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Wnt/β-Catenin Pathway Activation Mediates Adaptive Resistance to BRAF Inhibition in Colorectal Cancer

Guangming Chen[1,2], Chenxi Gao[1,2], Xuan Gao[1,2], Dennis Han Zhang[3], Shih-Fan Kuan[4], Timothy F. Burns[2,5], and Jing Hu[1,2]

Abstract

One of the most encouraging developments in oncology has been the success of BRAF inhibitors in *BRAF*-mutant melanoma. However, in contrast to its striking efficacy in *BRAF*-mutant melanomas, BRAF inhibitor monotherapy is ineffective in *BRAF*-mutant colorectal cancer. Although many studies on BRAF inhibitor resistance in colorectal cancer have focused on mechanisms underlying the reactivation of the EGFR/RAS/RAF/MEK/ERK pathway, the current study focuses on identifying novel adaptive signaling mechanisms, a fresh angle on colorectal cancer resistance to BRAF inhibition. We found that treatment with BRAF inhibitors (both current and next-generation BRAF inhibitors) upregulated the Wnt/β-catenin pathway in $BRAF^{V600E}$-mutant colorectal cancer cell lines through activating the cytoplasmic tyrosine kinase focal adhesion kinase (FAK). The results showed that FAK activation upon BRAF inhibitor treatment did not require EGFR or ERK1/2 activation, implying that BRAF inhibitor treatment-induced hyperactivation of Wnt signaling is "pathway reactivation"-independent. BRAF inhibition–induced Wnt pathway activation was further validated in preclinical models of $BRAF^{V600E}$-mutant colorectal cancer, including cell line xenograft model and a patient-derived xenograft model. Combined inhibition of BRAF/Wnt pathways or BRAF/FAK pathways exerted strong synergistic antitumor effects in cell culture model and mouse xenograft model. Overall, the current study has identified activation of the Wnt/β-catenin pathway as a novel fundamental cause of colon cancer resistance to BRAF inhibition. Our results suggest that although complete vertical pathway blockade is pivotal for effective and durable control of *BRAF*-mutant colorectal cancer, cotargeting parallel adaptive signaling—the Wnt/β-catenin pathway—is also essential. *Mol Cancer Ther; 17(4); 806–13. ©2017 AACR.*

Introduction

Approximately 8% to 15% of all colorectal cancer patients harbor activating mutations (mostly V600E mutation) of BRAF, a serine/threonine kinase that is downstream of KRAS and immediate upstream of MEK (1). Among subtypes of colorectal cancer, *BRAF*-mutant colorectal cancers are less responsive to chemotherapy, do not benefit from anti-EGFR therapy or BRAF inhibitor treatment, and have the highest mortality (2, 3). Adaptive pathway reactivation (reactivation of the EGFR/RAS/MEK/ERK pathway) through various mechanisms including feedback activation of EGFR contributes to *de novo*/acquired resistance in BRAF-mutant colorectal cancers (4). However, because these mechanisms are also involved in melanomas resistance to $BRAF^{V600E}$ inhibition (5), we believe the difference in the clinical results seen in melanoma patients versus in colorectal cancer patients could not solely relate to these mechanisms. Consistent with this, recent clinical study showed that the overall response rate to the triple EGFR/BRAF/MEK inhibition was 26% in *BRAF*-mutant colorectal cancer patients (6). Comparing with the 60% to 80% response rate in melanoma patients (7), there is still large room to improve, suggesting that the best possible treatment outcome cannot be realized by vertical pathway targeting only. The current study has uncovered a novel parallel "bypass" signaling mechanism that is independent of MAPK pathway reactivation: FAK-dependent hyperactivation of the Wnt/β-catenin pathway. Our results indicate that the Wnt/β-catenin pathway activation is a central driver of BRAF inhibitor resistance in colorectal cancer and that targeting of BRAF/Wnt pathways represents a novel effective approach to overcome BRAF inhibitor resistance in colorectal cancer patients.

Materials and Methods

Cell lines and cell culture

All cell lines were obtained from the ATCC prior to all experiments. To ensure authentication and consistency throughout the study, only low-passage cells (< passage 5–8) were used in the experiments. Cells were cultured in a 37°C humidified incubator containing 5% CO_2. HEK293T, SW48, HT29, RKO, and WiDr cells

[1]Department of Pharmacology and Chemical Biology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania. [2]University of Pittsburgh Cancer Institute, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania. [3]Dietrich School of Arts and Sciences, University of Pittsburgh, Pittsburgh, Pennsylvania. [4]Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania. [5]Division of Hematology/Oncology, Department of Medicine, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania.

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G. Chen and C. Gao contributed equally to this article.

Corresponding Author: Jing Hu, University of Pittsburgh Cancer Institute, 5117 Centre Avenue, Suite 2.42D, Pittsburgh, PA 15213. Phone: 412-623-2227; Fax: 412-623-7828; E-mail: huj3@upmc.edu

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were cultured in DMEM medium. DLD-1 cells and COLO741, LS411N, and COLO205 cells were cultured in RPMI-1640 medium. All cells were supplemented with 5% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin. Cell lines were authenticated using a short tandem repeat DNA profiling from the cell bank from which they were acquired. Cell lines were authenticated when initially acquired and every 6 months using a commercial vendor. Cell lines were tested for mycoplasma every 6 months using the MycoAlert Detection Kit (Lonza).

Western blot, antibodies, and reagents

Whole-cell lysates were prepared in lysis buffer (20 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1 mmol/L EDTA, 1% NP40, and 10% Glycerol), resolved by SDS-PAGE, and blotted with indicated antibodies. The following antibodies were used in this study: Anti–β-catenin, anti-FAK, anti–phospho-FAK (Tyr397), anti-ERK1/2, anti-SRC and anti–Integrin β1 (Cell Signaling Technology); anti-GSK3α/β, anti–phospho-GSK3α/β (Tyr279/216), anti–phospho-ERK1/2, anti–β-Actin, and anti-SP1 (Santa Cruz Biotechnology). SuperSignal West Pico Chemiluminescent Substrate and SuperSignal Western Blot Enhancer (Thermo Fisher Scientific) were used to enhance western signal when needed. Vemurafenib, dabrafenib, GDC-0879 (8), erlotinib, SCH772984 (9), LY3009120 (10), and ICG-001 (11) were purchased from Selleck Chemicals. PF-562271 (12) was purchased from MedKoo Biosciences. PLX7904 (13) was obtained from Plexxikon. Gelucire is a gift from Gattefosse. NE-PER Nuclear and Cytoplasmic Extraction Reagents from Thermo Fisher Scientific were used for subcellular fractionation according to the manufacturer's protocol.

