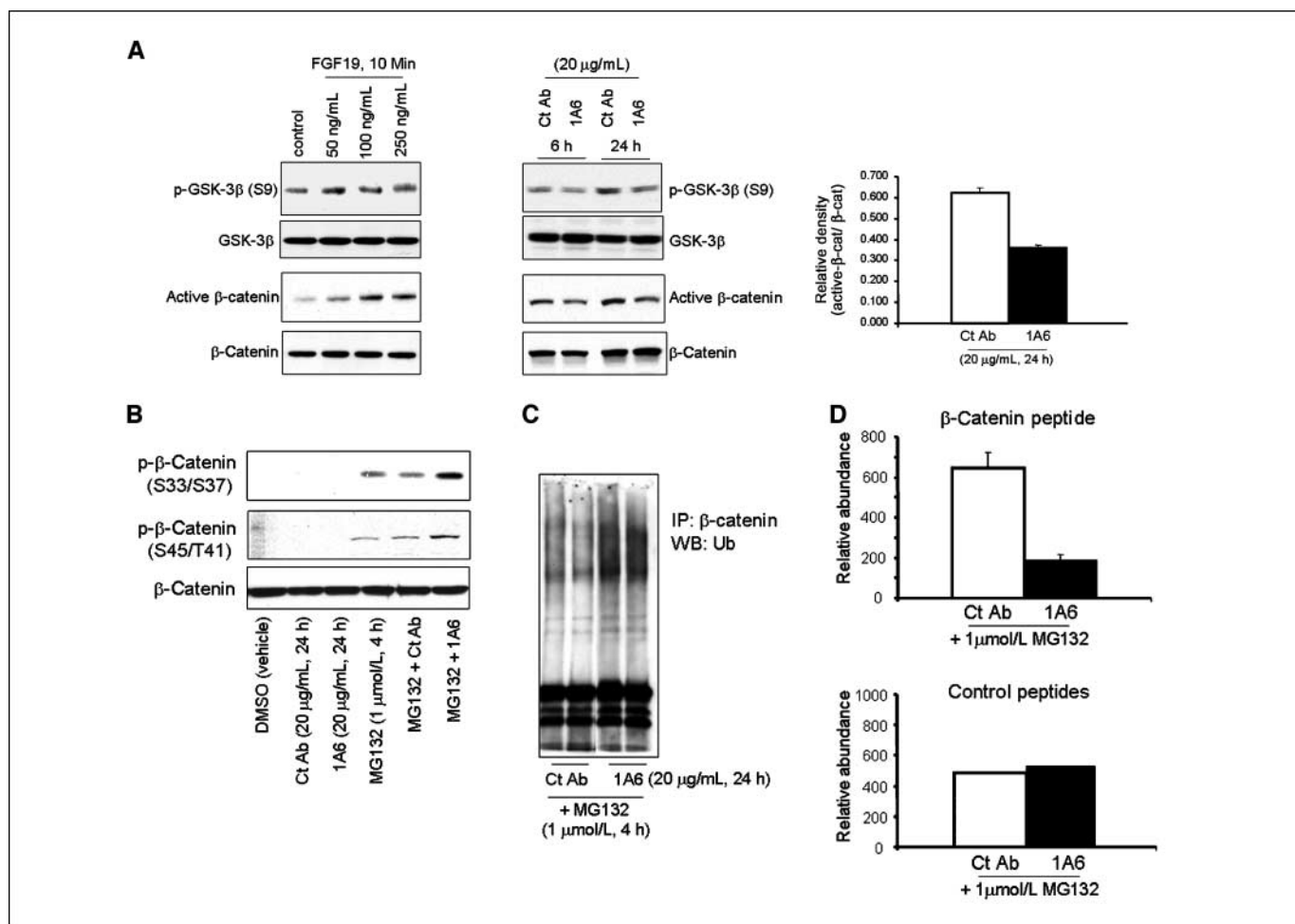


Figure 2. FGF19 increases p-GSK-3 β (S9) and activates β -catenin and anti-FGF19 antibody (1A6) abrogates this effect of FGF19 in HCT116 cells. Cells were treated with vehicle (control), FGF19 (50–250 ng/mL, 10 min), or control antibody (Ct Ab) or anti-FGF19 (1A6) antibody (both 20 $\mu\text{g/mL}$) for 6 and 24 h. *A, left*, p-GSK-3 β (S9) and active β -catenin levels were determined by immunoblotting. Respective blots were stripped and reprobed for total GSK-3 β and β -catenin levels. *Middle*, representative blots showing p-GSK-3 β (S9) and active β -catenin levels after control antibody or 1A6 treatment. *Right*, quantitative analysis of active β -catenin levels after 1A6 antibody treatment for 24 h ($P = 0.0006$) as determined by calculating the ratio between the active β -catenin and total β -catenin levels from three separate experiments. *Columns*, mean; *bars*, SE. *B*, representative blots showing β -catenin phosphorylation on S33/S37/S45 and T41 in cells pretreated with MG132 (1 $\mu\text{mol/L}$) for 4 h followed by treatment with either control or anti-FGF19 (1A6) antibody (both 20 $\mu\text{g/mL}$) for 24 h. Respective blots were stripped and reprobed for total β -catenin. *C*, a representative blot showing β -catenin ubiquitination levels in cells pretreated with MG132 (1 $\mu\text{mol/L}$) for 4 h followed by treatment with either control or anti-FGF19 (1A6) antibody (both 20 $\mu\text{g/mL}$) for 24 h as determined by immunoprecipitation and immunoblotting. *D*, indirect quantification of NH₂-terminal β -catenin phosphorylation levels using linear ion-trap MS. Data-dependent tandem MS on NH₂-terminal peptide from immunoprecipitated β -catenin from cells treated with MG132 followed by control antibody or 1A6 (*top*) was performed using a linear ion-trap instrument as described in Materials and Methods. Cross-correlation scores for each CID spectrum were generated and the relative abundance of peptides was determined. The data were normalized to the signal intensities of other nonrelated peptides that showed no difference in signal intensities from the treated and untreated samples (*bottom*).

