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Original Article

Impact of BMI1 expression on the apoptotic effect of paclitaxel in colorectal cancer

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Abstract: Colorectal cancer (CRC) is one of the most common cancers worldwide. Despite advances in treatment, no treatment modality specific for the different CRC phenotypes has been developed. BMI1 has been previously reported to play an important role in the regulation of cancer stem cells and cell cycle in CRC. However, the role of BMI1 in individualized treatment for CRC is largely unknown. In this study, we found that the apoptotic effect of paclitaxel is more profound in BMI1-depleted cells. The apoptotic effect is exerted by promoting caspase-8-independent apoptotic pathways after combination with paclitaxel in BMI1 knockdown cells. This effect could be totally recovered by pretreatment with caspase inhibitor compared with caspase-8 inhibitor alone. It has been reported that the levels of MCL-1 play a role in regulating cell resistance to paclitaxel treatment. Our data indicated that the downregulation of MCL-1 through the activation of GSK3beta and JNK is driven by BMI1 depletion. Consistent with in vitro data, a synergic anti-growth effect of BMI1 depletion with paclitaxel treatment was shown in vivo. In conclusion, paclitaxel has a stronger suppressive effect on tumor growth and proliferation in CRC with low BMI1 expression. Thus, in CRC patients, paclitaxel could be specifically indicated for patients with low BMI1 expression.

Keywords: BMI1, colorectal cancer, paclitaxel, MCL-1, protein kinase inhibitor

Introduction

Colorectal cancer (CRC) is the third most common cancer in the United States. Despite improvements in diagnostic and treatment modalities, CRC patients with metastasis still have poor survival, with a 5-year survival rate of only <20% [1, 2]. Surgery followed by adjuvant chemotherapy remains the standard treatment for patients with locally advanced CRC [3]. Conventional chemotherapy with 5-fluorouracil (5-FU) has been used for several decades; however, 40% of patients recur and die within 8 years even after treatment with surgery and 5-FU-based chemotherapy [4, 5]. Thus, there have been efforts to develop more effective treatments. Combination treatment with anti-angiogenic drugs has been suggested to enhance cytotoxicity and overcome chemoresistance by blocking angiogenesis and facilitating hypoxic environment [6-8].

Regorafenib, a small-molecule multiple kinase inhibitor, is widely used as the second-line treatment of metastatic CRC patients, based on phase 3 studies showing that it prolongs overall survival and progression-free survival [9, 10]. However, most patients with CRC develop resistance to these drugs [11, 12]. Several protein kinase inhibitors have been developed to block specific pathways associated with tumor proliferation and progression. Small-molecule kinase inhibitors have been established to be effective for the treatment of different malignancies, but several factors, including the tumor microenvironment, drug resistance, and tumor genetics, influence their clinical efficacy. Therefore, the most appropriate strategy for obtaining the op-

timal efficacy of protein kinase inhibitors has become an important issue in CRC.

BMI1 is an oncogene that belongs to the polycomb group, which functions as a transcriptional repressor [13]. The BMI1 gene is associated with several mechanisms that promote the development of hematologic malignancies and solid tumors, including tumorigenesis, blocking cell senescence, epithelial-mesenchymal transition, invasion and migration of cancer stem cells, and chemoresistance [13-15]. Aberrant expression of BMI1 has been detected in CRC, breast carcinoma, and hepatocellular carcinoma [16-18]. In CRC patients, the lower expression of BMI1 is associated with longer survival and favorable clinical outcome [19]. However, although there have been studies on the BMI1 gene, its implication in clinical practice, particularly treatment, has not been fully explored. This study aimed to investigate whether CRC sensitivity to protein kinase inhibitors can be improved by regulating BMI1 expression.

Materials and methods

Cell lines, reagents, and plasmids

HT-29 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. sh-BMI1 and sh-luciferase (sh-Luci) plasmids were from the National RNAi Core Facility (Academia Sinica, Taiwan). The sh-BMI1#1, #2, #3 sequence was as follows: 5'-CAGATTGGATCGGAAAGTAAA-3'; sh-BMI1#4, #5, #6 sequence: 5'-ATTGATGCCACAACCATAATA-3'; sh-Luci sequence: 5'-CTTCGAAATGTCCGTTCGGTT-3'. Anti-BMI1 was purchased from Bethyl Laboratories, Inc. Anti-cleaved caspases-3, cleaved caspase-8, Bcl-2, tubulin, and actin were purchased from Genetex (San Antonio, TX, USA). UNC0638 was purchased from Cayman Chemical (Ann Arbor, MI, USA).