Plasmid, transient transfection, lentivirus production, and infection

The murine FAK expression plasmid (pEGFP-C1-FAK-HA) was kindly provided by Dr. David D. Schlaepfer (University of California San Diego). Plasmid transient transfections were performed using PolyJet In Vitro DNA Transfection Reagent (SignaGen Laboratories) according to the manufacturer's instruction. Lentiviral-based shRNA plasmids were purchased from Sigma-Aldrich. Lentiviruses encoding shRNA for specific gene were produced in HEK293T cells by transfection of the lentiviral vector expressing shRNA with the third-generation packaging systems (Addgene). The media containing viral particles were filtered through syringe filters and subsequently used to infect target cells. Cell lines that stably expressed shRNA were established by puromycin selection.

Cell proliferation assays

A total of 3×10^3 cells were seeded per well into 96-well plates and incubated for 1 to 2 days before inhibitor treatment. Thereafter, the medium was changed, and the individual inhibitor or their combinations were added at the indicated concentrations. After 3-day treatment, cell viabilities were measured by a CCK-8 kit (Sigma-Aldrich) according to the manufacturer's protocol for 3 consecutive days. IC_{50} was calculated using excel. Cell growth rates were calculated as percentage of DMSO-treated control. The combined index (CI) was calculated according to the equation of $CI = DA/IC_{50}\ A + DB/IC_{50}\ B$. IC_{50} A and IC_{50} B are the concentrations of two inhibitors used alone to achieve the 50% growth inhibition, whereas DA and DB are the concentrations of two inhibitors used together to achieve the 50% growth. Strong synergism was defined as CI < 0.4. Each experiment was performed in triplicates and repeated at least 3 times.

Colony formation assay

HT-29 cells and LS411N cells were seeded at 2,500 cells/dish into 35 mm dishes 1 day prior to treatment. Inhibitors were added at the indicated concentrations. Cells were allowed to grow for 10 days with medium containing inhibitors replenished every 3 days. Colonies were visualized by crystal violet staining.

Animal experiments

Cell line xenograft experiments. All animal procedures were carried out according to protocols approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh. Mice were fed a standard diet (Purina LabDiet, diet ID 5P75). HT-29 cells were injected (3×10^6 cells per site) subcutaneously into both flanks of 4- to 5-week-old male athymic nude mice (Charles River Laboratories). Once tumors volume reached approximately 50 mm^3, mice were randomized into different groups and treated with indicated inhibitors. The following inhibitor concentrations were used in these experiments: Vemurafenib (75 mg/kg), PF562271 (30 mg/kg), ICG-001 (100 mg/kg), Trametinib (0.3 mg/kg). All inhibitors were formulated in 5% gelucire. For study evaluating therapeutic efficacy, the mice were treated with individual inhibitors or their combinations as indicated twice daily by oral gavage for 4 days per week for up to 10 weeks as indicated. The tumor volume was assessed by caliper measurements every week. For studying the molecular effect of inhibitors on tumor xenograft, mice were given indicated inhibitors twice daily by oral gavage for 4 consecutive days and sacrificed 96 hours after first drug administration. The tumors were then collected for Western blot.

PDX experiment. This experiment was conducted by Crown Bioscience. The protocol involving the care and use of animals in this study was approved by the Institutional Animal Care and Use Committee of Crown Bioscience. The primary human colorectal cancer xenograft model (CR2506, $BRAF^{V600E}$-mutant) fragment (2–3 mm in diameter) was inoculated subcutaneously at the right flank of each mouse. When the tumor volume reached 200 to 400 mm^3, mice was randomized into 3 groups and treated with vehicle or vemurafenib (75 mg/kg) for 4 days, twice daily by oral gavage. The mice were then sacrificed 96 hours after first drug administration. The tumors were collected, fixed in formalin, and then embedded in paraffin for further immunohistochemistry (IHC) experiments. The immunohistochemical analysis was performed as previously described (14). The following antibodies were used: anti–phosphor-FAK (Tyr397; Abcam; Cat# ab39967; 1:100 dilution); anti–β-catenin (Zymed; Cat# 18-0226; 1:200 dilution); anti-Sox9 (Cell Signaling Technology; Cat# 82630; 1:200 dilution). *In situ* hybridization (ISH) was conducted using the RNAscope 2.5 HD Reagent Kit and probe recognizing human LGR5 mRNA from Advanced Cell Diagnostics according to the manufacturer's protocols.

RNA extraction and quantitative RT-PCR analysis

Total RNA was extracted from untreated or vemurafenib-treated colon cancer cell lines using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. The DNase-treated RNA was reverse-transcribed using SuperScript III reverse transcriptase (Invitrogen). Quantitative PCR was carried out on the

MiniOpticon Real-Time PCR System (Bio-Rad Laboratories). The PCR thermal cycle conditions were as follows: denature at 95°C for 2 minutes and 40 cycles for 95°C, 15 seconds; 60°C, 1 minute. Melting curve analysis was performed to ensure the specificity of the PCR products. *β-Actin* was selected as internal reference gene. The following primers were used: *JAG1*, 5′-TCGCTGTATCTGTCCACCTG-3′ (forward) and 5′-AGTCACTGGCACGGTTGTAG-3′ (reverse); *DKK1*, 5′-CTCGGTTCTCAATTCCAACG-3′ (forward) and 5′-GCACTCCTCGTCCTCTG-3′ (reverse); *Axin2*, 5′-CAAGGGCCAGGTCACCAA-3′ (forward) and 5′-CCCCCAACCCATCTTCGT-3′ (reverse); *MMP7*, 5′- TGTATGGGGAACTGCTGACA-3′ (forward) and 5′-GCGTTCATCCTCATCGAAGT-3′ (reverse); *HIG2*, 5′-GTTGTGTGGGTGGGCTGT-3′ (forward) and 5′-GGTGGCCACAATGTCCATA-3′ (reverse); *β-actin*, 5′-TTGTTACAGGAAGTCCCTTGCC-3′ (forward) and 5′-ATGCTATCACCTCCCCTGTGTG-3′ (reverse).

