BATF2 Deficiency Promotes Progression in Human Colorectal Cancer via Activation of HGF/MET Signaling: A Potential Rationale for Combining MET Inhibitors with IFNs

Zebing Liu1,2,3,4, Ping Wei1,2,4, Yu Yang5, Wenli Cui1,2,4, Bing Cao1,2,4, Cong Tan1,2,4, Baohua Yu1,2,4, Rui Bi1,2,4, Kaiqin Xia1,2,4, Weixiang Chen1,2,6, Yiqin Wang1,2,4, Youyuan Zhang3, Xiang Du1,2,4, and Xiaoyan Zhou1,2,4

Abstract

Purpose: BATF2, a novel IFN-stimulated gene, inhibits tumor cell proliferation, invasion, and migration. The objectives of this study were to determine how BATF2 expression is associated with colorectal cancer progression and patient outcome, to investigate how BATF2 overexpression inhibits hepatocyte growth factor (HGF)/MET signaling, and to elucidate the rationale for combining MET inhibitors with IFN.

Experimental Design: BATF2 expression in colorectal cancer tissues was determined and correlated with colorectal cancer patient prognosis. Cultured colorectal cancer cells were used to investigate the effects of BATF2 overexpression on the malignant phenotype of colorectal cancer cells and HGF/MET signaling. Tumor xenograft models were used to validate the effects of BATF2 on colorectal cancer xenograft growth and assess the efficacy of the combination of MET inhibitors with IFNs in colorectal cancer.

Results: In colorectal cancer tissues, BATF2 was found to be significantly downregulated, and its expression negatively correlated with MET expression. Decreased BATF2 expression was associated with progression and shorter patient survival in colorectal cancer. BATF2 overexpression promoted apoptosis and inhibited proliferation, migration, and invasion in colorectal cancer cells, as well as dramatically blunted tumor xenograft growth. In addition, MET inhibitors in combination with IFNβ produced synergistic cytotoxicity both in vitro and in vivo.

Conclusions: Together, these novel findings suggest that BATF2, a tumor suppressor gene, is a potent negative regulator of HGF/MET signaling in colorectal cancer and may serve as a prognostic tumor marker. Furthermore, these results provide a rationale for combining MET inhibitors with IFNs in preclinical trials.

Introduction

Colorectal cancer is one of the leading causes of cancer-related death worldwide (1). Although surgical resection combined with adjuvant therapy displays efficacy at early stages of disease, subsequent relapse and metastasis often occur. At advanced stages, resistance to conventional therapies is frequent, and further therapeutic options for these patients are relatively limited. To conquer this resistance, multiple targeted drugs have been developed and tested in clinical trials. Therefore, to better understand the molecular mechanisms underlying colorectal cancer pathogenesis, it is crucial to identify new therapeutic targets to facilitate treatment decisions (2).

BATF2, also named SARI (suppressor of AP-1, regulated by IFN), can be induced by type I IFN in early stages (3). A novel tumor suppressor, BATF2 potently inhibits growth and induces apoptosis in malignant cells but not in normal cells. BATF2 mRNA expression was detected in nonmalignant cells of various lineages, including prostate and breast epithelial cells, pancreatic mesothelial cells, melanocytes, and astrocytes; however, BATF2 expression was not detected in diverse malignant tumor cell lines of the same tissue origin. Mechanistically, BATF2 overexpression inhibits DNA binding of activator protein-1 (AP-1) complexes and, consequently, AP-1–dependent gene expression (3). Furthermore, BATF2 deficiency was found in hepatocellular cancer (HCC) tissues and correlated with a shorter survival compared with those with normal BATF2 expression (4). Although these findings confirm that BATF2 functions as a potent tumor suppressor and predicts prognosis in some human malignancies, its role in tumorigenesis and progression requires further elucidation through laboratory and clinical studies.
Translational Relevance

Although HGF/MET signaling is frequently activated in human colorectal cancer, the mechanism by which the activation of this pathway regulates the progression of colorectal cancer in vivo has not been fully elucidated. Here, we report that BATF2, a novel tumor suppressor gene, is induced by IFNs and that expression of BATF2 progressively decreases with tumor progression and correlates with poor colorectal cancer patient prognosis. Mechanistically, BATF2 downregulates MET expression through its interaction with c-Jun/AP-1 (a key transcription factor targeting MET), thereby reducing MET promoter activity in colorectal cancer cells. Interestingly, MET inhibitors in combination with IFNβ produce a synergistic cytotoxicity both in vitro and in vivo, a rationale for the use of this combination is supported by the existence of the BATF2–c-Jun/AP-1–MET axis. This study suggests that IFNs, as an adjuvant in combination with MET inhibitors, may serve as a novel therapeutic strategy for patients with colorectal cancer.

