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SMK-17, a MEK1/2-specific inhibitor, selectively induces apoptosis in β-catenin-mutated tumors

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Although clinical studies have evaluated several MEK1/2 inhibitors, it is unlikely that MEK1/2 inhibitors will be studied clinically. BRAF mutations have been proposed as a responder marker of MEK1/2 inhibitors in a preclinical study. However, current clinical approaches focusing on BRAF mutations have shown only moderate sensitivity of MEK1/2 inhibitors. This has led to insufficient support for their promoted clinical adoption. Further characterization of tumors sensitive to MEK inhibitors holds great promise for optimizing drug therapy for patients with these tumors. Here, we report that β-catenin mutations accelerate apoptosis induced by MEK1/2 inhibitor. SMK-17, a selective MEK1/2 inhibitor, induced apoptosis in tumor cell lines harboring β-catenin mutations at its effective concentration. To confirm that β-catenin mutations and mutant β-catenin-mediated TCF7L2 (also known as TCF4) transcriptional activity is a predictive marker of MEK inhibitors, we evaluated the effects of dominant-negative TCF7L2 and of active, mutated β-catenin on apoptosis induced by MEK inhibitor. Indeed, dominant-negative TCF7L2 reduced apoptosis induced by MEK inhibitor, whereas active, mutated β-catenin accelerated it. Our findings show that β-catenin mutations are an important responder biomarker for MEK1/2 inhibitors.

onstant activation of the mitogen-activated protein kinase (MAPK) pathway due to aberrant activation of receptor tyrosine kinase and due to K-Ras mutations or BRAF mutations is common in human tumors and represents a major factor in abnormal cell growth[1]. Approximately 30% of all human tumors contain an activating Ras mutation[2]. Oncogenic V600E mutations in BRAF have been found in 66% of melanomas and in 69% of papillary thyroid tumors[3,4]. Furthermore, aberrant activation of the MAPK pathway correlates with tumor progression and poor prognosis in patients with various tumors. The constitutive expression of MEK1/2 is sufficient to induce transformation[5,6]. Targeting MEK1/2 with small-molecule inhibitors is an attractive treatment strategy, as all potentially aberrant oncogenic signaling upstream is preventable[7].

Furthermore, several MEK inhibitors (e.g., PD184352/CI-1040 and PD0325901) have been evaluated in clinical studies[8–10]. However, MEK inhibitors have met with limited clinical success in single-agent therapy.

Wnt signaling also plays a central role in cell proliferation and differentiation[11]. In the absence of a Wnt stimulus, β-catenin interacts with AXIN1/2, glycogen synthase kinase-3β (GSK-3β, encoded by GSK3B), and the adenomatous polyposis coli protein (APC). GSK-3β phosphorylates β-catenin and triggers its ubiquitination and degradation by β-Trcp[12]. Activation of the Wnt pathway inhibits GSK-3β-dependent phosphorylation of β-catenin and then stabilizes β-catenin. The form of β-catenin resulting from hypophosphorylation then translocates to the nucleus and interacts with TCF7L2, leading to increased expression of c-Myc or cyclin D1[13,14]. Mutations in β-catenin enhance its stability and promote the subsequent transactivation of TCF7L2; such transactivation is found in a wide variety of human tumors[15].