Luciferase reporter gene assay

Cells were seeded into 24-well plate 1 day prior to transfection. Super 8x TOPFlash or Super 8x FOPFlash (Addgene) was cotransfected with pRL-TK into the cells. Twenty-four hours after transfection, the cells were treated with Vemurafenib or DMSO for indicated time points. The luciferase assays were performed using the dual-luciferase reporter assay system (Promega) according to the manufacturer's instructions. Renilla luciferase activity was used as an internal control to normalize the firefly luciferase activity. Each assay was performed in triplicate and repeated at least 3 times. Data represent the mean ± SD of the relative ratio of TOP/FOP.

Statistical analysis

Data are presented as mean ± SD. The difference between two groups was evaluated using the Student *t* test (two tailed). The *P* values less than 0.05 were considered statistically significant.

Results and Discussion

BRAF inhibitor treatment hyperactivates the Wnt/β-catenin pathway in *BRAF*-mutant colorectal cancer cells

We approached the BRAF resistance problem from a previously unexplored angle: identifying BRAF inhibitor treatment-triggered molecular events that lead to opposing functional outcomes in melanoma cells versus colorectal cancer cells. Wnt/β-catenin signaling functions as a tumor suppressor in melanoma biology (15), and activation of the Wnt pathway is essential for the antitumor effects of BRAF inhibitors (16); however, in colorectal cancer, it is an established critical driving force behind tumor initiation and development. We found that within hours and sustained for days, treatment of *BRAF*-mutant HT-29 colorectal cancer cells with BRAF inhibitors (dabrafenib, GDC-0879, and vemurafenib) resulted in increase in β-catenin level in a time course–dependent manner (Fig. 1A). Our results showed that vemurafenib treatment–mediated induction of total and nuclear β-catenin which correlates with β-catenin activity was dependent on *BRAF*-mutation status, but was independent of microsatellite instability, microsatellite stable, *APC* (adenomatous polyposis coli) mutation status, or the sex of the colorectal cancer cells (Fig. 1B; Supplementary Fig. S1A and S1B). We next performed a TOPFlash/FOPFlash assay (a luciferase reporter assay of β-catenin–mediated transcriptional activation) and real-time PCR analysis to further validate vemurafenib treatment–induced activation of the Wnt/β-catenin pathway. The results of the TOPFlash/FOPFlash assay showed that vemurafenib treatment strongly activated β-catenin transcriptional activity in *BRAF*-mutant cells but not in *BRAF*-WT cells (Fig. 1C, top), whereas the real-time PCR analysis confirmed that mRNA expressions of Wnt-target genes including *JAG1*, *DKK1*, *AXIN2*, *MMP7*, and *HIG2* were elevated in HT-29 cells (Fig. 1C, bottom). Treatment with Wnt pathway–specific inhibitor ICG-001 (ICG-001 antagonizes Wnt/β-catenin/TCF-mediated transcription by specifically binding to CREB-binding protein) sensitized *BRAF*-mutant colorectal cancer cells, but not *BRAF*-WT (DLD-1 cells) and RKO (β-catenin–independent) cells, to BRAF inhibitor treatment (Fig. 1D), suggesting that Wnt pathway activation is functionally significant. Together, the above findings provide the first evidence that BRAF inhibitor treatment activates the Wnt/β-catenin pathway in *BRAF*-mutant colorectal cancer cells.

BRAF inhibitor treatment upregulates Wnt/β-catenin signaling through FAK activation

We next explored how the Wnt/β-catenin pathway hyperactivation was achieved upon BRAF inhibition. We recently reported that focal adhesion kinase (FAK, a cytoplasmic tyrosine kinase) stabilizes β-catenin by phosphorylating glycogen synthase kinase 3α/β at tyrosine 279/216 (GSK3α/$\beta^{Y279/Y216}$; ref. 14). In this study, we found that treatment with vemurafenib or GDC-0879 time course-dependently activated FAK (represented as phosphorylated FAK at Y397) accompanied by increased levels of phosphorylated GSK3α/$\beta^{Y279/Y216}$ and β-catenin in *BRAF*-mutant but not WT colorectal cancer cells (Fig. 2A and Supplementary Fig. S2A). To confirm that it was activation of FAK that mediated the accumulation of β-catenin, we tested if inhibition of FAK prevents BRAF inhibitor–induced β-catenin increase. The results showed that shRNA knockdown of FAK (Fig. 2B) reduced basal levels of β-catenin and blocked BRAF inhibitor treatment-induced β-catenin accumulation. FAK-mediated activation of β-catenin signaling was further supported by the results of TOPFlash/FOPFlash assay showing that FAK knockdown strongly suppressed β-catenin–dependent transcriptional activity (Fig. 2C). Reconstitution of the FAK-knockdown HT-29 cells with murine FAK restored the level of β-catenin to the level comparable with WT cells (Supplementary Fig. S2B), thus confirming that FAK activation is responsible for the upregulation of the Wnt/β-catenin pathway. It has been reported that melanoma-associated fibroblasts can drive resistance to the BRAF inhibitor PLX4720 by stimulating matrix production/remodeling, and, consequently, survival signaling in melanoma cells via integrin β1, SRC, and FAK (17, 18). Cotargeting of FAK and BRAF inhibited ERK reactivation and synergized to suppress melanoma tumor growth in mice (17). Interestingly, however, FAK activation upon BRAF inhibitor treatment is independent of integrin β1 and SRC in *BRAF*-mutant colorectal cancer cells (Supplementary Fig. S2C). FAK inhibitor PF562271 exerted similar antitumor effect as IGC-001: treatment with PF562271 sensitized *BRAF*-mutant colorectal cancer cells, but not *BRAF*-WT (DLD-1 cells) and β-catenin–independent RKO cells, to BRAF inhibitor treatment (Supplementary Fig. S3), hence validating the functional significance of FAK activation in BRAF inhibitor resistance.