Quantitative reverse transcriptional PCR (RTqPCR)

Total RNA extraction was conducted using the RNeasy mini kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. Equal amounts of RNA were used to synthesize the first-strand cDNA using the RT^2 First Strand Kit (Qiagen). Real-time polymerase chain reaction (RT-PCR) was performed using SYBR Green on an RT-PCR System (Applied Biosystems, Foster City, CA). BMI1-forward (5'-GCTGGTTGCCCATTGACAG-3') and BMI1-reverse (5'-CACACATCAGGTGGGGAT-3'); GAPDH-forward (5'-GAGTCAACGGATTTGGTCGT-3'); and GAPDH-reverse (5'-TGTGGTCATGAGTCCTTCCA-3') were used.

Flow cytometry for cell cycle analysis

HT-29 cells were transfected with sh-BMI1, harvested via trypsinization, and fixed with 70% ice-cold ethanol overnight at -20°C. On the following day, the cell pellet was resuspended in propidium iodide (PI)-staining buffer (50 µl/ml PI, RNAse A, Beckman Coulter, Brea, CA) and incubated for 15 min at 37°C for further cell cycle analysis. Cell cycle distribution was analyzed via FACS Calibur (BD Biosciences, San Diego, CA) using ModFit software.

Cell viability test

We used 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay to assess cell viability. We seeded 3000 cells in 96-well plates. Cells were treated with indicated drugs for 72 h. After inoculation, cells were then incubated with 0.5 mg/ml MTT at 37°C for 2 h. The medium was replaced with 100 µl of dimethyl sulfoxide per well to dissolve the precipitates. Colorimetric analysis using a 96-well microplate reader (BioTek Instruments) was performed at a wavelength of 490 nm.

Xenograft of tumor-bearing SCID mice

Parental HT-29 and two sh-BMI1 HT-29 cells ($2.5*10^6$ cells) were suspended in 30 µl of Hank's balanced salt solution and injected subcutaneously into the left hind leg of each 7-week-old nonobese diabetic/severe combined immunodeficient mouse (SCID). After 2 weeks, five mice of each set were grouped and injected i.p. with 1 mg/kg paclitaxel three times weekly. After 3 weeks, mice were sacrificed for further analysis. The tumor volume was calculated using the equation: tumor volume = (length × width^2)/2. All experiments were performed in accordance with the animal care and use guideline of Kaohsiung Medical University (Taiwan), and the study was approved number by the Animal Care and Use Committee of Kaohsiung Medical University.

Immunohistochemistry

Mouse tumor samples were cut into 4-µm-thick sections, deparaffined in xylene as previously described [25], and then stained with anti-