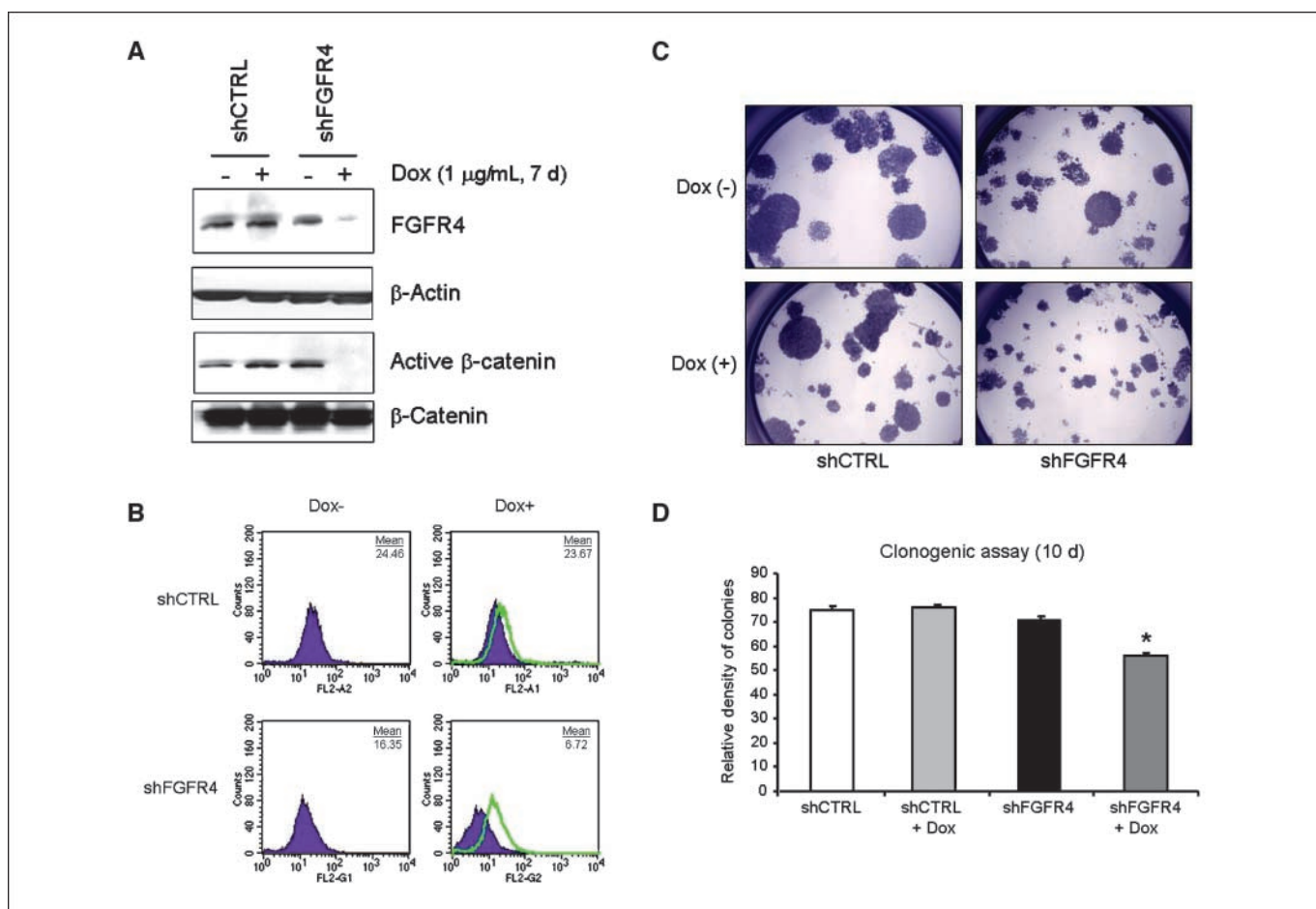


Figure 3. FGFR4 knockdown suppresses FGFR4 protein and active β -catenin expression and reduces colony formation *in vitro*. FGFR4 knockdown vectors were constructed by designing and cloning shRNA sequences into pShuttle-H1 inducible vector system and transferring the H1-shRNA cassette into pHUSH-GW by a Gateway (Invitrogen) recombination reaction. The cDNAs were transfected and stable cells were generated using puromycin in HCT116 cells. shCTRL and shFGFR4 stable cells were treated with or without doxycycline (Dox; 1 μ g/mL) and FGFR4 and active β -catenin levels were determined by immunoprecipitation and immunoblotting. **A**, representative blots showing FGFR4 and active β -catenin levels from three separate experiments. **B**, FACS analysis of anti-FGFR4 antibody binding to shCTRL and shFGFR4 stable cells treated with doxycycline (green) and without doxycycline (solid blue). **C**, shCTRL and shFGFR4 stable cell clones (100 cells per well) were seeded in 96-well culture plates in the presence and absence of doxycycline (1 μ g/mL) and incubated for 10 d. Colonies were stained in 0.5% crystal violet. Representative images of stained colonies from each group studied. **D**, quantitative analysis of relative density of stained colonies in a fixed area from a replicate of 10 wells. Columns, mean; bars, SE. *, P values shCTRL-Dox (-) vs shCTRL-Dox (+) = 0.0824; shCTRL-Dox (-) vs shFGFR4-Dox (-) = 0.334; shCTRL-Dox (-) vs shFGFR4-Dox (+) = 0.0097; shCTRL-Dox (+) vs shFGFR4-Dox (+) = 3.1124E-07; shFGFR4-Dox (-) vs shFGFR4-Dox (+) = 0.0005.

increased phosphorylation on the NH₂ terminus of β -catenin in 1A6-treated cells.

FGFR4 knockdown using shRNA significantly reduces active β -catenin levels and decreases clonogenicity *in vitro*. To determine whether inhibition of FGFR4 receptor would mimic the effect of FGF19 inhibition with 1A6 antibody, we analyzed FGFR4 and shCTRL HCT116 stable cell lines for active β -catenin levels. Quantification of relative density of FGFR4 protein expression levels in shCTRL stable cells did not show any significant change with (261.6 ± 17.5) and without (261.75 ± 11.1) doxycycline