We tested whether the results seen *in vitro* can be extended to *in vivo*. The results of the HT-29 cell line xenograft experiments showed that BRAF inhibitor treatment substantially elevated β-catenin level, whereas treatment with FAK inhibitor PF562271

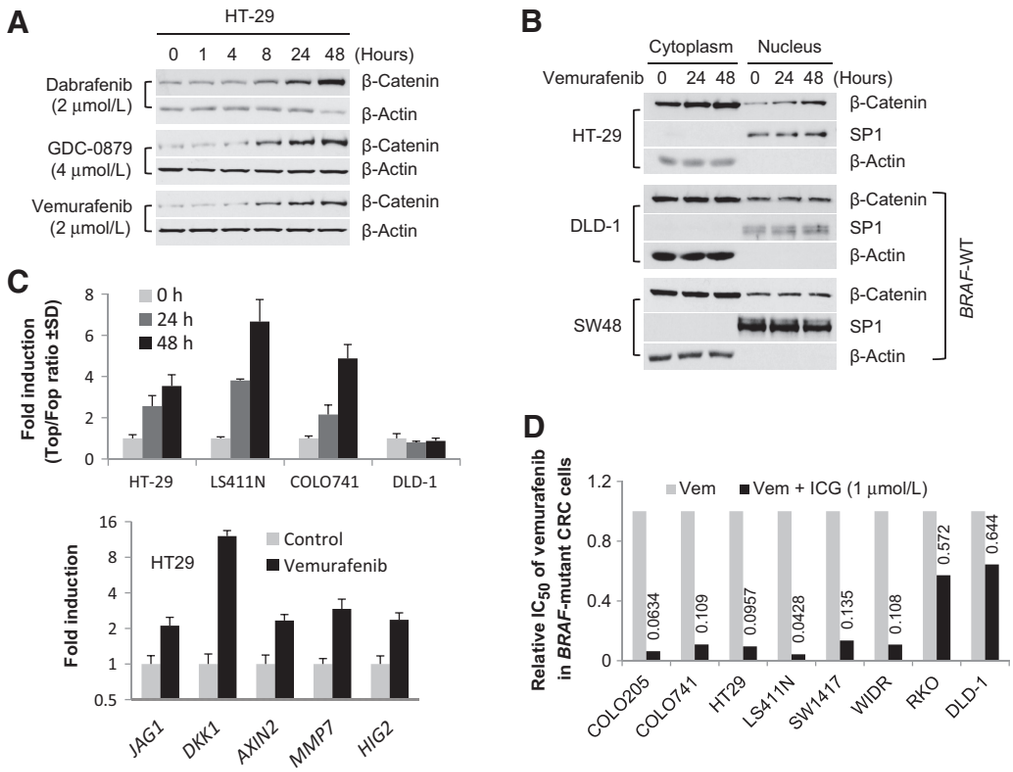

Figure 1.

BRAF inhibitors activate Wnt/β-catenin signaling in *BRAF*-mutant colorectal cancer cells. **A,** HT-29 cells were treated with BRAF inhibitors dabrafenib (2 μmol/L), GDC-0879 (4 μmol/L), or vemurafenib (2 μmol/L) for indicated times. The whole-cell lysates were used for Western blot with indicated antibodies. **B,** Cytoplasmic and nuclear fractions of colorectal cancer cells (*BRAF*-mutant or *BRAF*-wild type) treated with 2 μmol/L vemurafenib for indicated hours were used for immunoblotting with indicated antibodies. **C,** Top plot: Super 8x TOPFlash or Super 8x FOPFlash was cotransfected with pRL-TK into HT-29, LS411N, and COLO741 cells. Twenty-four hours after transfection, the cells were treated with 2 μmol/L vemurafenib for 24 or 48 hours. The luciferase assays were performed using the dual-luciferase reporter assay system. Data are presented as mean ± SD. Bottom plot: mRNA from HT-29 cells treated with 2 μmol/L vemurafenib for 48 hours was isolated for quantitative RT-PCR. β-Actin was used as a control. Data are presented as mean ± SD. **D,** IC_{50} of vemurafenib for indicated colorectal cancer cell lines was determined with CCK-8 assay in the presence or absence of 1 μmol/L β-catenin activation antagonist ICG-001.

completely blocked vemurafenib treatment–induced FAK activation and induction of β-catenin in xenograft tumors (Fig. 2D). We further examined FAK-mediated Wnt pathway activation in a patient-derived xenograft (PDX) model ($BRAF^{V600E}$ mutation and microsatellite stable). H&E staining results confirmed that the histology of the PDX tissues recapitulated the features of the histopathology of primary *BRAF*-mutant colorectal cancer tissues (Supplementary Fig. S4A). The results of the IHC and ISH showed that vemurafenib treatment elevated the levels of β-catenin (IHC), p-FAK^{Y397} (IHC), and the Wnt pathway targets SOX9 (IHC) and leucine-rich repeat-containing G-protein–coupled receptor 5 (LGR5; ISH) in PDX tumors (Supplementary Fig. S4A and S4B). Together, these results confirmed BRAF inhibition–induced FAK-dependent Wnt/β-catenin pathway activation *in vivo*. Of note, in contrast to FAK induction in the tumor stroma in melanoma (17), the current study found that FAK activation appeared to occur mainly in tumor cells.