An earlier report documented that AP-1 overexpression (c-Jun plus c-Fos) dramatically increased MET promoter activity (5). Blocking the hepatocyte growth factor (HGF)/MET pathway by MET inhibitors, monoclonal antibodies, or siRNA potently inhibits tumor xenograft formation and growth rates in many malignancies (6). MET is an upstream regulator of multiple pathways, including PI3K/Akt, Ras/MAPK, STAT, and NF-kB, that contribute to tumor initiation and progression and correlate with outcome in many tumors (7, 8). Although the presence of gene amplification, activating point mutations, and ligand autocrine activation has been described, by far, the most frequent MET alteration in human tumors is overexpression (9, 10). In colorectal cancer, many studies have demonstrated that MET overexpression is associated with the development of distant metastases and a shorter metastasis-free survival (11). Consequently, MET activation is considered to be crucial for the acquisition of metastatic potential. This viewpoint is supported by the observation that the MET gene is very rarely amplified in primary colorectal cancer, while an enhanced copy number can be found in liver metastatic lesions derived from colorectal cancers (12). Furthermore, many targeted MET inhibitors have been developed and show tumor inhibitory effects, especially in combination with other drugs or inhibitors, in many human malignancies (13–15).

In the current study, we first detected BATF2 mRNA and protein expression in Chinese colorectal tumor patient samples at different disease stages and investigated their clinicopathologic association as well as prognostic value. Then, considering the role of BATF2 in the inhibition of tumor initiation and progression, we studied the regulatory mechanism of BATF2 in colorectal cancer cell lines. We found that BATF2 overexpression by a lentivirus attenuated MET expression in colorectal cancer cells. Thus, the inhibitory effect of the BATF2 activator IFN and the MET inhibitor PF-04217903 on colorectal cancer cells was evaluated both in vitro and in vivo.

Materials and Methods

Patients and samples
A total of 222 paired tumor samples and matched adjacent nontumor tissues were collected from patients with colorectal cancer (median age 65 years, range 26–88) at Fudan University Jinshan Hospital between 2004 and 2012. The fresh tissues were immediately immersed in liquid nitrogen after surgical resection and then frozen at −80°C until RNA and protein extraction was performed. Another 148 paraffin-embedded, archived colorectal cancer samples were collected for the immunohistochemical (IHC) assay. In addition, 46 adenoma tissues and 40 high-grade intrapitelial neoplasia (HGIN) tissues were collected from patients who had undergone a biopsy. All samples were collected with informed consent according to the Internal Review and Ethics Boards of the Fudan University Jinshan Hospital (Shanghai, China).

Quantitative real-time PCR: general protocol
Total RNA was extracted using TRIzol (Invitrogen) and then purified using the RNeasy Plus Kit (Qiagen). To synthesize first-strand cDNA using M-MLV Reverse Transcriptase (Fermentas), reverse transcription was performed with 2 μg of total RNA. The cDNA was then subjected to quantitative real-time PCR (qPCR) to evaluate the relative mRNA levels of BATF2 and GAPDH (as an internal control) with the corresponding primer pairs (BATF2 forward: 5′AGACCCCAAGGAGCAACAA3′ and reverse: 5′CAGGG-CGACGGTGTGC3′; GAPDH forward: 5′ATGCGGAAGTGAAGGGCAGTC3′ and reverse: 5′GCTGATCATCAGGCGTGT-GTCC3′). Gene-specific amplification of BATF2 was determined using an ABI 7900HT (PE Applied Biosystems) qPCR machine. For relative quantification, target gene mRNA expression was normalized to the geometric mean of the internal control gene, GAPDH. The data were analyzed using the comparative threshold cycle method (2−ΔΔCt).

Immunohistochemistry

IHC analysis was performed as previously described (16, 17). BATF2, MET and Ki-67 were detected using anti-BATF2 (Abcam), anti-MET (Cell Signaling Technology) and anti-Ki-67 (Santa Cruz Biotechnology) antibodies, respectively. Biotinylated anti-rabbit secondary antibody and a DAB kit were purchased from DAKO. The negative control consisted of replacing the primary antibody with 0.1% BSA/PBS.

BATF2 expression was scored using an immunoreactive scoring scale and was evaluated by two independent pathologists who had no prior knowledge of the patients’ clinical information. In our scoring system, both the percentage of positive cells and the staining intensity were taken into account. Briefly, the percentage of positive staining was scored as 0 (0%, no positive cells), 1 (<25% positive cells), 2 (26–50% positive cells), 3 (51–75% positive cells), or 4 (>75% positive cells). The intensity of immunostaining was scored as 0 (no positive staining), 1 (weakly stained), 2 (moderately stained), or 3 (strongly stained). A final immunoreactive score was determined by the sum of the positive proportion and the staining intensity. The final score was clustered into four groups: −, ≤2 total points; +, 3–4 total points; ++, 5–6 total points; and ++++, 7 total points. In this study, − and + represent low expression, whereas + + and + +++ indicate high expression (4). The scoring criteria for MET and Ki-67 were similar to those described in recent studies of gastric cancer and thyroid cancer, respectively (16, 17).