Although MAPK and Wnt signals are important intracellular signaling pathways, the mechanism of their crosstalk is not yet fully elucidated. In this study, we classified human tumor cell lines as either sensitive or resistant to a MEK inhibitor, as determined by apoptosis induction. We show that mutated β-catenin in tumor cells promotes MEK inhibitor-induced apoptosis. Our results suggest that β-catenin mutations are a novel predictive marker of MEK inhibitors.
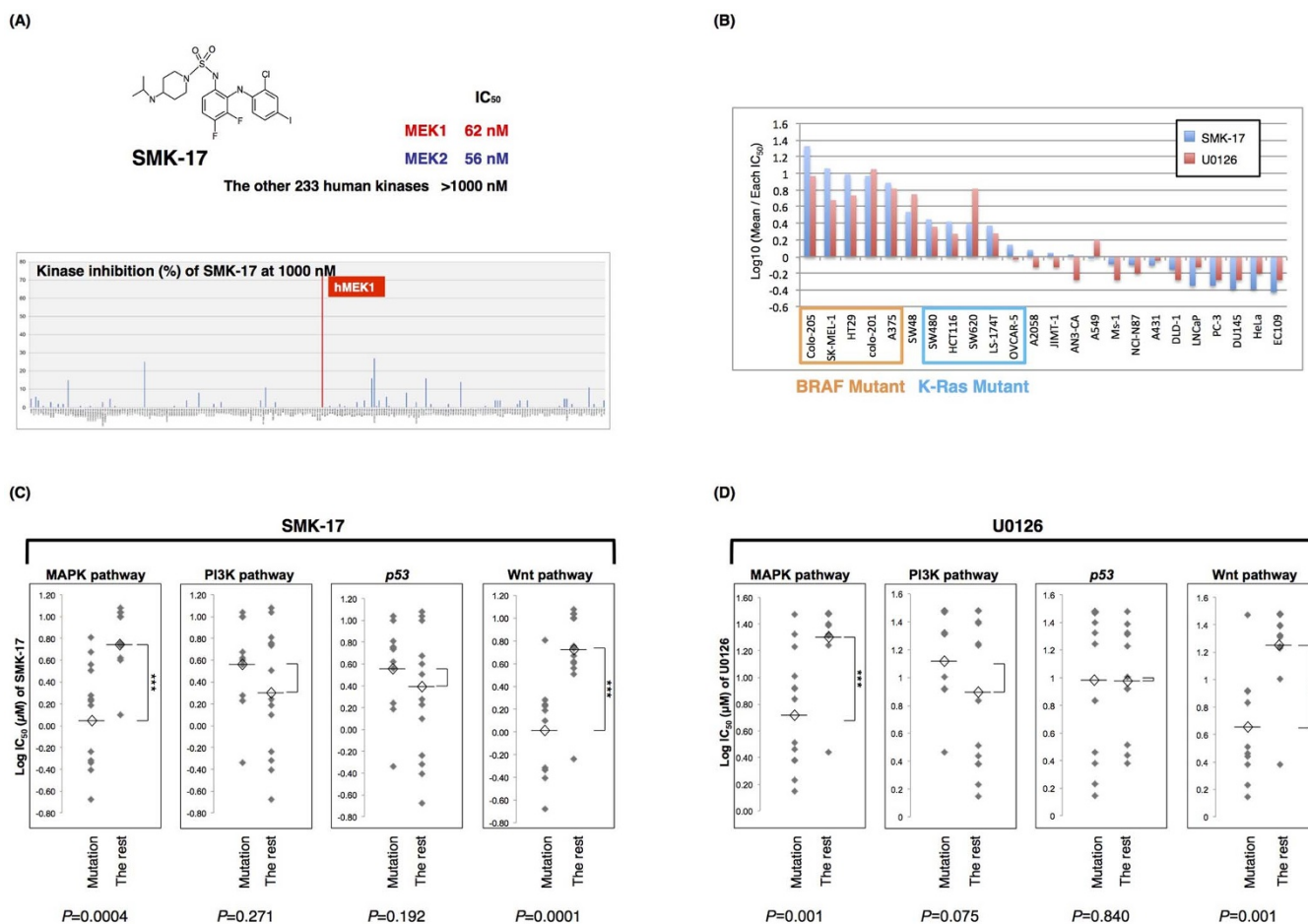
Figure 1 | Anti-tumor activities of SMK-17 *in vitro*. (A) The chemical structure of SMK-17, its IC$_{50}$ values for the cell-free kinase reactions of human MEK1 and MEK2, and kinase inhibition profiling on 233 human kinases at 1000 nM of SMK-17. (B) IC$_{50}$ values for a panel of tumor cell lines harboring several oncogenic mutations treated with the MEK inhibitors, SMK-17 and U0126. Cell numbers were determined 72 h after compound treatment. IC$_{50}$ values were calculated from dose-response curves, as described in the Materials and Methods. The figures show the differential growth inhibition pattern of the compounds against the 24 tumor cell lines tested. The log y-axis shows the difference between the mean of the log IC$_{50}$ for the 24 cell lines and the log IC$_{50}$ for each cell line. Values are the mean of three independent experiments. (C) SMK-17 and (D) U0126 selectively inhibit the proliferation of MAPK-mutated tumor cell lines. Scatter plots show the log IC$_{50}$ of mutated cell line and the rest one in the MAPK, PI3K, *p53*, and Wnt pathway (in a left-to-right fashion). *P* values were obtained by performing a Student's *t*-test for group comparisons.