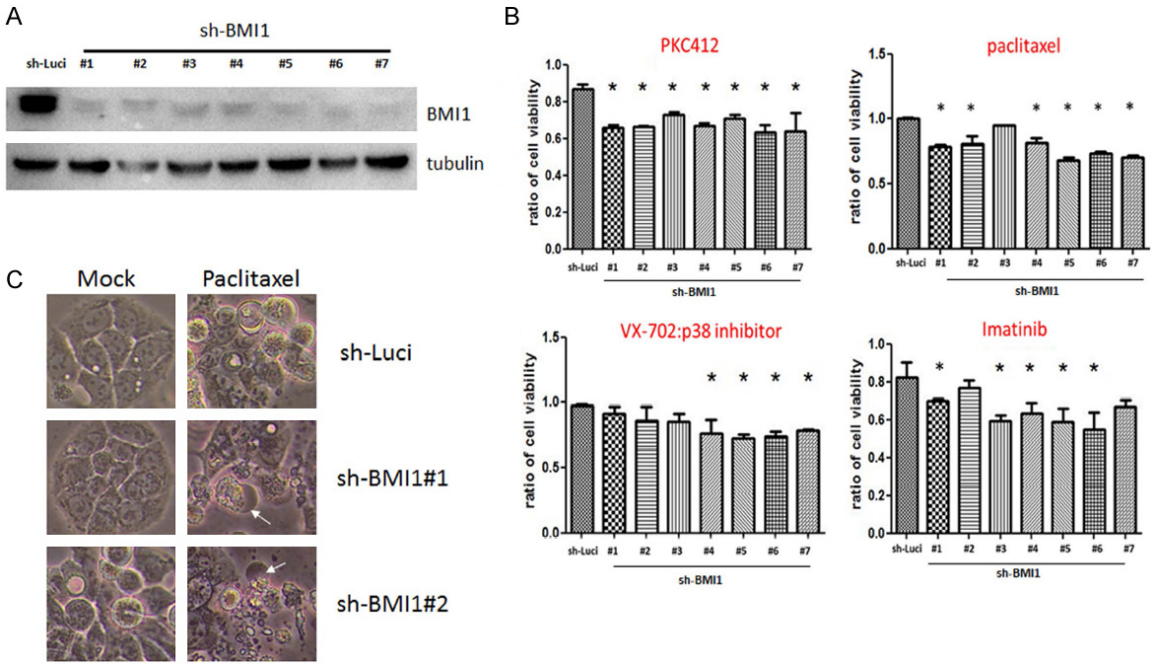

Figure 1. BMI1 knockdown enhances the cytotoxicity of paclitaxel in CRC cells. A. The level of BMI1 protein was determined using western blot analysis. B. Cytotoxicity of indicated protein kinase inhibitor to HT-29 cells. Cells were treated with protein kinase inhibitor (1 μM PKC412; 10 nM paclitaxel; 10 μM VX-702; 10 μM Imatinib) as indicated for 72 hours, and cell viability was determined via the MTT assay. The experiments were repeated three times. Between-group comparisons were performed using one-way ANOVA (*, $P<0.05$). C. Morphology of apoptosis was visualized under a light microscope. White arrowheads in the pictures indicate the nuclei of apoptotic cells.

MCL1 Ab (1:20), anti-p-GSK3beta Ab (1:100), anti-γ-H2AX Ab (1:1000), and anti-Ki-67 Ab (1:150) following the manufacturer's protocol. Afterward, the samples were incubated for 30 min at 25°C with secondary antibodies in the Envision system (Dako, Denmark). Finally, sections were counterstained with hematoxylin and analyzed under a microscope.

Statistical analysis

All experiments were performed in triplicate. Data are expressed as mean ± SD. Multiple comparisons were evaluated using one-way analysis of variance, and between-group comparisons were conducted using two-tailed Student's t-test.

Results

BMI1 depletion increases the cytotoxicity of paclitaxel in CRC cells

To clarify the role of BMI1 in cellular response to protein kinase inhibitors in CRC cell lines, we first established stably BMI1-depleted cells among HT-29 cells (**Figure 1A**). Consequently, we tested the cytotoxicity of BMI1 inhibition with small molecules reported in the Library of Pharmacologically Active Compounds, in a cell viability assay. We evaluated 143 protein kinase inhibitors, including 31 included in ongoing clinical trials. We found that only four protein kinase inhibitors, namely, paclitaxel, PKC412, VX-702, and imatinib, had an inhibitory effect in BMI1-depleted cells (**Figure 1B**). Notably, the BMI1-depleted cells exhibited more prominent apoptosis after treatment with paclitaxel (**Figure 1C**). Collectively, the apoptotic effect was more profound in CRC cells with low BMI1 expression treated with paclitaxel.

Combination of paclitaxel and depleted BMI1 expression promote extrinsic apoptotic signaling pathways and hinder cell survival in CRC

Caspase cascade plays a vital role in programmed cell death, which involves several initiator caspases (e.g., caspase 8) and executioner caspases (e.g., caspase 9). Caspase-3 is a convergence of the intrinsic and extrinsic

apoptotic pathways, and its activation leads to DNA fragmentation, membrane blebbing, and then apoptosis [20]. Therefore, we then investigated whether the levels of caspase change in BMI1-depleted cell influence the efficacy of paclitaxel. As shown in **Figure 2A**, the activation of caspase 3 was more increased in BMI1-depleted cells after treatment with paclitaxel compared with untreated control cells. However, there was no profound inhibitory effect in other protein kinase inhibitors. To further clarify the role of BMI1 in apoptosis, we analyzed the cytotoxicity of paclitaxel in parental HT-29 and BMI1-depleted HT-29 cells using flow cytometry. As shown in **Figure 2B**, the amount of cells in the early and late apoptotic phase was increased in two BMI1-depleted cells (sh-Luci: 10.87%; sh-BMI1#1: 15.46%; sh-BMI1#2: 19.59%). These data indicated that BMI1 knockdown further enhances the apoptotic effect of paclitaxel in CRC. To further clarify these findings, we studied the contribution of caspases in paclitaxel-mediated apoptosis of BMI1-deficient cells. Flow cytometry analysis showed that the apoptotic effect could be reversed by pretreatment with 50 μM zVAD-fmk, a pan caspase inhibitor, without caspase-8 inhibitor (Z-IETD-FMK) alone (**Figure 2C**). These findings imply that, combined with BMI1 deficiency, paclitaxel may contribute to caspase 8-independent cell death. These results suggested that intrinsic apoptotic pathways might play a role in paclitaxel-induced apoptosis of BMI1-depleted cells.