treatment. However, FGFR4 expression levels were significantly reduced in doxycycline-treated shFGFR4 stable cells (163.25 ± 7.4 , $P = 0.003$). Non-doxycycline-treated shFGFR4 stable cells also showed a slight reduction (230.88 ± 17.3) in FGFR4 expression, indicating some leakiness (Fig. 3A). FACS analysis for FGFR4 in doxycycline-treated shFGFR4 stable cells showed a significant reduction in FGFR4 expression versus doxycycline-treated shCTRL stable cells or shFGFR4 without doxycycline

treatment (Fig. 3B). Leakiness of the TetR-IRES cassette was also apparent with FACS analysis. Immunoblot analysis of cell lysates from shFGFR4 stable cells (that showed effective FGFR4 expression knockdown) showed a significant decrease in active β -catenin levels when compared with shCTRL stable cells (Fig. 3A), clearly indicating involvement of FGF19 in modulating β -catenin signaling in colon cancer cells. Similar reduction in active β -catenin levels were observed in other shFGFR4 clones (Supplementary Fig. S3). Furthermore, analysis of FRS2 phosphorylation in shCTRL and shFGFR4 stable cell clones shows that the steady-state phosphorylation levels are decreased in FGFR4 knockdown cells (shFGFR4 + doxycycline) when compared with controls (shCTRL \pm doxycycline and shFGFR4 without doxycycline treatment; Supplementary Fig. S4).

Next, to determine whether FGFR4 knockdown has any biological effect, we assessed cell proliferation and colony-forming ability using these stable cells. The rate of cell proliferation as determined by BrdUrd incorporation into cellular DNA only shows

a very modest change between shCTRL cells and shFGFR4 stable cells in the presence of doxycycline without reaching statistical significance (Supplementary Fig. S5). However, shFGFR4 stable cells treated with doxycycline show a significant reduction in colony-forming ability when compared with shCTRL stable cells treated with and without doxycycline ($P = 0.0015$ and 0.00001 , respectively; Fig. 3C and D). In addition to the larger number of colonies formed, the phenotype of colonies of shCTRL stables is more robust and larger in size than those of FGFR4 knockdown stable cells (Fig. 3C).