Overexpression of cyclin D1, which is a well-known downstream target of Wnt/β-catenin pathway, mediated BRAF inhibitor resistance in *BRAF*-mutant melanoma and associated with *BRAF* mutation in colorectal cancer (19, 20). We tested whether vemurafenib could induce cyclin D1 expression. We found that cyclin D1 was slightly induced in vemurafenib-treated HT-29 cells (Supplementary Fig. S5). Treatment with FAK or β-catenin inhibitor overcame this induction, suggesting cyclin D1 induction as a potential downstream signaling of β-catenin activation that contributes to BRAF inhibitor resistance.

Activation of the Wnt/β-catenin pathway upon BRAF inhibitor treatment is independent of ERK pathway reactivation

Because ERK reactivation appeared to coincide with FAK activation in vemurafenib-treated HT-29 cells (Fig. 2A), we tested whether FAK activation is ERK1/2 reactivation dependent. The results showed that treatment of the cells with ERK inhibitor SCH772984, EGFR inhibitor erlotinib, or MEK inhibitor trametinib blocked ERK reactivation but failed to inhibit vemurafenib treatment–induced FAK activation, β-catenin accumulation, and β-catenin–dependent transcriptional activation (Fig. 3A and B), implying that FAK-mediated activation of the Wnt/β-catenin pathway upon BRAF inhibitor treatment is independent of MAPK pathway reactivation. Therefore, activation of Wnt/β-catenin pathway is a parallel "bypass" mechanism of BRAF inhibitor resistance in colorectal cancer.

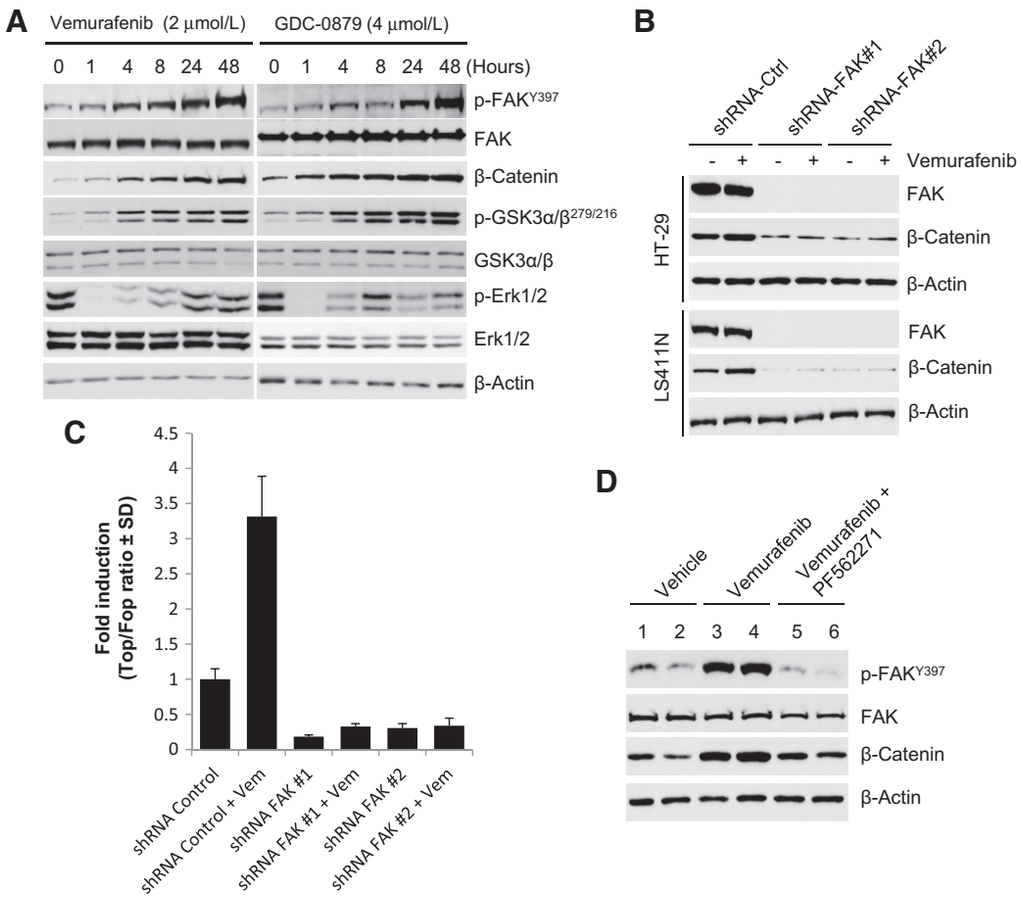

Figure 2.

FAK activation mediates BRAF inhibitor–induced Wnt pathway activation. **A,** The whole-cell lysates from HT-29 cells treated with vemurafenib (2 μmol/L) or GDC-0879 (4 μmol/L) for indicated times were used for immunoblotting with indicated antibodies. **B,** The whole-cell lysates from HT-29 or LS411N shRNA control cells or FAK knockdown cells treated with DMSO or 2 μmol/L vemurafenib for 24 hours were used for immunoblotting with indicated antibodies. **C,** Super 8x TOPFlash or Super 8x FOPFlash was cotransfected with pRL-TK into HT-29 cells. Twenty-four hours after transfection, the cells were treated with DMSO or 2 μmol/L vemurafenib for 24 hours. The luciferase assays were performed using the dual-luciferase reporter assay system. Data are presented as mean ± SD. **D,** Nude mice carrying HT-29 xenografts were treated with vehicle, vemurafenib (75 mg/kg), or a combination of vemurafenib and PF-562271 (30 mg/kg) twice daily by oral gavage for 4 days. The tumors were collected for Western blot with indicated antibodies.

Cotargeting of BRAF/Wnt pathways or BRAF/FAK pathways exerts synergistic antitumor effects *in vivo*

Consistent with the results that targeting Wnt pathway or FAK pathway sensitized *BRAF*-mutant colorectal cancer cells to BRAF inhibitor treatment (Fig. 1D and Supplementary Fig. S3), the combination of either a Wnt pathway inhibitor IGC-001(Fig. 4A and B) or a FAK inhibitor PF562271 (Fig. 4A and C) exerted strong synergistic antitumor effects with vemurafenib in *in vitro* cell culture model and in *in vivo* xenograft model. Combination of either IGC-001or PF562271 with vemurafenib abrogated colony formation of *BRAF*-mutant colorectal cancer cells (Supplementary Fig. S6). In HT29 xenograft model (Fig. 4B and C), triple inhibition of BRAF/MEK/Wnt pathway or BRAF/MEK/FAK leads to a sustained antitumor effect, validating the importance of complete vertical pathway blockade and simultaneous inhibition of colorectal cancer–specific parallel Wnt signaling.