Paclitaxel inhibits intrinsic apoptotic signaling pathways in BMI1-deficient CRC cells

Members of the Bcl-2 family proteins harbor similar conserved sequences in Bcl-2 homology (BH) domains (BH1-BH4), which have diverse functional effects on different member proteins [21]. The pro-survival proteins, such as BCL-2, BCL-w, Bfl-1/A1, and MCL-1, ensure cell survival, whereas the pro-apoptotic proteins including Bim, Bid, Bad, Bak, and Bax induce cell death [22, 23]. MCL-1, a pro-survival protein of the Bcl-2 family, has anti-apoptotic capability and serves as the main regulator of cell death [24]. BCL-2 targets the phosphorylate protein kinase Raf-1 and inactivates cell death signaling pathways [25, 26]. As shown in **Figure 2**, paclitaxel main affected intrinsic apoptotic signaling pathways in BMI1-deficient CRC cells. Next, we determined whether BMI1 regulates the expression of the Bcl-2 family. Here, we found no significant change in the amount of pro-apoptotic regulators, including Bid, Bad, Bak, and Bax, after BMI1 depletion (**Figure 3A**). Considering previous results, the regulatory mechanism of BMI1 may be assumed to affect the function or stability of pro-survival proteins, but not that of pro-apoptotic proteins. Further, we found lower MCL-1 and BCL-2 expression after BMI1 depletion. Moreover, the pro-survival proteins MCL-1 was suppressed more profoundly after treatment with paclitaxel in BMI1-depleted cells (**Figure 3B**). However, the inhibitory effect was not observed in gene expression. Eliminating BMI1 did not lead to lower mRNA expressions of MCL-1 (**Figure 3C**). These results raise an interesting question of the protein stability significance of the MCL-1 in BMI1 deficient cells. To answer this question, we used cycloheximide (CHX), a protein synthesis inhibitor, to measure the degradation of the MCL-1 in BMI1 knockdown cells. As shown in **Figure 3D**, MCL-1 stabilization is affected in BMI1 depleted cells for the indicated periods of time. Evidences indicated that GSK-3β mediated phosphorylation of Ser^{155} and Ser^{159} on MCL-1 in conjunction with Thr^{163}, then destabilized MCL-1, and hindered the anti-apoptotic function of MCL-1 [27]. Further, the initial "priming" phosphorylation of Thr^{163} mediated by JNK (C-Jun N-terminal kinase) was a prerequisite for Ser^{159} phosphorylation [28]. JNK1 also plays a role in the regulation of BCL-2. The JNK pathway induced phosphorylation and inactivation of BCL-2 [29]. Supporting our assumption, we found that the phosphorylation form of JNK and GSK-3β increased expressions in sh-BMI1 HT29 cells compared with parental HT-29 cells (**Figure 3E**). Overall, these results indicated that BMI1 downregulation induced cell apoptosis and impeded cell survival by affecting the stability of pro-survival proteins, and the synergically suppressive effect was more profound in paclitaxel treatment.

Inhibition of BMI1 combined with paclitaxel treatment retards cell proliferation and tumor growth in vivo

To further investigate the role of BMI1 in colorectal tumor growth in vivo, studies were performed in SCID mice. As shown in **Figure 4A** and **4B**, BMI1 knockdown resulted no significant difference in tumor size compared with that in HT-29 parental cells. In addition, the