FGFR4 knockdown stable cells show reduced tumor growth *in vivo*. Because we found that 1A6 affects β -catenin signaling *in vitro*, we next investigated whether inhibition of the FGF19/FGFR4 pathway affects growth of tumor xenografts *in vivo* using shFGFR4 stable cells. As determined by tumor volume measurements, FGFR4 knockdown significantly reduced tumor growth when compared with shCTRL stable controls (day 21: $62 \pm 6\%$

reduction, $n = 8$; Fig. 4A). Further analyses of tumor tissues for FGFR4 and active β -catenin levels showed a significant reduction in FGFR4 expression, FRS2 phosphorylation, and active β -catenin levels in shFGFR4 + doxycycline group versus shCTRL + doxycycline group (Fig. 4B). These results correlated well with the tumor volumes. This is also consistent with our recent study showing that 1A6 antibody treatment of colon cancer cell tumor xenografts (HCT116, Colo201) in athymic nude mice significantly reduces tumor growth versus control antibody treatment group and that this was associated with substantially reduced FGFR4 phosphorylation and active β -catenin levels in the 1A6-treated group versus control (24). Similar reduction in tumor growth was observed in animals inoculated with other clones of shFGFR4 stable cells and treatment with doxycycline (data not shown). Histologic analyses of ki67 nuclear antigen in tissue sections of xenograft tumors showed a significant decrease in the number of ki67-positive nuclei in FGFR4 knockdown tumors versus shCTRL