Results

SMK-17 inhibited cell proliferation in tumor cell lines with activated K-Ras or BRAF mutations. SMK-17 was a potent and highly selective MEK1/2 inhibitor with an IC$_{50}$ of 62 and 56 nM, respectively (Figure 1A). Several studies have reported a wide range of sensitivity toward the anti-proliferative effects of MEK1/2 inhibitors[16]. As we have previously confirmed, MEK1/2 inhibition by SMK-17 without off-targeting kinases has remarkably high selectivity[17]; thus, we examined the effect of SMK-17 on several types of human tumor cell lines. As shown in Figure 1B, cell lines with BRAF mutations, including colo-205, SK-MEL-1, HT-29, colo-201, and A375 cells, were sensitive to SMK-17. Cell lines with K-Ras mutations, such as SW480, HCT 116, SW620, LS-174T, and OVCAR-5 cells, were moderately sensitive to SMK-17. Scatter plots showing the log IC$_{50}$ of cell lines with mutations in the MAPK pathway, including mutations in K-Ras or BRAF, revealed that these cell lines were completely sensitive to SMK-17 (Figure 1C). We similarly analyzed the effect of SMK-17 in cells with mutations in the PI3K pathway (including mutations in PI3K or PTEN), p53, and the Wnt pathway including APC and β-catenin. Significant differences were not observed in cell lines harboring PI3K and p53 mutations. On the other hand, there were significant differences in sensitivities between cell lines with Wnt pathway mutations and the

other cell lines (Figure 1C). Similar results were obtained when another MEK inhibitor, U0126, was used instead of SMK-17 (Figure 1D).