The next generation of RAF inhibitors—paradox-breaking RAF inhibitors (13) and a pan-RAF inhibitor targeting both monomeric and dimeric RAF (10)—have recently been developed. These inhibitors possess much more potent MAPK inhibition activities and may provide enhanced safety and efficacy over first generation of BRAF inhibitors. We examined if treatment with these next generation of inhibitors also activates the Wnt/β-catenin pathway. The results showed that treatment with LY3009120 (a new pan-RAF inhibitor) or PLX7904 (a new paradox-breaking RAF inhibitor) did not induce ERK reactivation but activated FAK and induced β-catenin accumulation similarly as the current generation of BRAF inhibitors (Supplementary Fig. S7A and S7B). As anticipated, Wnt pathway inhibitor IGC-001 and FAK inhibitor PF562271 exerted strong synergistic inhibitory effects with these next-generation BRAF inhibitors (Supplementary Fig. S7C). Given these findings, we speculated that *BRAF*-mutant colorectal tumors will exhibit similar levels of resistance to second-generation BRAF inhibitors as to current inhibitors. BGB-283 is another recently developed next-generation RAF inhibitor (a novel RAF kinase and EGFR inhibitor). Although BGB-283 displayed potent antitumor activity in *BRAF*-mutant colorectal cancer in both cell line–derived and primary human

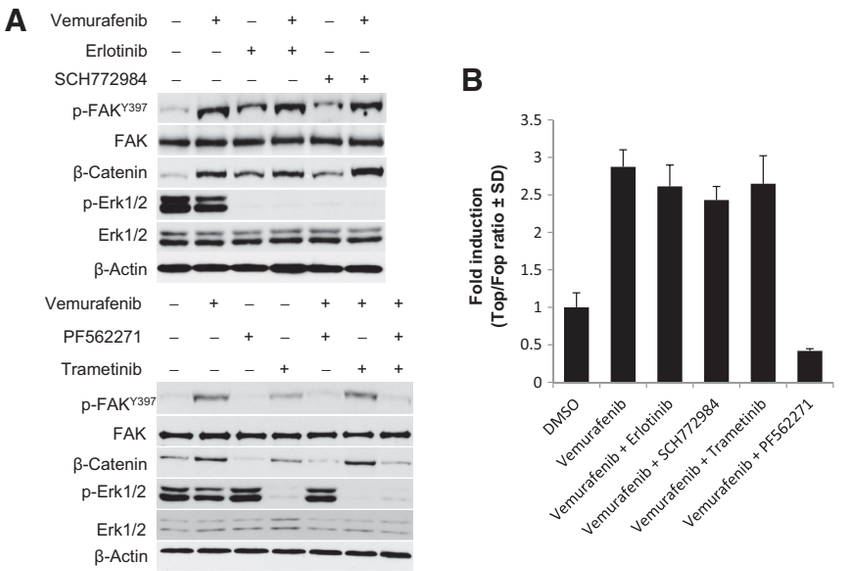

Figure 3.
FAK and Wnt pathway activation is independent of ERK pathway reactivation. The concentrations of inhibitors used in the experiments are as follows: BRAF inhibitor vemurafenib (2 μmol/L), EGFR inhibitor erlotinib (10 μmol/L), ERK inhibitor SCH772984 (1 μmol/L), MEK inhibitor trametinib (1 μmol/L), and FAK inhibitor PF562271 (2 μmol/L). **A,** The whole-cell lysates from HT-29 cells treated with indicated inhibitors alone or in combinations for 24 hours were used for immunoblotting with indicated antibodies. **B,** Super 8x TOPFlash or Super 8x FOPFlash was cotransfected with pRL-TK into HT-29 cells. Twenty-four hours after transfection, the cells were treated with indicated inhibitors alone or in combinations for 24 hours. The luciferase assays were performed using the dual-luciferase reporter assay system. Data are presented as mean ± SD.