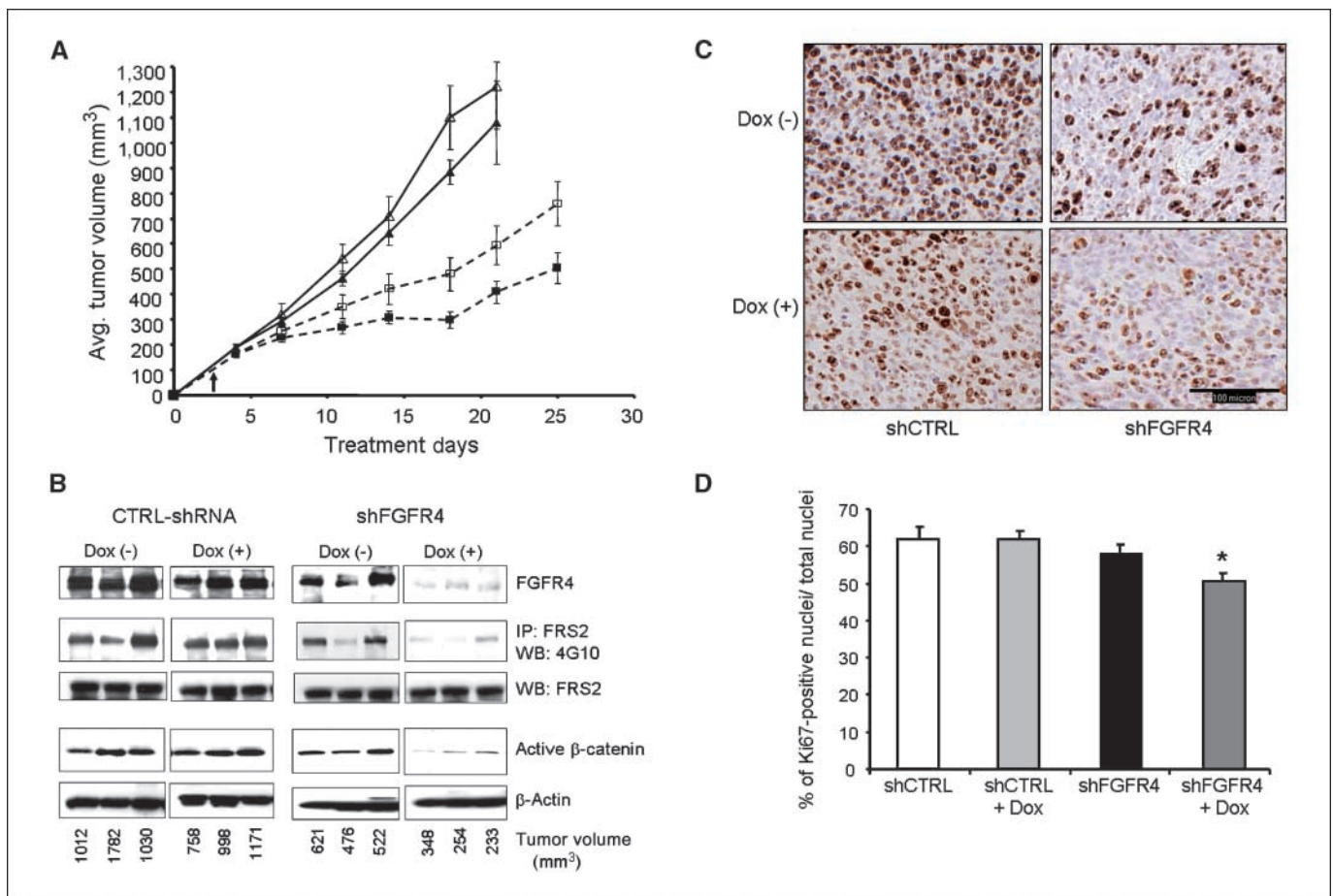


Figure 4. FGFR4 knockdown suppresses FGF19/FGFR4 signaling pathway and reduces tumor growth *in vivo*. **A**, athymic mice were s.c. inoculated with 5×10^6 shCTRL or shFGFR4 stable cells. Mice received 5% sucrose (\square , \triangle) only or 5% sucrose plus 1 mg/mL doxycycline (\blacksquare , \blacktriangle) for control (solid line) and FGFR4 knockdown (broken line) cohorts, respectively. Tumors were measured with calipers and mice were weighed twice a week. Eight mice were used for each treatment group. Points, mean tumor volume; bars, SE. P values: shCTRL-Dox (-) vs shCTRL-Dox (+) = 0.226; shCTRL-Dox (-) vs shFGFR4-Dox (+) = $1.00573E-06$; shCTRL-Dox (+) vs shFGFR4-Dox (+) = $4.17868E-06$; shFGFR4-Dox (-) vs shFGFR4-Dox (+) = 0.0152. **B**, shCTRL and shFGFR4 stable cells xenograft tissues treated with and without doxycycline were homogenized in modified RIPA buffer and analyzed for FGFR4 expression, FRS2 phosphorylation, and active β -catenin levels using immunoprecipitation and immunoblotting as detailed in Materials and Methods. Representative blots showing FGFR4 expression, FRS2 phosphorylation, total FRS2 levels, active β -catenin, and total β -catenin levels in three randomly selected animal tissues from each treatment group. **C**, ki67 immunostaining of xenografted tumor tissue sections of shCTRL and shFGFR4 stable cell lines. Images of representative fields from shCTRL-Dox (-) (top left), shCTRL-Dox (+) (bottom left), shFGFR4-Dox (-) (top right), and shFGFR4-Dox (+) (bottom right) groups at $\times 100$ magnification. **D**, quantitative analysis of number of ki67-positive nuclei/negative nuclei ($n = 6$). Columns, mean; bars, SE. *, P values: shCTRL-Dox (-) vs shCTRL-Dox (+) = 0.978; shCTRL-Dox (-) vs shFGFR4-Dox (+) = 0.0170; shCTRL-Dox (+) vs shFGFR4-Dox (+) = 0.027; shFGFR4-Dox (-) vs shFGFR4-Dox (+) = 0.0644.