SMK-17 induced apoptosis in active β-catenin-mutant cell lines. We evaluated A375, HT-29, HCT 116, colo-205, and SW48 cells, which are representative cell lines that are highly sensitive to SMK-17 (i.e., an IC$_{50}$ of less than 2.0 μM), with respect to the effect of SMK-17 on the MAPK pathway, cell cycle, and apoptosis induction. SMK-17 inhibited ERK1/2 phosphorylation in all cell lines in a dose-dependent manner (Figure 2A), as determined by analysis of intracellular MEK1/2 activity. Treatment with 1 μM SMK-17, which almost completely inhibited ERK1/2 phosphorylation, induced G1 cell cycle arrest in BRAF-mutant A375 and HT-29 cells (Figure 2B). However, even at concentrations of up to 10 μM, SMK-17 did not induce apoptosis in BRAF-mutant A375 and HT-29 cells, as shown by the increase in the sub-G1 population, as determined via flow cytometry (Figure 2B). On the other hand, treatment of cells with SMK-17 at the minimum effective concentrations for the inhibition of ERK1/2 phosphorylation induced apoptosis in BRAF-mutant colo-205 and K-Ras-mutant HCT 116 cell lines, as shown by the presence of cleaved PARP (Figure 2A). SMK-17-induced apoptotic cell death was further
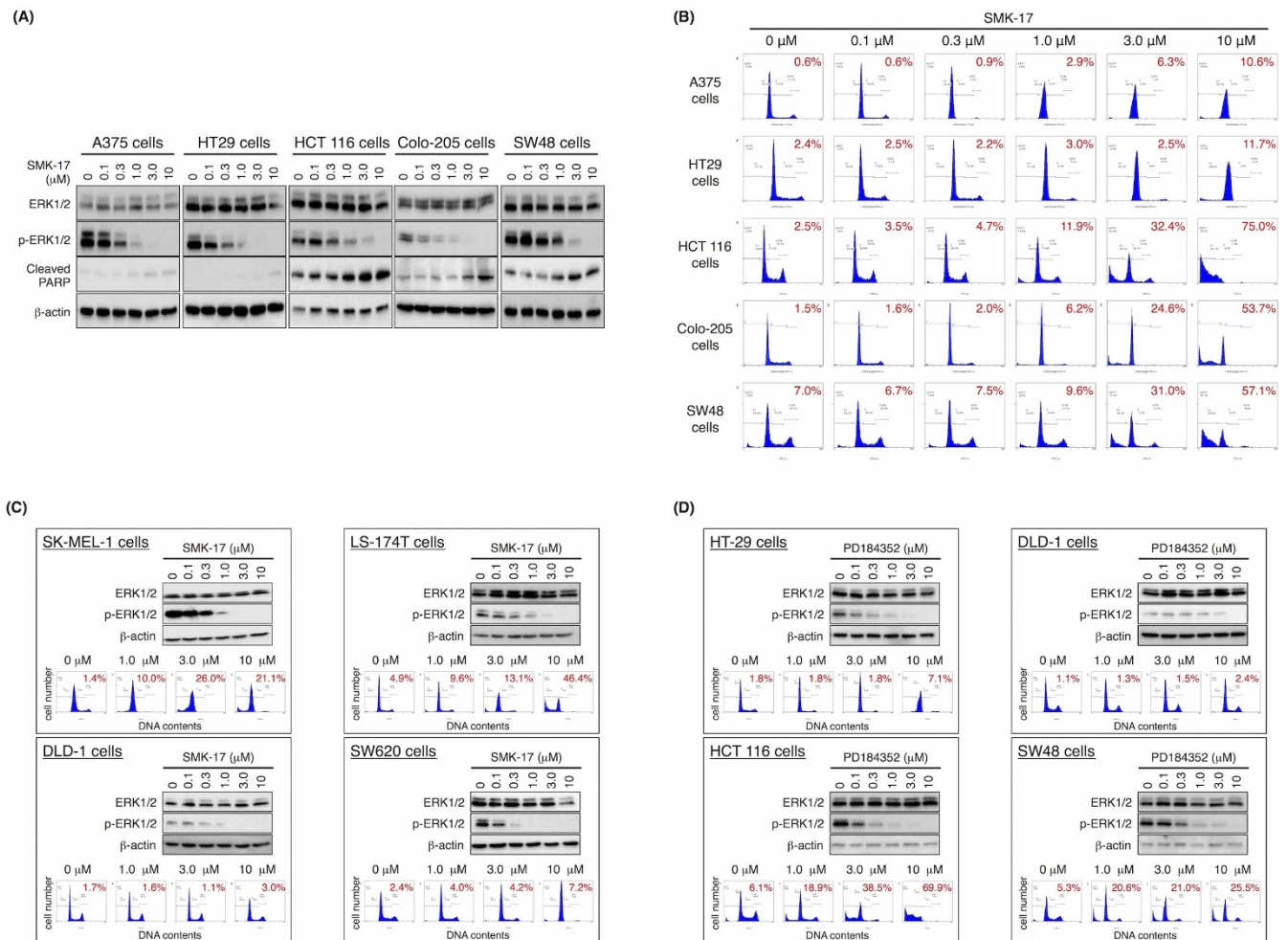
Figure 2 | MEK inhibitors selectively induced apoptosis in β-catenin-mutated cell lines. (A) SMK-17 selectively induced apoptosis in β-catenin-mutated cell lines at the lowest effective concentration. Cells were treated with the indicated concentrations of SMK-17 and, after 24 h, cell lysates were probed for ERK1/2, phospho-ERK1/2 (p-ERK1/2), cleaved PARP, and β-actin expression via western blot. (B) SMK-17 induced G1 arrest in β-catenin wild-type cells and apoptosis in β-catenin-mutant cells. Cells were treated with the indicated concentrations of SMK-17. After 48 h, the sub-G1 and G1 populations were analyzed by flow cytometry. The percentage of the sub-G1 population (red) is indicated in each histogram. (C) SMK-17 induced apoptosis in β-catenin-mutant cells. Cells were treated with the indicated concentrations of SMK-17. After 24 h, cell lysates were probed for ERK1/2 and phospho-ERK1/2 (p-ERK1/2) by western blot. After 48 h, the sub-G1 and G1 populations were analyzed by flow cytometry. (D) PD184352 induced apoptosis in β-catenin-mutant cells. Cells were treated with the indicated concentrations of PD184352. After 24 h, cell lysates were probed for ERK1/2 and phospho-ERK1/2 (p-ERK1/2) by western blot. After 48 h, the sub-G1 and G1 populations were analyzed by flow cytometry.