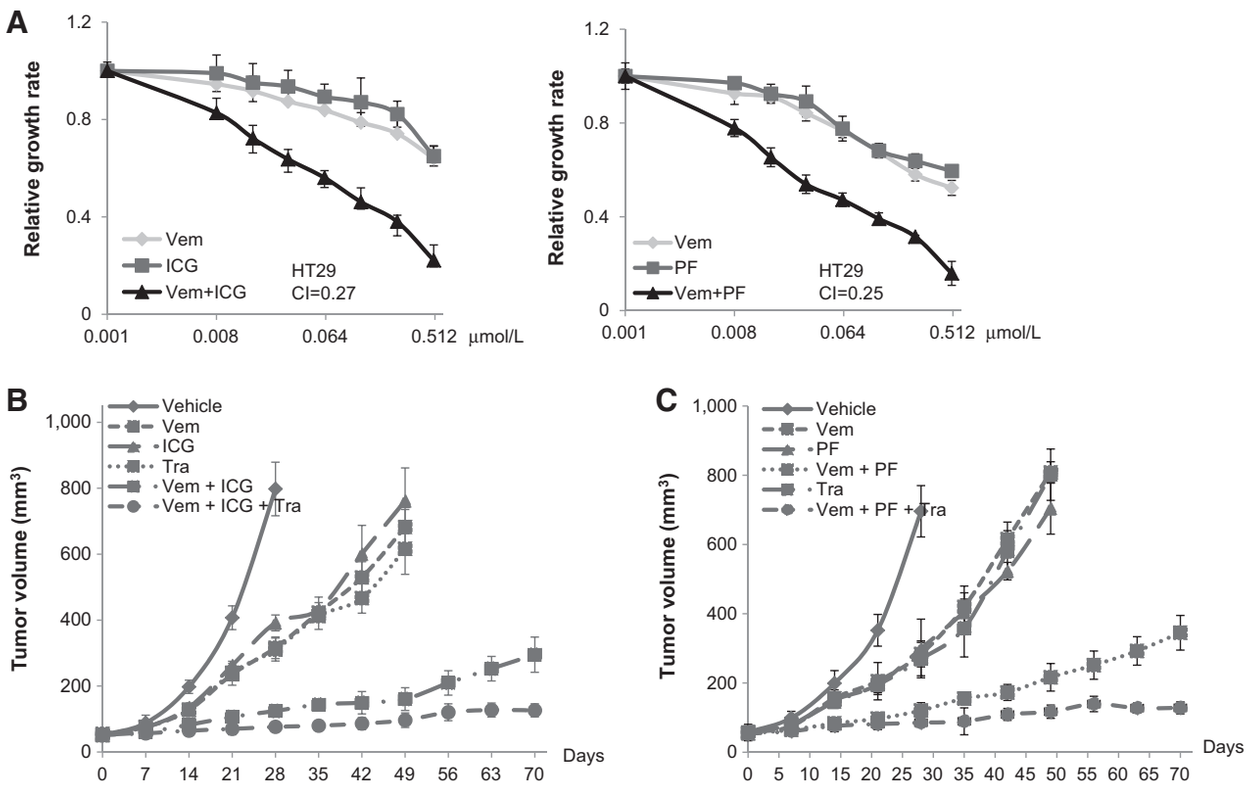

Figure 4.
In vitro and *in vivo* synergistic antitumor effects between BRAF inhibitor, Wnt pathway inhibitor, and FAK inhibitor. Vem, vemurafenib; ICG, ICG-001; PF, PF562271; Tra, trametinib. **A,** HT-29 cells were treated with indicated inhibitors alone or in combinations at various concentrations for 3 days. The maximum concentration for vemurafenib and PF562271 was 0.5 μmol/L, for ICG-001 was 5 μmol/L, and a serial twofold dilution was used for each inhibitor. Cells proliferation was measured by the CCK-8 assay. The calculation of CI values was described in Materials and Methods. Cell growth rates were calculated as percentage of control cells (DMSO treatment). Data was presented as mean ± SD. **B** and **C,** HT-29 cells (3×10^6 cells per site) were injected subcutaneously into both flanks of 4- to 5-week-old male athymic nude mice. When the xenografts volume reached approximately 50 mm^3, mice were randomized into different groups (5 mice per group) and administrated with vemurafenib (75 mg/kg), PF562271 (30 mg/kg), ICG-001 (100 mg/kg), trametinib (0.3 mg/kg), or in combination as indicated, twice daily by oral gavage for 4 days per week for up to 10 weeks. The tumor volume was assessed by caliper measurement every week. Values are mean ± SD.

colorectal tumor xenografts bearing $BRAF^{V600E}$ mutation (21), the most recent phase IB study (a phase IB study of RAF dimer inhibitor BGB-283 in patients with B-RAF or K-RAS/N-RAS mutated solid tumors by Jayesh Desai, AACR Annual Meeting 2017, http://webcast.aacr.org/console/player/34342?mediaType=audio&) confirmed our prediction that next generations of BRAF inhibitors, as monotherapy, are ineffective in the treatment of *BRAF*-mutant colorectal cancer patients, presumably due to the Wnt/β-catenin pathway activation.

How Wnt/β-catenin pathway hyperactivation contributes to BRAF inhibitor resistance remains unclear. Phenotypic plasticity plays an crucial role in the escape of cancer cells from targeted therapy (22). Given that cancer stem cell (CSC) plasticity confers therapeutic resistance and tumor recurrence (23) and that high Wnt activity functionally designates the colon CSC population (24), it is highly possible that hyperactivation of Wnt signaling upon BRAF inhibitor treatment enhances colon cancer stemness by stem cell pool increase, which in turn contributes to resistance to the therapy. The results showed that vemurafenib treatment elevated the levels of the Wnt pathway targets SOX9 and LGR5 in PDX tumors (Supplementary Fig. S4B). CSCs are a dynamic population continuously shaped by intrinsic and microenvironmental signals and thus are highly dependent on contextual conditions (25). LGR5 positivity is commonly used to define crypt stem cell and stem-like cells in colorectal cancer (26, 27). The increased numbers of LRG5-positive cells upon BRAF inhibitor treatment could suggest an increased stem cell pool, which could result from conversion of nonstem cells to CSCs driven by hyperactivated Wnt signaling and/or stem cell proliferation. Future studies are needed to further understand these potential mechanisms.

In summary, the current study has identified a novel "bypass" mechanism—Wnt/β-catenin pathway hyperactivation—as a potential root cause for BRAF inhibitor resistance in colorectal cancer. Cotargeting of FAK-dependent Wnt/β-catenin pathway activation could ultimately lead to new solutions to overcome BRAF resistance with great potential to dramatically change the outlook for *BRAF*-mutant colorectal cancer patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: G. Chen, C. Gao, T.F. Burns, J. Hu
Development of methodology: G. Chen, C. Gao, X. Gao, D.H. Zhang
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): G. Chen, C. Gao, X. Gao, D.H. Zhang, S.-F. Kuan
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): G. Chen, C. Gao, X. Gao, D.H. Zhang, T.F. Burns, J. Hu
Writing, review, and/or revision of the manuscript: C. Gao, J. Hu
Study supervision: C. Gao, J. Hu