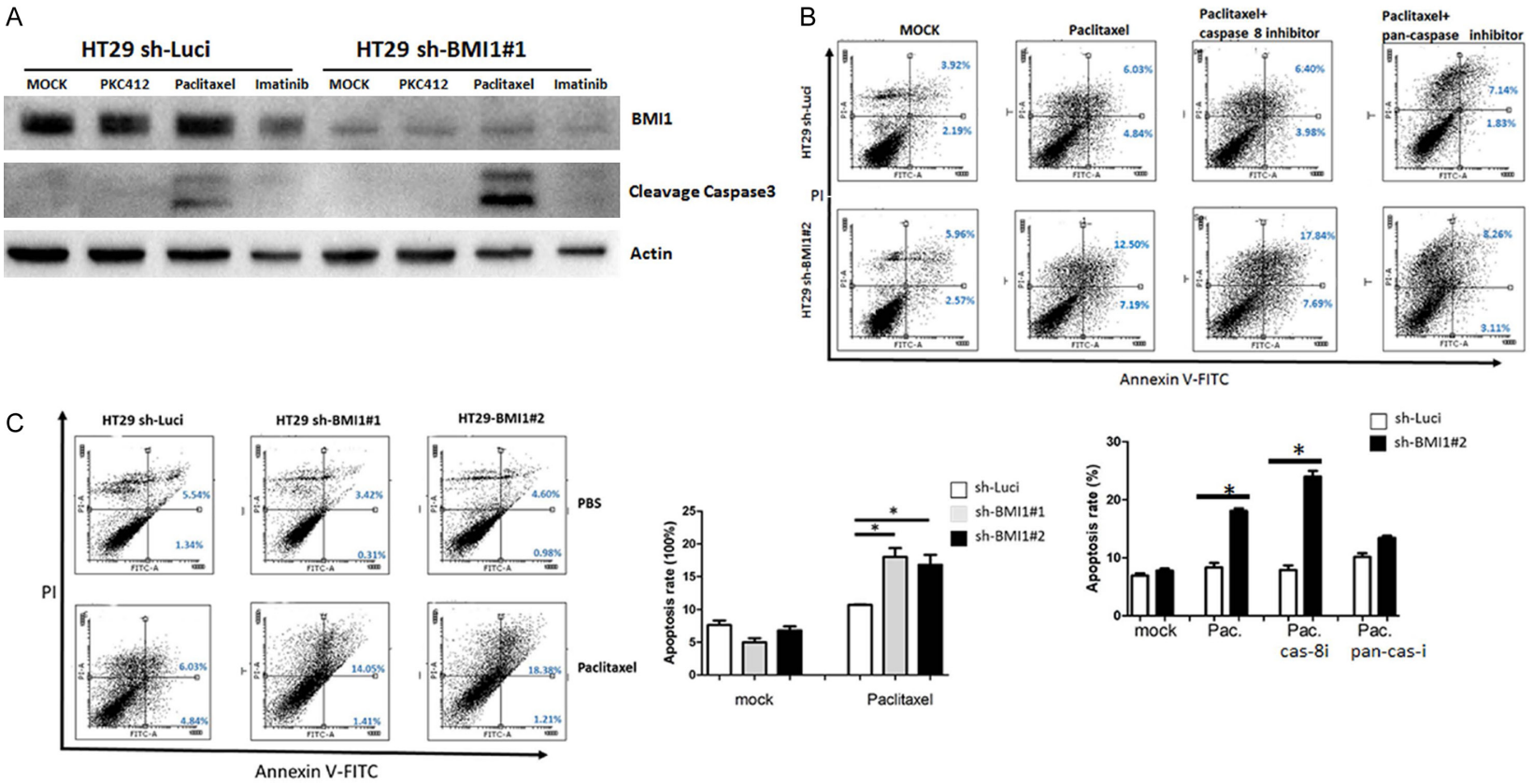

Figure 2. Paclitaxel has stronger effect on apoptotic signaling pathways in BMI1-deficient cells. A. Protein levels that cleaved caspase-3 and BMI1 were determined in cells treated with indicated inhibitors using western blot analysis. B. The percentage of apoptotic cells treated with paclitaxel was detected via dual staining with annexin V-FITC and PI in HT-29 cell lines. HT-29 BMI1-deficient (sh-BMI1) cell lines were analyzed via flow cytometry. The experiments were repeated three times. Between-group comparisons were performed using one-way ANOVA (*, P<0.05). C. The percentage of apoptotic cells treated with paclitaxel combined with caspase-8 inhibitor (Z-IETD-FMK) or pan-caspase inhibitor (zVAD-fmk) was detected via dual staining with Annexin V-FITC and PI in HT-29, and HT-29 BMI1-deficient (sh-BMI1) cell lines were analyzed via flow cytometry. The experiments were repeated three times. Between-group comparisons were performed using one-way ANOVA (*, P<0.05).