confirmed by the detection of a sub-G1 population in HCT 116 and colo-205 cells (Figure 2B). However, the SW48 cell line, which does not carry mutations in either the K-Ras or BRAF gene, still underwent apoptosis under conditions in which ERK1/2 phosphorylation is inhibited by SMK-17 (Figures 2A and 2B). These results indicate that the apoptosis-inducing ability of SMK-17 is independent of the status of the MAPK pathway in tumor cells.

Therefore, we focused on the differences between the cell lines that underwent either G1 arrest or apoptosis by examining the β-catenin status of cells; cell lines that underwent apoptosis after SMK-17 treatment commonly harbored a mutation of β-catenin activation. The dependence of SMK-17-induced apoptosis on such mutation was further explored. Other cell lines also harboring such mutation, specifically, SK-MEL-1 and LS-174T cells, also underwent apoptosis when treated with SMK-17, as indicated by the increased cell population at the sub-G1 peak (Figure 2C). However, DLD-1 and SW620 cells, which harbor a K-Ras mutation but not a mutation of β-catenin activation, did not undergo apoptosis under conditions in which the ERK1/2 phosphorylation was completely inhibited by SMK-17 (Figure 2C). Next, we examined whether another MEK inhibitor also

selectively induced apoptosis in cell lines harboring activating mutations in β-catenin. As shown in Figure 2D, 1.0 μM PD184352 completely inhibited ERK1/2 phosphorylation and induced cell cycle arrest at the G1 phase in HT29 cells. Similarly, PD184352 also inhibited ERK1/2 phosphorylation in DLD-1 cells without leading to a significant increase in the sub-G1 population (Figure 2D). On the other hand, PD184352 inhibited ERK1/2 phosphorylation at concentrations of 0.3 μM and higher in HCT 116 cells and gradually induced a dose-dependent increase in the sub-G1 population. Similar results were obtained in SW48 cells, indicating that MEK inhibitors selectively induced apoptosis in cell lines harboring active mutations in β-catenin.

Activating mutations in β-catenin are associated with SMK-17-induced apoptosis. To test the hypothesis that active mutations in β-catenin may be responsible for apoptosis mediated by MEK inhibitor, we transfected the active form of β-catenin (S37A, S45A) and EGFP expression plasmids into A375 cells harboring wild-type β-catenin and evaluated SMK-17-induced apoptotic cell death by analyzing the levels of cleaved PARP via western blotting. As