Cell culture and treatments
The colorectal cancer cell lines HT-29, LoVo, Caco-2 (SIBS), SW480, SW620, and HCT116 (ATCC) were cultured according
to the standard protocols provided by ATCC. Six cell lines using for in vitro and in vivo assays were tested and authenticated by STR assay (CST/TCCAS, China). Cell lines were subjected to different treatments as follows: (i) incubation with IFNβ (R&D Systems) or dimethyl sulfoxide (DMSO; Sigma-Aldrich) as a vehicle control; (ii) incubation with PF-04217903 (Selleckchem) or DMSO as a vehicle control; (iii) incubation with HGF (R&D Systems) or DMSO as a vehicle control; (iv) transfection with siMET (Qiagen) or a scrambled sequence as a control siRNA; and (v) infection with Lenti.BATF2 or a lentivirus containing a scrambled sequence as a negative control.

**Construction of lentivirus and plasmid**

To achieve stable BATF2 overexpression, cells were transduced with the indicated lentiviral particles, followed by puromycin (Sigma-Aldrich) selection. A lentivirus containing a scrambled sequence was used as a negative control. The BATF2 expression plasmid was created by cloning BATF2 cDNA into pcDNA3.1, and the empty vector was used as a control.

**Transient transfection and luciferase assays**

Cells were grown at a density of 1 x 10^5 per well in 6-well culture plates and transfected with Lipofectamine 2000 transfection reagent (Invitrogen) and plasmid DNA for 24 hours. For luciferase assays, cells were transiently cotransfected with reporter gene constructs (MET/pGL3Luc) or with mutant control constructs (lacking a c-Jun binding site with the MET promoter). MET (-c-Jun/AP-1)/pGL3-Luc, and pRL-TK-luc plasmid encoding Renilla luciferase. Cells were incubated for 24 hours and then infected with Lent.BATF2 or negative control for another 24 hours, after which point the activities of the firefly and Renilla luciferases were determined using a dual-luciferase reporter assay system (Promega).

**Cell proliferation and apoptosis studies**

Cell proliferation was monitored using the CCK8 (Dojindo) assay. Apoptosis was detected using the dual staining Annexin V/PE-7AAD Apoptosis Detection Kit (BD Biosciences) on a Cytomics FC500 flow cytometer (Beckman Coulter). The percentage of apoptotic cells in each quadrant was calculated using CXP Software. Each experiment was performed in triplicate.

**Wound-healing and cell invasion assays**

Wound-healing and cell invasion assays were performed as described previously (18, 19). The Transwell plates and Matrigel were purchased from BD Biosciences.

**Immunofluorescence staining**

Cells (2 x 10^5) were grown on chamber slides and infected with Lent.BATF2 or negative control. After 24 hours, the cells were fixed, permeabilized, and blocked routinely. After incubation with primary antibody (rabbit anti-MET, Cell Signaling Technology) at room temperature for 2 hours and incubation with Alexa Fluor 546 (red) goat anti-rabbit secondary antibody (Invitrogen) at room temperature for 1 hour, DAPI (Invitrogen) was used to stain the DNA. Finally, the slides were mounted, and the cells were visualized under a Leica inverted fluorescence microscope.

**Western blot and coimmunoprecipitation**

The frozen colorectal cancer samples or harvested cells were washed with cold PBS and then lysed in RIPA lysis buffer, and the lysates were cleared by centrifugation (14,000 rpm) at 4°C for 15 minutes. Western blot analysis was performed as previously described (17). The following primary antibodies were used for analysis: Flag, CCN1 and BATF2 (Abcam); Ki-67 (Santa Cruz Technology); MET, p-MET, Akt, p-Akt, ERK 1/2, p-ERK 1/2, PARP, caspase-3, c-Jun, WAF1, and MMP2 (Cell Signaling Technology); and GAPDH, MMP3, MMP7, and MMP9 (Proteintech Group). The anti-rabbit IgG and anti-mouse IgG secondary antibodies were purchased from Cell Signaling Technology. GAPDH served as a loading control.

For the coimmunoprecipitation (co-IP) assay, whole-cell lysates were obtained. After prewashing with lysis buffer, Protein G Dynabeads (Invitrogen) were incubated with antibodies at room temperature for 10 minutes. The beads were then mixed with the cell lysate supernatants overnight at 4°C. The complex was eluted using elution buffer (glycine HCl, pH 3.0), followed by heating for 10 minutes at 70°C.