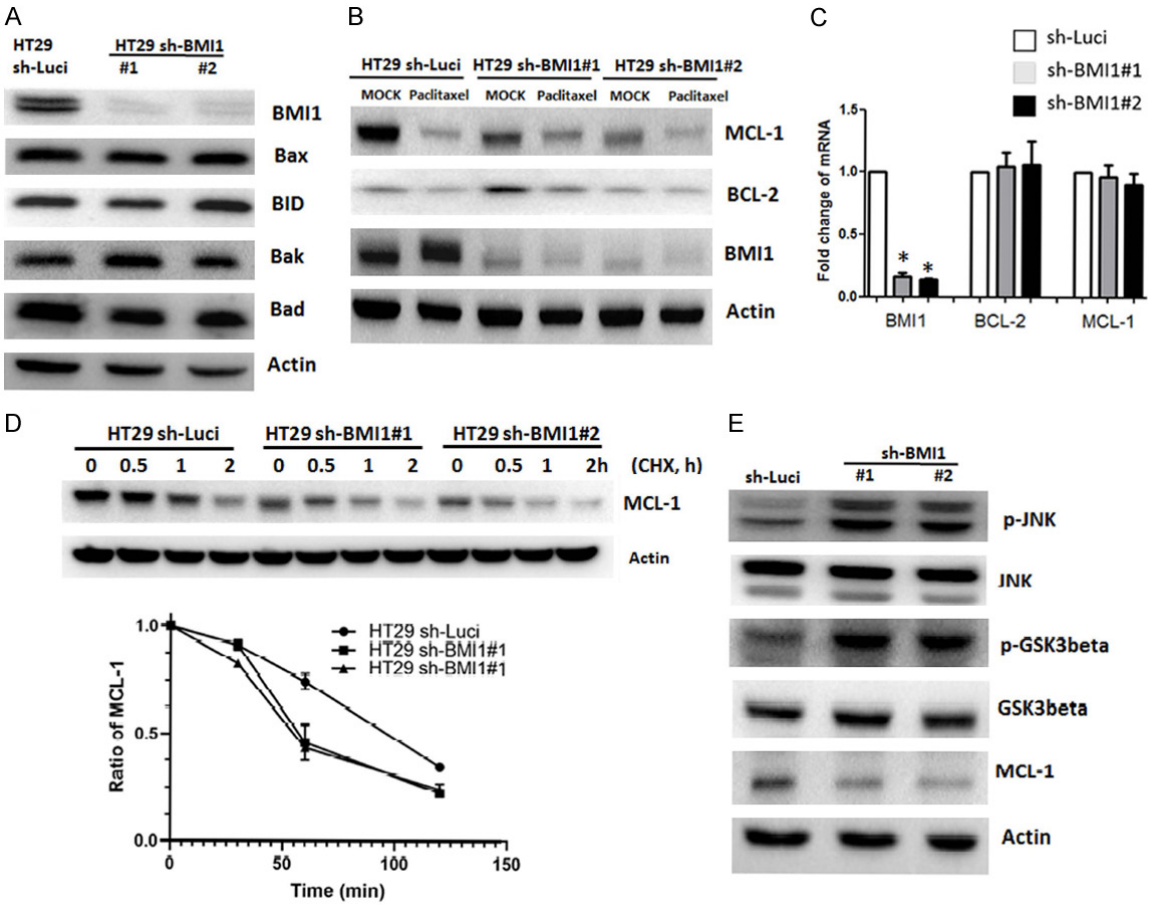

Figure 3. BMI1 deficiency induces activation of GSK3beta and JNK in modulating the phosphorylation of MCL-1. A. Protein levels of Bak, Bad, Bax, BID, and BMI1 in cells treated with paclitaxel, determined using western blot analysis. B. Protein levels of MCL-1, BCL-2, and BMI1 in cells treated with paclitaxel, determined using western blot analysis. C. Expression of BCL-2 and MCL-1 mRNA in HT-29-sh-Luci, HT-29-sh-BMI1#1 cells, and HT-29-sh-sh-BMI1#2 cells determined using real-time qPCR. Columns represent the mean results from PCR assays performed in triplicate and normalized to GAPDH (*, $P<0.05$). D. Densitometry was utilized to quantify MCL-1 protein levels after normalization with Actin to obtain the percentage of MCL-2 degradation (mean ± SD; n=3). Error bars indicate SD. E. Protein levels in HT-29-sh-Luci, HT-29-sh-BMI1#1 cells, and HT-29-sh-sh-BMI1#4 cells analyzed via western blot analysis.

inhibitory effect in BMI1-depleted CRC cells was intensified with paclitaxel treatment. Immunohistochemistry (IHC) staining showed that, compared with paclitaxel alone, the combination of paclitaxel treatment and BMI1 depletion better restrained the expression of Ki-67 as a cell proliferation marker (**Figure 4C**). Notably, we found decreased MCL-1 expression and increased activation of γ-H2AX and p-GSK3beta (Ser9) in BMI1-depleted cells treated with paclitaxel.