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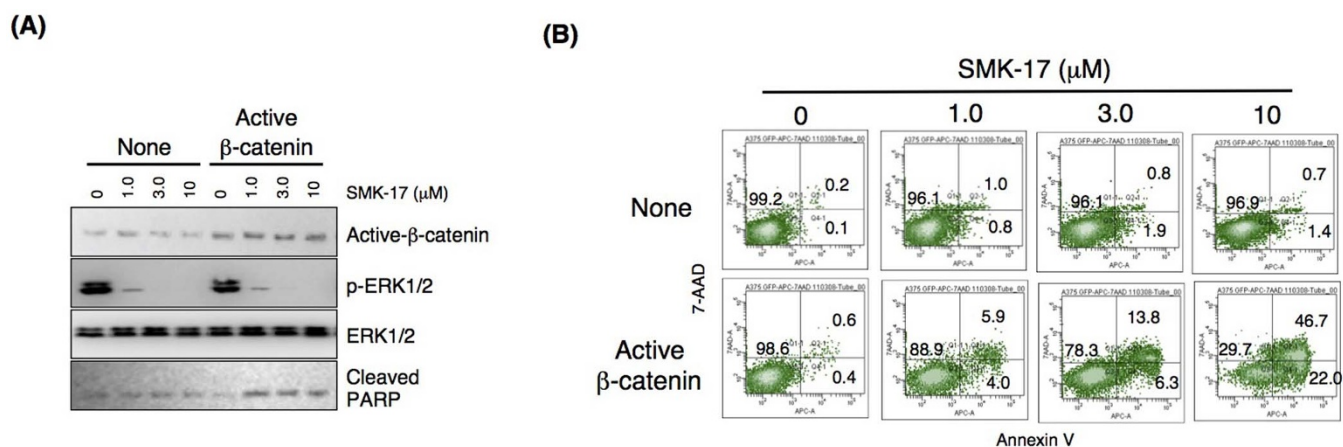
Figure 3 | Overexpression of the active form of β-catenin induced apoptosis in SMK-17-treated A375 cells. A375 cells were transfected with the control vector or with a vector for the active form of β-catenin. Transfected cells were treated with DMSO or SMK-17 (1.0, 3.0, and 10 μM). After 48 h, (A) cell lysates were probed for ERK1/2, phospho-ERK1/2 (p-ERK1/2), and cleaved PARP by western blot. (B) Cells were stained with annexin V/APC and 7-AAD and then analyzed by flow cytometry, with gates set to include only EGFP-positive cells. Apoptotic cells were defined as being annexin V-positive.

shown in Figure 3A, expression of the active form of β-catenin did not affect SMK-17-induced inhibition of ERK1/2 phosphorylation, although apoptosis was observed after SMK-17 treatment in A375 cells. To quantitatively detect early apoptosis and late apoptosis induced by SMK-17, active β-catenin/EGFP-transfected A375 cells were stained with annexin V/APC and 7-AAD and then analyzed by flow cytometry, with the gates set to include only EGFP-positive cells. As shown in Figure 3B, SMK-17 at concentrations of up to 10 μM did not affect the proportion of annexin V^+/7-AAD$^-$ cells (early apoptosis) and annexin V^+/7-AAD$^+$ cells (late apoptosis) in control, EGFP-expressing A375 cells. On the other hand, the percentage of not only early apoptotic, but also of late apoptotic A375 cells expressing the active form of β-catenin increased dose-dependently. At 10 μM SMK-17, 22.0% of A375 cells expressing active β-catenin were early apoptotic, and 46.7% were late apoptotic. In contrast 1.4% of control EGFP-expressing A375 cells were early apoptotic and 0.7% were late apoptotic. These results indicate that expression of the active form of β-catenin is required for SMK-17-induced apoptosis.

TCF7L2 activity is related to apoptosis induced by MEK inhibitor in β-catenin-mutant cell lines. To show that SMK-17-induced