**Tumor xenograft models**

Colorectal cancer xenografts were generated as previously described (20, 21). Female 6- to 8-week-old BALB/c nude mice (Beijing HFK Bioscience) were randomly divided into two groups (n = 6 per group) and anesthetized by intraperitoneal injection of ketamine (100 mg/kg). Subsequently, the mice were s.c. injected with 5 x 10^6 colorectal cancer cells infected with either Lent.BATF2 or negative control in 0.05 mL of PBS. Tumor volumes were estimated by measuring two dimensions [length (a) and width (b)], with the volume calculated as V = ab^2/2 (20). At the end of each experiment, the tumors were excised and frozen in liquid nitrogen or fixed in 4% neutral paraformaldehyde solution for Western blot and IHC analyses, respectively.

In addition, the same xenografts were generated as described above. When the tumors reached 68–185 mm^3, the mice were randomly divided into four groups (n = 6 per group). Different groups received different treatments. We treated the mice every other day intraperitoneally with vehicle, IFNβ (2 x 10^6 IU/kg), PF-04217903 (20 mg/kg), or both IFNβ and PF-04217903 for 18 days. Tumor volume estimation and the procedures at the end of the experiment were performed as previously described.

**Statistical analysis**

Statistics are presented as the mean ± SD and compared using unpaired two-tailed Student t tests or one-way ANOVA. The χ^2 test was used to analyze the relationship between BATF2 protein expression and clinicopathologic status. Kaplan–Meier survival curves were plotted, and a log-rank test was performed. Correlation coefficients were investigated using the Spearman rank correlation test. A P value < 0.05 was considered to be significant.

**Results**

**BATF2 deficiency promotes colorectal cancer progression and correlates with a worse patient prognosis**

To confirm BATF2 expression in colorectal cancer tissues, we examined BATF2 mRNA and protein expression in paired non-tumor (N) and tumor (T) tissue samples derived from colorectal cancer patients. Examined by qPCR, BATF2 mRNA was 3-fold lower in tumor tissues than in nontumor tissues (Fig. 1A).
Western blot analyses also showed that BATF2 protein expression was substantially stronger in nontumor tissues than in tumor tissues derived from colorectal cancer patients (Fig. 1B). Immunohistochemistry for BATF2 expression in colorectal cancer tissues indicated that BATF2-positive cells were rarely observed among colorectal cancer cells (Fig. 1C). However, strong and intermediate immunostaining was observed in the adjacent non-neoplastic epithelia and adenomas, respectively (Fig. 1C). In addition, the ratio of BATF2-positive cases gradually decreased when nontumor tissue progressed to invasive carcinoma (Fig. 1D).

We next correlated the rate of low BATF2 expression with the clinicopathologic status of colorectal cancer patients (Supplementary Table S1). The ratio of decreased BATF2 expression increased with increasing aggressive tumor biology, as defined by depth of tumor invasion, lymphatic and/or venous invasion, and nodal involvement. However, there was no observed difference in BATF2 expression with respect to age, gender, tumor location, histologic type, differentiation, distant metastasis, proliferation index Ki-67, and TNM stage. The Kaplan–Meier survival curve with a median follow-up period of 38 months demonstrated that patients with low BATF2 expression, also noted elsewhere (4), have dramatically shorter survival than those with high BATF2 expression (Fig. 1E). Multivariate Cox regression analysis showed that low BATF2 expression correlated with poor colorectal cancer patient prognosis (hazard ratio, 0.519; \( P = 0.037 \)), independent of other clinical covariates (Supplementary Table S2), and was an independent prognostic factor for colorectal cancer.

**BATF2 arrests cell proliferation and induces apoptosis in colorectal cancer cell lines**

To determine the functional effects of BATF2 on the biological behaviors of colorectal cancer cells, we first detected basal BATF2 mRNA and protein expression in six colorectal cancer cell lines and representative fresh tissue of normal colon mucosa by qPCR and Western blot analyses, respectively. As shown in Fig. 2A and B, BATF2 expression was significantly decreased, even to undetectable levels. Then, we constructed a replication-incompetent lentivirus expressing BATF2 (Lenti.BATF2). LoVo and SW620 cells were infected with Lenti.BATF2 (100 pfu/cell). Lenti.BATF2 dramatically arrested cell proliferation and downregulated the protein expression of the proliferation marker Ki-67 in LoVo and SW620 cells (Fig. 2C–E). Flow cytometry analyses of the effect of Lenti.BATF2 on apoptosis of LoVo and SW620 cells showed that Lenti.BATF2 induced earlier apoptosis in both LoVo cells (Lenti.BATF2, 22.3%; NC, 5.5%; mock, 6.4%) and SW620 cells (Lenti.BATF2, 8.9%; NC, 2.7%; mock, 4.2%) and increased the frequency of apoptotic cells by approximately 3.5- and 3.2-fold, respectively (Fig. 2F–H). In addition, as caspase-3 and its downstream target PARP are pivotal mediators involved in apoptosis, we assessed whether Lenti.BATF2 also caused their activation. Moreover, BATF2 promoted caspase-3 and PARP cleavage (Fig. 2I).
BATF2 inhibits migration and invasion in colorectal cancer cell lines