Discussion

Our data indicated that the downregulation of MCL-1 through the activation of GSK3beta and JNK is driven by BMI1 depletion. Paclitaxel is an antitumor agent widely used in different solid tumors. It mainly targets microtubules to affect its stabilization and induce cell cycle arrest and promotes antitumor immunity [30]. Paclitaxel is the first-line chemotherapeutic for breast and ovarian cancer. Meanwhile, it has unsatisfactory efficacy in CRC and induces drug resistance. Several mechanisms of resistance have been reported including mutation of tubulin, cellular total antioxidant capacity, and overexpression of P-glycoprotein [31-33]. However, the kinase inhibitory effect has not been clarified in previous studies. The 70 kDa ribosomal S6 kinase (p70S6K) plays a crucial role in cell growth and survival. Paclitaxel induces phosphorylation of p70S6K at both serine and threonine residues and inactivates this kinase in a concentration- and time-dependent manner to

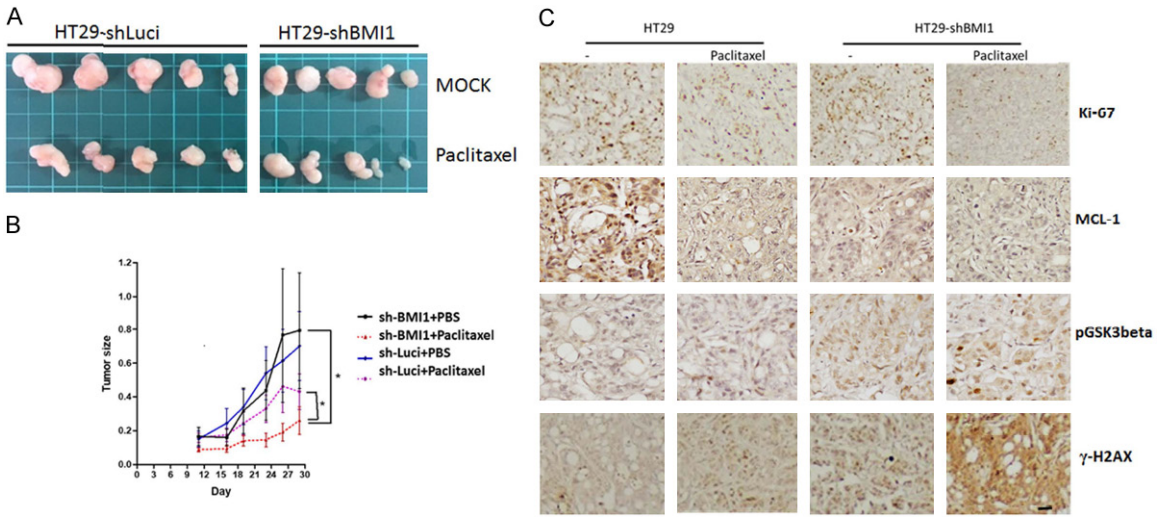

Figure 4. Bmi1 deficiency combined with paclitaxel retards tumor growth in vivo. A. Paclitaxel treatment in BMI1-deficient cells leads to the reduction of tumor growth. Representative images of HT-29-sh-Luci and HT-29-sh-BMI in SCID mice. B. Quantitative analysis of tumor size after injection in designated cells at 30 days. Statistical comparisons were conducted using one-way ANOVA (*, P<0.05). C. Representative staining for Ki-67, γ-H2AX, MCL-1, and p-GSK3beta in tumors with mock and paclitaxel treatment groups. Original magnification: × 40, scale bar: 10 μm.

interfere cell proliferation [34]. To date, protein kinase inhibitors still have unsatisfactory efficacy in CRC. Regorafenib, a small-molecule multikinase inhibitor, prolonged median overall survival by only 1.4 months in patients with metastatic CRC; further, some patients developed hand-foot skin reactions, fatigue, and diarrhea [10]. Thus, improving treatment response to protein kinase inhibitors in CRC has become a concern in recent years.