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References

1. Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, et al. Mutations of the BRAF gene in human cancer. Nature 2002;417:949–54.
2. Phipps AI, Limburg PJ, Baron JA, Burnett-Hartman AN, Weisenberger DJ, Laird PW, et al. Association between molecular subtypes of colorectal cancer and patient survival. Gastroenterology 2015;148:77–87e2.
3. Sinicrope FA, Shi Q, Smyrk TC, Thibodeau SN, Dienstmann R, Guinney J, et al. Molecular markers identify subtypes of stage III colon cancer associated with patient outcomes. Gastroenterology 2015;148:88–99.
4. Corcoran RB. New therapeutic strategies for BRAF mutant colorectal cancers. J Gastrointest Oncol 2015;6:650–9.
5. Spagnolo F, Ghiorzo P, Queirolo P. Overcoming resistance to BRAF inhibition in BRAF-mutated metastatic melanoma. Oncotarget 2014;5:10206–21.
6. Atreya CE, Van Cutsem E, Bendell JC, Andre T, Schellens JH, Gordon MS, et al. Updated efficacy of the MEK inhibitor trametinib (T), BRAF inhibitor dabrafenib (D), and anti-EGFR antibody panitumumab (P) in patients (pts) with BRAF V600E mutated (BRAFm) metastatic colorectal cancer (mCRC). J Clin Oncol 33, 2015(suppl; abstr 103).
7. Flaherty KT, Puzanov I, Kim KB, Ribas A, McArthur GA, Sosman JA, et al. Inhibition of mutated, activated BRAF in metastatic melanoma. N Engl J Med 2010;363:809–19.
8. Hoeflich KP, Herter S, Tien J, Wong L, Berry L, Chan J, et al. Antitumor efficacy of the novel RAF inhibitor GDC-0879 is predicted by BRAFV600E mutational status and sustained extracellular signal-regulated kinase/mitogen-activated protein kinase pathway suppression. Cancer Res 2009;69:3042–51.
9. Morris EJ, Jha S, Restaino CR, Dayananth P, Zhu H, Cooper A, et al. Discovery of a novel ERK inhibitor with activity in models of acquired resistance to BRAF and MEK inhibitors. Cancer Discov 2013;3:742–50.
10. Peng SB, Henry JR, Kaufman MD, Lu WP, Smith BD, Vogeti S, et al. Inhibition of RAF isoforms and active dimers by LY3009120 leads to anti-tumor activities in RAS or BRAF mutant cancers. Cancer Cell 2015;28:384–98.
11. Emami KH, Nguyen C, Ma H, Kim DH, Jeong KW, Eguchi M, et al. A small molecule inhibitor of beta-catenin/CREB-binding protein transcription [corrected]. Proc Natl Acad Sci U S A 2004;101:12682–7.
12. Roberts WG, Ung E, Whalen P, Cooper B, Hulford C, Autry C, et al. Antitumor activity and pharmacology of a selective focal adhesion kinase inhibitor, PF-562,271. Cancer Res 2008;68:1935–44.
13. Zhang C, Spevak W, Zhang Y, Burton EA, Ma Y, Habets G, et al. RAF inhibitors that evade paradoxical MAPK pathway activation. Nature 2015;526:583–6.
14. Gao C, Chen G, Kuan SF, Zhang DH, Schlaepfer DD, Hu J. FAK/PYK2 promotes the Wnt/beta-catenin pathway and intestinal tumorigenesis by phosphorylating GSK3beta. eLife 2015;4.
15. Chien AJ, Moore EC, Lonsdorf AS, Kulikauskas RM, Rothberg BG, Berger AJ, et al. Activated Wnt/beta-catenin signaling in melanoma is associated with decreased proliferation in patient tumors and a murine melanoma model. Proc Natl Acad Sci U S A 2009;106:1193–8.
16. Biechele TL, Kulikauskas RM, Toroni RA, Lucero OM, Swift RD, James RG, et al. Wnt/beta-catenin signaling and AXIN1 regulate apoptosis triggered by inhibition of the mutant kinase BRAFV600E in human melanoma. Sci Signal 2012;5:ra3.
17. Hirata E, Girotti MR, Viros A, Hooper S, Spencer-Dene B, Matsuda M, et al. Intravital imaging reveals how BRAF inhibition generates drug-tolerant microenvironments with high integrin beta1/FAK signaling. Cancer Cell 2015;27:574–88.
18. Sharma R, Fedorenko I, Spence PT, Sondak VK, Smalley KS, Koomen JM. Activity-based protein profiling shows heterogeneous signaling adaptations to BRAF inhibition. J Proteome Res 2016;15:4476–89.

19. Smalley KS, Lioni M, Dalla Palma M, Xiao M, Desai B, Egyhazi S, et al. Increased cyclin D1 expression can mediate BRAF inhibitor resistance in BRAF V600E-mutated melanomas. Mol Cancer Ther 2008;7:2876–83.
20. Saridaki Z, Papadatos-Pastos D, Tzardi M, Mavroudis D, Bairaktari E, Arvanity H, et al. BRAF mutations, microsatellite instability status and cyclin D1 expression predict metastatic colorectal patients' outcome. Br J Cancer 2010;102:1762–8.
21. Tang Z, Yuan X, Du R, Cheung SH, Zhang G, Wei J, et al. BGB-283, a novel RAF kinase and EGFR inhibitor, displays potent antitumor activity in BRAF-mutated colorectal cancers. Mol Cancer Ther 2015;14:2187–97.
22. Emmons MF, Faiao-Flores F, Smalley KS. The role of phenotypic plasticity in the escape of cancer cells from targeted therapy. Biochem Pharmacol 2016;122:1–9.
23. Doherty MR, Smigiel JM, Junk DJ, Jackson MW. Cancer stem cell plasticity drives therapeutic resistance. Cancers 2016;8.
24. Vermeulen L, De Sousa E Melo F, van der Heijden M, Cameron K, de Jong JH, Borovski T, et al. Wnt activity defines colon cancer stem cells and is regulated by the microenvironment. Nat Cell Biol 2010; 12:468–76.
25. Zeuner A, Todaro M, Stassi G, De Maria R. Colorectal cancer stem cells: from the crypt to the clinic. Cell Stem Cell 2014;15:692–705.
26. Clevers H. The intestinal crypt, a prototype stem cell compartment. Cell 2013;154:274–84.
27. Hirsch D, Barker N, McNeil N, Hu Y, Camps J, McKinnon K, et al. LGR5 positivity defines stem-like cells in colorectal cancer. Carcinogenesis 2014;35:849–58.