A wound-healing assay was used to evaluate the effect of BATF2 on cancer cell migration. As shown in Fig. 3A–D, compared with the mock and negative control, BATF2 overexpression significantly blocked LoVo and SW620 cell migration by approximately 70% and 60%, respectively. An invasion assay was performed in Transwell chambers containing 8-μm pore size inserts, the upper surfaces of which were coated with Matrigel matrix. LoVo and SW620 cells were suspended in serum-free media and plated on the upper chambers. After 12 hours of culture, BATF2 overexpression sharply inhibited LoVo and SW620 cell invasion by approximately 65% and 70%, respectively (Fig. 3E–H). Furthermore, as matrix metalloproteinases (MMP) play a key role in tumor invasion, we determined whether BATF2 overexpression resulted in MMP inhibition. It was found that Lenti.BATF2 downregulated MMP2 and MMP7, but not MMP3 and MMP9, protein expression to a large extent (Fig. 3I–K).

BATF2 downregulates MET expression by inhibiting transcription and inversely correlates with MET expression in colorectal cancer

Activator protein-1 (AP-1) plays a critical role in regulating cell proliferation and malignant transformation (22). BATF2 interacts with c-Jun, a key component of AP-1, resulting in the inhibition of DNA binding of AP-1 complexes. Consequently, BATF2 selectively inhibits AP-1–dependent gene expression. An earlier report documented that AP-1 overexpression (c-Jun plus c-Fos) dramatically increased MET promoter activity (5). On the basis of this observation, we first detected MET expression in the presence of BATF2 both at the transcriptional and translational level after treatment.
with HGF. Infection with Lenti.BATF2 significantly blunted baseline and HGF-induced MET expression (Fig. 4A). In addition, the use of immunofluorescence analysis to determine the location and expression level of MET in LoVo cells infected with Lenti.BATF2 demonstrated that MET localization to the plasma membrane was sharply attenuated by BATF2 overexpression (Fig. 4B). In addition, we also investigated the effects of BATF2/c-Jun/AP-1 on the levels of CCN1 and WAF1, which play key roles in the progression of a diverse range of human malignancies (23, 24). Western blots showed that the expression of WAF1 was significantly increased by BATF2 overexpression in colorectal cancer cells, but CCN1 levels did not change (Supplementary Fig. S1). Moreover, several studies have shown that activation of HGF/MET signaling promotes the progression of many tumors via PI3K/Akt and/or MAPK/ERK (20, 25). Our Western blot data revealed that BATF2 overexpression significantly attenuated the levels of phosphorylated Akt (p-Akt) in both LoVo and SW620 cells but only slightly decreased phosphorylated ERK 1/2 (p-ERK 1/2) in LoVo cells (Fig. 4C).

We also examined MET protein expression to confirm the association between BATF2 and MET in colorectal cancer tissues. As detected by IHC, MET protein expression was gradually enhanced in tissues from nontumors that progressed to invasive carcinomas (Supplementary Fig. S2A and S2B). There was a negative correlation between BATF2 and MET protein expression ($r = -0.306; P < 0.001$) in colorectal cancer (Fig. 4D and E). In addition, the Kaplan–Meier survival curve indicated that patients with MET overexpression showed a significantly shorter survival than those with low MET expression (Supplementary Fig. S2C). Multivariate Cox regression analysis showed that MET overexpression was not independent of other clinical covariates (HR, 1.621; $P = 0.134$; Supplementary Table S3). Furthermore, we constructed a MET-specific siRNA (siMET) and transfected LoVo and SW620 cells to...
confirm the reliance of proliferation, migration, and invasion on MET transcription. The results (Supplementary Figs. S3 and S4) were concordant with previously published results (26).