Regulating BMI1 expression has been an attractive approach in CRC because BMI1 expression is correlated with prognosis and several mechanisms in genetic modification [14, 19]. Previous studies showed that over 50% of tissues in colon cancer showed higher BMI1 expression than the corresponding normal tissue [19]. Further, BMI1 is a repressive transcriptional factor that blocks the binding of RNA polymerase and transcriptional factors, leading to the regulation of stem cell and epithelial-mesenchymal transition [14]. Regulating BMI1 not only manipulates the behavior of cancer stem cell but also arrests the cell cycle to completely eliminate the tumor [35]. However, there is currently no therapeutic agent that directly targets the BMI1 gene or protein.

Apoptosis plays a crucial role in carcinogenesis and cancer progression. BMI1 regulates the apoptosis of cancer cells [36]. BMI1 knockdown suppresses cancer cell proliferation and colony formation [36, 37] and increases susceptibility to chemotherapy and radiation therapy [38, 39]. The molecular mechanisms of BMI1 function have been elucidated in previous studies. However, the precise regulatory mechanisms by which BMI1 influences apoptosis are still unclear. In nasopharyngeal cancer, BMI1 depletion led to the downregulated expression of phospho-AKT and anti-apoptotic protein BCL-2 but induced (upregulated) the expression of the pro-apoptotic protein Bax [40]. In addition, BMI1 also suppressed apoptosis by activating NF-κB signaling and angiogenesis [41]. BMI1 knockdown promoted Noxa expression, which is a pro-apoptotic BH3-only member of the Bcl-2 family of proteins [42].

In our study, BMI1 depletion activated the intrinsic apoptotic signaling pathway in triggering the activation of caspase 3. BMI1 modulated the expression of the anti-apoptotic regulators MCL-1 in the anti-apoptotic protein stability instead of their gene expressions. However, it did not influence pro-apoptotic proteins (i.e., BH3-family protein, including Bim, Bid, Bad, Bak, and Bax). With respect to protein function, the regulation of MCL-1 expression is focused on phosphorylation and inactivation of protein function. The upstream regulators GSK-3β and JNK promote the phosphorylation of anti-apop-

totic regulators [29, 43]. Based on our findings, we hypothesized that BMI1 knockdown may enhance phosphorylated GSK-3β and JNK expression, promoting MCL-1 phosphorylation. The phosphorylation of different phosphoresidues in MCL-1 influences protein stability, dimerization, and function [43]. The anti-apoptotic regulators lose their function either via degradation or inactivation.

To clarify the mechanism by which BMI1 is involved in the apoptosis pathway, we first compared the extent of apoptosis between four groups, namely, the control group, paclitaxel alone, paclitaxel plus caspase 8 inhibitor, and paclitaxel plus total caspase inhibitor, in both HT29 and shBMI1-HT29 cells via flow cytometry. We found increased cell death in paclitaxel treatment; however, the apoptosis was not restored after treatment with paclitaxel plus caspase 8 inhibitor. Surprisingly, combining a total caspase inhibitor with paclitaxel overcame apoptosis, implying that the treatment of paclitaxel in sh-BMI1 CRC cells generates intrinsic apoptosis. Similar results were found with respect to tumor growth assessed via IHC staining.

Interestingly, high BMI1 expression was noted in 27.7%-74.5% of CRC patients who had worse clinical outcomes and prognosis [19, 44, 45]. These in vitro and in vivo findings suggest that CRC patients with high BMI1 expression have limited sensitivity to apoptosis-inducing treatment and may require BMI1 inhibitors (e.g., PT209) to enhance cancer cell vulnerability to protein kinase inhibitors. Meanwhile, CRC patients with low BMI1 expression who have relatively active intrinsic and extrinsic apoptosis pathways would show better response to paclitaxel.

In conclusion, the results indicate that paclitaxel has a stronger suppressive effect on tumor growth and proliferation in CRC with low BMI1 expression. Specifically, we found a synergic therapeutic effect between protein kinase inhibitors, particularly paclitaxel, and BMI1 downregulation. This synergic effect not only induces intrinsic and extrinsic cell apoptosis but also inhibits cancer cell survival in vivo and in vitro. Further, BMI1 regulates the intrinsic apoptotic pathway by modulating the stability of pro-survival proteins MCL-1, instead of pro-apoptotic proteins. Collectively, these results indicate that CRC treatment may be individualized according to BMI1 expression, with paclitaxel being specifically indicated for patients with low BMI1 expression.

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Disclosure of conflict of interest

None.

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