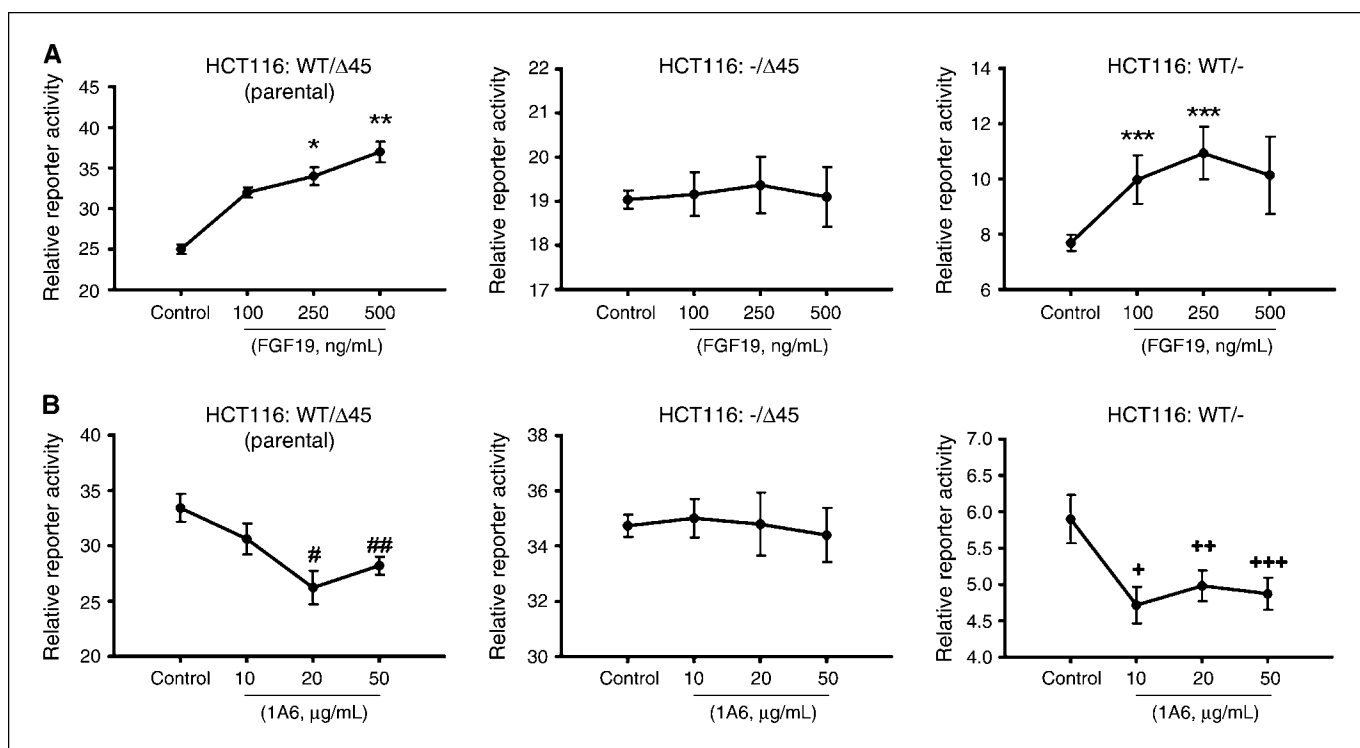


Figure 5. FGF19 increases β -catenin/TCF-4-regulated transcription activity and inhibition of endogenous FGF19 with 1A6 antibody reduces β -catenin/TCF-4-regulated transcription activity. HCT116 cells harboring either WT/ Δ 45 (parental), $-/\Delta$ 45, or WT/ $-$ β -catenin were cotransfected with the TOP/FOP glow, pCAN LEF1, Renilla reporter constructs and treated with and without FGF19 (100–500 ng/mL; A) or 1A6 antibody (20–50 μ g/mL; B) for 6 h. Luciferase activity was measured using the dual luciferase assay system and the mean TOP/FOP ratios were normalized to Renilla luciferase activity. Relative reporter activity from three separate experiments performed in duplicate. Points, mean; bars, SE. *P* values vs control: *, *P* = 0.005; **, *P* = 0.018, both ***, *P* = 0.05. #, *P* = 0.009; ##, *P* = 0.008; +, *P* = 0.022; ++, *P* = 0.037; +++, *P* = 0.025.

tumors. Non-doxycycline-treated shFGFR4 group also showed a small reduction in ki67-positive nuclei versus shCTRL group (Fig. 4C and D). This is consistent with our FACS and immunoblotting data that indicate leakiness of TetR-IRES cassette. Immunoreactivity for cleaved caspase-3 did not show any significant change between any groups studied (Supplementary Fig. S6).

FGF19 induces β -catenin/TCF-4-regulated transcriptional activity. To ascertain whether FGF19 activates β -catenin/TCF-4-regulated transcription activity, we assessed TCF/LEF reporter activity in response to FGF19 using HCT116-WT/ Δ 45 (parental) and its $-/\Delta$ 45 and WT/ $-$ derivatives (23). Interestingly, FGF19 treatment significantly increased TCF/LEF reporter activity in parental and WT/ $-$ cells but not in mutant ($-/\Delta$ 45) derivative (Fig. 5A). Likewise, inhibition of endogenous FGF19 with 1A6 antibody significantly reduced the TCF/LEF reporter activity in parental and WT/ $-$ cells but not in mutants ($-/\Delta$ 45; Fig. 5B), indicating that FGF19 modulates mostly WT β -catenin. To further confirm whether FGFR4 signaling affects β -catenin transcriptional activity, we used HEK293 stably overexpressing FGFR4 (inset in Supplementary Fig. S7) and determined TCF/LEF reporter activity. Activation of FGFR4 with either FGF1 or FGF19 induced TCF/LEF reporter activity when compared with untreated controls (Supplementary Fig. S8). FGF1 or FGF19 treatment did not induce any reporter activity in control vector-transfected HEK293 stable cells versus untreated control (Supplementary Fig. S7).

Inhibition of FGF19/FGFR4 reduces β -catenin target gene expression *in vitro* and *in vivo*. Because FGF19 inhibition reduced active β -catenin levels and increased targeting β -catenin

for ubiquitination and proteasomal degradation, we next sought to determine whether 1A6 affects β -catenin target gene expression levels using real-time quantitative PCR. As shown in Fig. 6, 1A6 treatment consistently reduced cyclin D1, CD44, c-jun, Cox-2, and UPAR mRNA expression levels at 6 hours when compared with the control antibody-treated cells. Similar results were obtained from shRNA xenograft tissues showing reduced β -catenin target gene expression in FGFR4 knockdown group versus control (Supplementary Fig. S8).

Discussion

Increasing evidence suggests that functional cross-talk exists between regulatory and signaling molecules. Our studies explore the potential involvement of FGF19 and its receptor FGFR4 in the regulation of the E-cadherin/catenin system. In the present study using colon cancer cells (HCT16, Colo201), we examined the effect of FGF19 and the effect of anti-FGF19 blocking antibody on E-cadherin and β -catenin binding and the activation of β -catenin signaling both *in vitro* and *in vivo*.