**BATF2 inhibits MET promoter function via interaction with c-Jun/AP-1**

The associations between BATF2 and MET, in vivo and in vitro, prompted us to investigate the mechanism by which BATF2 arrests MET transcription. We asked whether MET promoter activity could be inhibited by BATF2 overexpression and its interaction with c-Jun/AP-1. LoVo and SW620 cells were transfected with a MET/pGL3-Luc (a truncated fragment, ~2 kb, of the MET promoter cloned into the pGL3 basic vector) and then infected with Lenti.BATF2. BATF2 dramatically inhibited the negative control and HGF-induced MET promoter activity within 24 hours in two colorectal cancer cell lines (Fig. 5A).
and B). Further analysis showed that LoVo cells transfected with MET (c-Jun/AP-1)/pGL3-Luc, containing a mutant MET promoter, decreased this activation and inhibition (Fig. 5C). Compared with cells transfected with WT MET/pGL3-Luc (WT Promoter) or AP-1 mutant MET (AP-1)/pGL3-Luc (Mt Promoter) for 24 hours and then infected with Lenti.BATF2 or NC for another 24 hours, and luciferase assays were performed as described in the Materials and Methods section. HGF (40 ng/mL) was added 4 hours before the luciferase assays. C, LoVo cells were transfected with WT MET/pGL3-Luc (WT Promoter) or AP-1 mutant MET (AP-1)/pGL3-Luc (Mt Promoter) for 24 hours and then infected with Lenti.BATF2 or NC for another 24 hours, and luciferase assays were performed as described in the Materials and Methods section. HGF (40 ng/mL) was added 4 hours before the luciferase assays were conducted. Three independent experiments were performed, and the data represent the mean ± 5D; *, P < 0.05. D, reciprocal coimmunoprecipitation (co-IP) showing endogenous coimmunoprecipitation of BATF2 and c-Jun/AP-1. Whole cell extracts of LoVo (infected with Lenti.BATF2 or NC) were subjected to immunoprecipitation with anti-Flag-BATF2 antibody, followed by Western blot analysis for c-Jun, and similar whole cell extracts were subjected to immunoprecipitation with anti-c-Jun, followed by Western blot analysis with anti-Flag-BATF2. The results are representative of three independent experiments.

Figure 5. BATF2 reduces MET promoter activity in colorectal cancer cells by inhibiting AP-1 activation. A and B, the indicated cells were transfected with wild-type (WT) MET/pGL3Luc, and after a 24-hour incubation, transfected cells were infected with Lenti.BATF2 or NC for another 24 hours. Luciferase assays were performed as described in the Materials and Methods section. HGF (40 ng/mL) was added 4 hours before the luciferase assays. C, LoVo cells were transfected with WT MET/pGL3-Luc (WT Promoter) or AP-1 mutant MET (AP-1)/pGL3-Luc (Mt Promoter) for 24 hours and then infected with Lenti.BATF2 or NC for another 24 hours, and luciferase assays were performed as described in the Materials and Methods section. HGF (40 ng/mL) was added 4 hours before the luciferase assays were conducted. Three independent experiments were performed, and the data represent the mean ± 5D; *, P < 0.05. D, reciprocal coimmunoprecipitation (co-IP) showing endogenous coimmunoprecipitation of BATF2 and c-Jun/AP-1. Whole cell extracts of LoVo (infected with Lenti.BATF2 or NC) were subjected to immunoprecipitation with anti-Flag-BATF2 antibody, followed by Western blot analysis for c-Jun, and similar whole cell extracts were subjected to immunoprecipitation with anti-c-Jun, followed by Western blot analysis with anti-Flag-BATF2. The results are representative of three independent experiments.

IFNβ enhanced the inhibitory effect induced by MET inhibition in colorectal cancer cells.

IFNs exert potent antitumor effects and have been used clinically to treat diverse solid tumors and hematologic malignancies as a monotherapy or as an adjuvant to chemo- or radiotherapy (27). In LoVo and SW620 cells, BATF2 protein and mRNA expression levels were induced by IFNβ in a dose-dependent manner, with maximal expression observed at 48 hours (for protein expression) and 6 to 8 hours (for mRNA expression) after IFNβ treatment (1,000 U/mL), decreasing sharply thereafter (Supplementary Fig. S5A–S5E). On the basis of our findings that BATF2 can dramatically suppress MET expression and an earlier report that PF-04217903 only
partially inhibited HGF-mediated cell proliferation in colorectal cancer cells (28), we evaluated whether the BATF2 inducer IFNβ in combination with the MET inhibitor PF-04217903 would produce a synergistic effect on cell apoptosis and proliferation in colorectal cancer cells. First, the half-maximal inhibitory concentration (IC50) of IFNβ and PF-04217903 in LoVo and SW620 cells was determined by CCK8 assays (Supplementary Fig. S6A–S6D). Consequently, compared with IFNβ or MET inhibitor PF-04217903 treatment alone, the combination treatment consistently synergistically induced apoptosis in both LoVo cells (combination, 20.4%; PF-04217903, 10.1%; IFNβ, 5.1%) and SW620 cells (combination, 20.9%; PF-04217903, 15.9%; IFNβ, 8.3%; Supplementary Fig. S7A), increasing the frequency of apoptotic cells by approximately 2.5- and 1.5-fold, respectively (Supplementary Fig. S7B and S7C). Moreover, the combination dramatically inhibited cell proliferation in both LoVo and SW620 cells (Supplementary Fig. S7D) and suppressed the level of phosphorylated MET (Supplementary Fig. S7E).