apoptosis could be regulated by the enhancement of β-catenin-dependent transcriptional activation, A375 cells were co-transfected with the TOPFlash and *Renilla* luciferase plasmids. Transfected cells were treated with 50 ng/mL recombinant Wnt3a for 24 h, and their luciferase activity was measured. Wnt3a was found to accelerate TCF7L2 transcription in A375 cells (Figure 4A). SMK-17-induced apoptosis in untreated and Wnt3a-treated A375 cells was analyzed by western blotting for cleaved PARP. We found that levels of cleaved PARP increased after combination treatment with SMK-17 and Wnt3a relative to levels after treatment with either agent (Figure 4B). To further confirm that TCF7L2 transcriptional activity is involved in SMK-17-induced apoptosis, we next examined the effect of TCF7L2 downregulation on SMK-17-induced death of HCT 116 cells harboring an active mutation of β-catenin. As shown in Figure 5A, TCF7L2 transcriptional activity was successfully reduced after dominant-negative TCF7L2 (DN-TCF7L2) plasmid transfection (0.061 to 0.033). DN-TCF7L2-transfected cells were then treated with SMK-17 and stained with annexin V/APC and 7-AAD for the analysis of apoptosis via flow cytometry. SMK-17-induced apoptosis in control EGFP-expressing HCT 116 cells (at 10 μM SMK-17, 23.2% were annexin V^+/7-AAD$^-$ and 17.1% were annexin V^+/7-AAD$^+$) was greatly diminished by the expression of
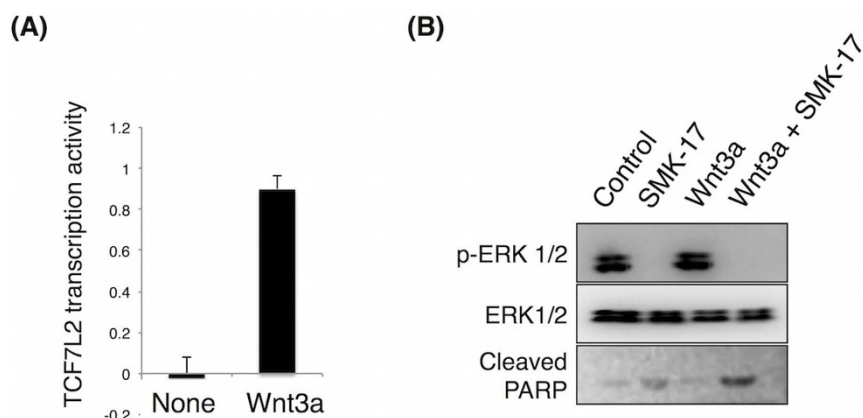
Figure 4 | Activation of Wnt/β-catenin signaling by Wnt3a induced apoptosis in A375 cells. (A) A375 cells were co-transfected with the TOPFlash and *Renilla* luciferase plasmids. Transfected cells were treated with 50 ng/mL Wnt3a for 24 h, and their luciferase activity was measured. Wnt signaling activity in terms of TCF7L2 transcription was monitored by using the TOPFlash/continuous *Renilla* luciferase assay. The ratio of the firefly luciferase intensity of TOPFlash to that of *Renilla* luciferase was used as an indicator of TCF7L2 transcriptional activity. (B) Cells were treated with either DMSO or with 10 μM SMK-17, with or without 50 ng/mL recombinant Wnt3a, for 48 h. Cell lysates were probed for ERK1/2, phospho-ERK1/2 (p-ERK1/2), and cleaved PARP via western blot. Cleaved PARP was observed after combination treatment with SMK-17 and Wnt3a.
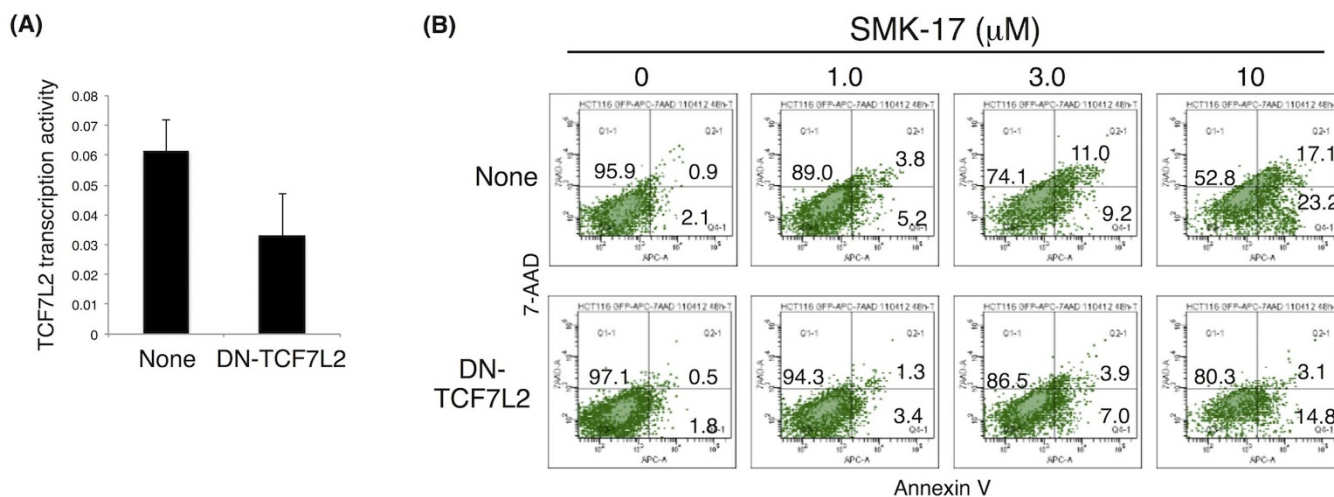
Figure 5 | Expression of DN-TCF7L2 reduced SMK-17-induced apoptosis in HCT 116 cells. HCT 116 cells were transfected with DN-TCF7L2. (A) Luciferase activity was monitored through co-transfection with the TOPFlash and *Renilla* luciferase plasmids. (B) HCT 116 cells expressing DN-TCF7L2 were treated with the indicated concentrations of SMK-17 for 48 h and stained with annexin V/APC and 7-AAD. Cells were analyzed by flow cytometry with gates including only EGFP-positive cells.