β -Catenin is downstream in the Wnt signaling pathway and plays important roles in the structural organization and function of cadherins by linking cadherins to the actin cytoskeleton through α -catenin (30, 31). The formation and maintenance of stable cell-cell adhesion is dependent on the association E-cadherin with β -catenin and α -catenin and loss of E-cadherin expression or function in epithelial carcinomas is associated with the loss of cell-cell adhesion and of tumors to invasive and/or metastatic state

(32). Moreover, the regulation of cadherin-mediated adhesion is suggested to be regulated by tyrosine phosphorylation/dephosphorylation of β -catenin (5). In the present study, we found that exogenous FGF19 increases tyrosine phosphorylation of β -catenin and this was inversely proportional to the loss of E-cadherin- β -catenin binding in HCT116 cells, indicating that FGF19 could possibly potentiate colon tumor growth and metastasis. Ezzat and co-workers have shown that treatment of mice bearing GH4 pituitary tumor xenografts with FGFR4 tyrosine kinase inhibitor results in recovery of membranous N-cadherin and reduces tumor growth, suggesting that FGFR4-N-cadherin plays an important role in neoplastic cell growth and invasiveness (33). In contrast, El-Hariry and co-workers have shown that FGF1 and FGF2 enhance association of catenins with E-cadherin in a panel of pancreatic adenocarcinoma cell lines (34), indicating that FGFs could elicit a cell-type-specific response.

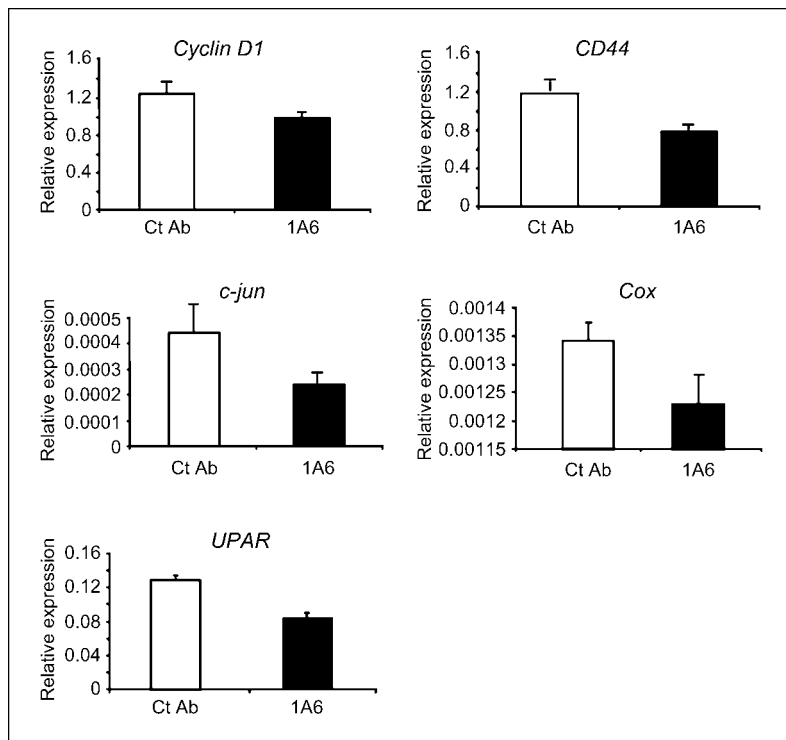
In normal cells, β -catenin is phosphorylated and subsequently ubiquitinated and degraded by the 26S proteasomal system and this degradation is initiated by phosphorylation at S45, T41, S37, and S33 at its NH₂-terminal region (35, 36). When Wnt signaling is inactive, β -catenin forms a complex with casein kinase 1 α (CKI α), GSK-3 β , and a scaffold protein Axin, resulting in CKI α -mediated phosphorylation of β -catenin S45 and this phosphorylation at S45 serves as a priming phosphorylation event for GSK3 β (37–39). The HCT116 cell line expresses FGF19, FGFR4, full-length APC, p53, and heterozygous mutations of β -catenin, harboring both wild-type and deletion mutant (Ser⁴⁵) forms of β -catenin (40). The Colo201 cell line has both somatic APC mutations and β -catenin mutations and the mutated (A-to-G in codon 287) sequence in β -catenin is believed not to conform to any known consensus phosphorylation site (41). As reported earlier, we found both parental (WT/ Δ 45) and mutant β -catenin ($-/\Delta$ 45) cells having higher β -catenin/TCF4-regulated transcription activity when compared with WT/ $-$ cells

(23). Moreover, FGF19 substantially increased the TCF/LEF reporter activity only in HCT116 parental (WT/ Δ 45) and WT/ $-$ cells but not in mutant ($-/\Delta$ 45) derivative. This was also consistent with 1A6 treatment reducing TCF/LEF reporter activity in HCT116 parental (WT/ Δ 45) and WT/ $-$ cells without having any effect on mutant ($-/\Delta$ 45) cells, indicating that WT β -catenin is accessible for modulation in these cells. In addition, our experiments in Colo201 cells showing that β -catenin (S33/S37) is phosphorylated upon 1A6 antibody treatment and dephosphorylated after FGF19 stimulation find support from a previous study showing β -catenin transcriptional activation in these cells through the dephosphorylation of S37/T41 residues in the NH₂ terminus of β -catenin (42).