BATF2 inhibited tumorigenesis and IFNβ in combination with PF-04217903 produced synergistic inhibitory effects on colorectal cancer in vivo

The in vitro findings led us to investigate whether BATF2 overexpression arrests tumor xenograft growth and whether IFNβ in combination with a MET inhibitor produces synergistic inhibitory effects in vivo. To achieve this objective, LoVo cells were infected with Lent.BATF2 or a negative control. The infected cells were
then engrafted onto 12 BALB/c nude mice (6 mice per group) to monitor tumor growth. BATF2 overexpression significantly decreased LoVo colorectal cancer xenograft growth by approximately 72% compared with the negative control (Fig. 6A and B). We next compared the inhibitory effects of Lenti.BATF2 in vivo by immunohistochemical staining for Ki-67 and MET protein. Infec-
tion with Lenti.BATF2 significantly decreased the expression of Ki-
67 and MET protein (Fig. 6C). In addition, we examined whether
in vivo
cancer tumors immunohistochemical staining for Ki-67 and MET protein. Infec-
tion with Lenti.BATF2 significantly decreased the expression of Ki-
67 and MET protein (Fig. 6C). In addition, we examined whether
in vivo
cancer tumors reached the size of $6 \times 10^6$ U/kg), PF-04217903 (20 mg/kg), or both IFN
however, the combination signi-
tumor growth by approximately 34% and 45%, respectively;
Table S4). Compared with animals treated with DMSO, both $2 \times 10^6$ U/kg IFN$\beta$ and 20 mg/kg PF-04217903 modestly suppressed tumor growth by approximately 34% and 45%, respectively; however, the combination significantly inhibited tumor growth by approximately 91% (Fig. 6D and E). Apart from tumor size, we also detected the effects of those treatments on the target protein MET and its phosphorylation status, as well as the proliferation marker Ki-67, in vivo. The results were concordant with their in vitro counterparts (Fig. 6F and G).

**Discussion**

BATF2 has a broader expression profile that includes hematopoietic and nonhematopoietic tissues (29). The current study provides new pieces of evidence regarding the role of BATF2 in colorectal cancer progression, negative regulation of HGF/MET signaling, and elucidation of potential mechanisms of IFNs as adjuvants to MET inhibitor chemotherapy.

First, we found that BATF2 mRNA and protein expression were decreased in both colorectal cancer tissues and cell lines compared with nontumor tissues. The results from immuno-
histochemistry showed that decreased BATF2 expression in colorectal cancer was dramatically associated with more aggressive disease. Furthermore, survival analysis revealed that reduced BATF2 protein expression correlated with a poor colorectal cancer patient prognosis. In addition, BATF2 expression gradually decreased as the normal colorectal epithelia pro-
gressed to carcinoma, which indicates that the loss of BATF2 may be a potential early event in the malignant transformation of colorectal cancer. Recent evidence also suggests that decreased BATF2 expression is associated with an inferior outcome in hepatocellular carcinoma and oral tongue squamous cell carcino-
noma (4, 30). Our findings combined with recent evidence potently support the notion that BATF2 plays a critical role in controlling tumor initiation and subsequent progression and could be used as a candidate marker for colorectal cancer diagnosis and prognosis.

Second, we found a novel pathway comprising BATF2 and c-Jun/AP-1 that inhibits canonical HGF/MET signaling and identi-
fied BATF2 as an independent factor in cell proliferation inhibi-
tion in colorectal cancer. We confirmed that BATF2 significantly blunted cell proliferation and induced cell apoptosis in colorectal cancer cells. Our findings are consistent with the results of Su and colleagues, which supports BATF2 as a potent inhibitory factor that arrests cell growth in prostate cancer, glioma, and melanoma cells (3). Furthermore, BATF2 overexpression significantly inhib-
ited cell migration and invasion. In addition to the in vitro findings, we observed that BATF2 dramatically inhibited colorectal cancer xenograft growth in the in vivo model. Overall, BATF2 inhibited cell growth and induced cell apoptosis, as well as arrested migration and invasion.

As a classical RTK signaling pathway, HGF/MET is associated with initiation and progression in many human tumors (e.g., gastric cancer, colorectal cancer and breast cancer; refs. 16, 31, 32). In this context, we first confirmed that BATF2 overexpression in colorectal cancer cells downregulated MET expression and decreased MET phosphorylation levels. In addition, a negative association between BATF2 and MET was detected by IHC in colorectal cancer tissues. Consequently, the results from IHC confirmed our assumption that BATF2 expression adversely correlated with MET expression.

Earlier studies have shown that the PI3K/Akt and MAPK/ERK pathways are downstream targets of MET (20, 25). Here, we show that BATF2 mainly blunted HGF-induced activation of Akt, but not ERK. These findings further confirm that BATF2 is a negative regulator of HGF/MET signaling. The results from qPCR revealed that BATF2 downregulated MET expression by inhibiting tran-
scription. Mechanistically, BATF2 overexpression dramatically impaired activator protein (AP)-1 function via the direct binding with c-Jun (a critical component of AP-1). Our findings are consistent with the results from a recent study, which confirmed the interaction between BATF2 and downstream targets of MET (data not shown). Overall, BATF2 suppressed MET transcription by impairment of MET promoter activity via direct binding with c-Jun/AP-1 in colorectal cancer cells.

Third, we found a potential functional mechanism to elucidate the effects of the combination of MET inhibitors with IFNs. IFNs, apart from their function as antiviral infection agents, exert a variety of inhibitory effects on cell growth, apoptosis, and angiogenesis. (38). IFNs induce growth inhibition by a variety of pathways that involve many IFN-stimulated genes (27). BATF2 is one of these genes and can be induced by IFN$\beta$, which indicates that BATF2 may be a key component involved in IFN signaling. In the current study, BATF2 protein expression was induced by IFN$\beta$ in a dose-dependent manner, with maximal levels observed 48 hours after IFN$\beta$ treatment, fol-
lowed by sharp decreases thereafter. As mentioned above, many inhibitors have been developed to target MET and have shown potent efficacy in preclinical trials. We investigated whether IFN$\beta$ in combination with the MET inhibitor PF-04217903 could produce synergistic cytotoxicity. In vitro, we observed...
that both IFNβ and PF-04217903 modestly inhibited colorectal cancer cell proliferation; however, the combined treatment with IFNβ and PF-04217903 significantly suppressed colorectal cancer cell proliferation. This result was further supported by our findings in vivo that IFNβ in combination with PF-04217903 more efficiently suppressed colorectal cancer xenograft growth than treatment with IFNβ or PF-04217903 alone. Our data together suggest that IFNβ can be used as an adjuvant to MET inhibitors as an anticancer strategy.

In conclusion, BATF2 displays potent tumor suppressor functions in human colorectal cancer. In this study, we demonstrated that a mechanism by which this gene performs these profound effects in colorectal cancer is by inhibiting MET transcription and arresting c-Jun. The MET promoter is regulated by AP-1–binding elements, and BATF2 suppresses AP-1–binding activity by interacting with the key transcription factor c-Jun. These findings support a rationale to combine a MET inhibitor with IFNβ, which may provide a novel antitumor therapeutic strategy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: Z. Liu, P. Wei, Y. Yang, W. Cui, C. Tan, R. Bi, K. Xia, X. Zhou
Development of methodology: Z. Liu, P. Wei, Y. Yang, W. Cui, C. Tan, R. Bi, K. Xia, X. Zhou
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Liu, P. Wei, Y. Yang, W. Cui, B. Cao, C. Tan, B. Yu, R. Bi, K. Xia, W. Chen, X. Zhou
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Z. Liu, P. Wei, Y. Yang, W. Cui, C. Tan, R. Bi, K. Xia, Y. Zhang, X. Du, X. Zhou
Writing, review, and/or revision of the manuscript: Z. Liu, X. Zhou
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Z. Liu, Y. Wang, X. Zhou
Study supervision: X. Zhou

Acknowledgments
The authors thank Dr. Zhang (Cancer Institute, Fudan University Shanghai Cancer Center, Shanghai, China) for her valuable assistance with the flow cytometric analyses; Dr. Wang (Department of Radiation Oncology, Fudan University Shanghai Cancer Center, Shanghai, China) for providing technical advice about the invasion assays; and Dr. Wu (Department of Medical Oncology, Fudan University Shanghai Cancer Center, Shanghai China) for his valuable assistance performing the qPCR analysis.

Grant Support
This work was supported by grants from the Natural Science Foundation of China (81272630, 81201836, and 81302163) and the Shanghai Pu Ding New Area Science and Technology Development fund (PKJ2013-Y08).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received June 22, 2014; revised January 12, 2015; accepted January 13, 2015; published OnlineFirst March 9, 2015.
CD82 and suppresses the Wnt/β-catenin signal via ISGylation of β-catenin.


Clinical Cancer Research

BATF2 Deficiency Promotes Progression in Human Colorectal Cancer via Activation of HGF/MET Signaling: A Potential Rationale for Combining MET Inhibitors with IFNs

Zebing Liu, Ping Wei, Yu Yang, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-14-1564

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2015/07/17/1078-0432.CCR-14-1564.DC1

Cited articles
This article cites 38 articles, 10 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/21/7/1752.full.html#ref-list-1

Citing articles
This article has been cited by 2 HighWire-hosted articles. Access the articles at:
/content/21/7/1752.full.html#related-urls

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

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

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