The present study also elucidated the mechanism of 1A6-inhibitory effect on β -catenin signaling by showing that 1A6 targets β -catenin for proteasomal degradation by increased Ser/Thr phosphorylation in the NH₂-terminal region of β -catenin and ubiquitination of β -catenin using both immunoblotting and ion-trap MS. Although Wang and co-workers have shown that in HCT116 cells, phosphorylation of β -catenin at S45 is not required for phosphorylation of residues S33/S37 or T41 (43), our data showing 1A6 increases β -catenin ubiquitination in parental HCT116 cells (WT/ Δ 45) and that 1A6 treatment does not affect TCF/LEF reporter activity in mutant ($-/\Delta$ 45) cells indicate that the mutant form is unlikely to be ubiquitinated and proteasomally degraded.

FGFR4 knockdown using shRNA significantly reduced active β -catenin levels *in vitro* and *in vivo*, further validating the effect of FGF19/FGFR4 pathway inhibition on β -catenin. In the present study (*in vitro*), we found that FGFR4 knockdown has very little effect on cell proliferation but has a substantial effect on clonogenicity. In addition, our immunohistochemistry data show that FGFR4 knockdown-xenograft tumor tissues exhibit reduced ki67-positive nuclei, but show no change in cleaved caspase-3

Figure 6. Anti-FGF19 antibody reduces β -catenin target gene transcription levels in colon cancer cells. HCT116 cells were grown in the presence of serum and treated with either control or 1A6 antibody (20 μ g/mL) for 6 h. Relative expression levels of β -catenin target genes (*cyclin D1*, *CD44*, *c-jun*, *Cox-2*, and *UPAR*) were analyzed by Taqman analysis. Analyses of data were performed using Sequence Detector 1.6.3 (PE Applied Biosystems) and results were normalized to RPL19.



levels. These results suggest that the reduced tumor growth in the FGFR4 knockdown group could be due to reduced clonogenic ability rather than increased apoptosis. The shRNA stables used in the xenograft studies were derived from parental HCT116 cells (WT/ Δ 45) and the data showing that 1A6 has no effect on mutant β -catenin-mediated TCF/LEF transcriptional activity suggest that the inability of FGFR4-shRNA to completely inhibit tumor growth *in vivo* is mostly due to the presence of mutant β -catenin.

In addition, our data show a steady-state level of FRS2 phosphorylation in the xenografted tumors and in shCTRL stable cells (*in vitro*) and inhibition of FGF19 signaling with either 1A6 antibody (24) or FGFR4 knockdown with shRNA reduces this FRS2 phosphorylation level, indicating an autocrine regulation of the FGF/FGFR4 loop. Our recent study showed that in addition to reduced active β -catenin levels, FRS2 and extracellular signal-regulated kinase 2 (ERK2) phosphorylation were also decreased in 1A6-treated xenograft tumor tissues (24), indicating possible involvement of more than one signaling pathway. Nevertheless, earlier studies have also reported cross-regulation of Wnt signaling pathway by mitogen-activated protein kinases (44, 45). Therefore, more studies are under way to ascertain the possibility of FRS2-ERK-mediated priming of GSK3 β during FGF19/FGFR4-mediated activation of β -catenin signaling pathway.

Although several lines of evidence suggest that Wnt regulate FGF genes (including *FGF16*, *FGF18*, *FGF20*), whether β -catenin regulates FGF19 remains unknown (4, 6, 46, 47). Inhibition of β -catenin using siRNA completely abolished FGF19 expression in HCT116 cells,⁴ indicating that β -catenin regulates FGF19 expres-

sion in colon cancer cells. Sporadic reports suggest that FGFs regulate Wnt-target genes (48, 49). In support of our data showing reduced active β -catenin levels and TCF/LEF reporter activity, we have shown that blocking FGF19/FGFR4 signaling also significantly reduces β -catenin target genes (*cyclin D1*, *CD44*, *c-jun*, *Cox-2*, and *UPAR*) *in vitro* and *in vivo*. Using more than one cell line, we show that FGF19 modulates β -catenin signaling pathway both *in vitro* and *in vivo*, therefore suggesting that FGF19/FGFR4-mediated activation of β -catenin signaling is not a cell type-specific response. Our studies showing a molecular link between FGF19 and β -catenin signaling pathway is further supported by an earlier report showing that Wnt signaling is attenuated in FGFR1 $^{-/-}$ mice (50). Taken together, our present findings suggest that the inactivation of FGF19 and/or FGFR4 could be beneficial in rescuing cells from deregulated signaling of β -catenin and therefore for the treatment of colon cancer and other malignancies involving interaction of FGF19 and FGFR4.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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⁴ R. Pai, D.M. French, unpublished observation.

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