DN-TCF7L2 (at 10 μM SMK-17, 14.8% were annexin V$^+$/7-AAD$^+$ and 3.1% were annexin V$^+$/7-AAD$^+$; Figure 5B). Thus, SMK-17-induced apoptosis positively correlated with TCF7L2 transcriptional activity.

Involvement of c-Myc-mediated apoptosis gene expression in SMK-17-induced apoptosis of β-catenin-mutant cells. As β-catenin-mediated increase in TCF7L2 transcriptional activity is involved in SMK-17-induced apoptosis, we next examined the possibility that SMK-17-induced apoptosis in β-catenin-mutant cells is mediated by c-Myc, whose promoters are directly activated by TCF7L2. To do this, HCT 116 cells were transiently transfected with *c-myc* siRNA, and their cell viability following SMK-17 treatment was examined. As shown in Figure 6A, SMK-17 dose-dependently decreased both c-Myc and phosphorylated ERK1/2 levels (Figure 6A). Furthermore, as the PARP cleavage and sub G1 population detected by flow cytometry analysis show, silencing c-Myc expression suppressed SMK-17-induced apoptosis. Indeed, as shown in Figure 6B and 6C, Wnt3a-induced PARP cleavage and sub G1 population in SMK-17-treated A375 cells were also inhibited by the knockdown of c-Myc. These results indicate that c-Myc is a key regulator involved in apoptosis induced by MEK inhibitor when the Wnt pathway is activated, for example, during Wnt3a stimulation or when β-catenin is mutated.

Apoptosis induction of SMK-17 in β-catenin-mutated xenograft models. Because SMK-17 selectively induced apoptosis in cell lines harboring activating mutations in β-catenin *in vitro*, we next examined the ability of SMK-17 to induce apoptosis *in vivo*. Active β-catenin-mutant tumor cells (SW48 and colo-205 cells) and wild-type β-catenin tumor cells (A375 and HT-29 cells) were injected subcutaneously into NOD-SCID mice (SW48 cells) or nude mice (other cell lines) to establish xenograft models. SMK-17 or the control vehicle was then administered by oral gavage. We confirmed the inhibitory activity of SMK-17 toward intratumor MEK by monitoring levels of phosphorylated ERK1/2 in SW48 and A375 cells *in vivo* after a single dose of SMK-17 (Supplemental Figure S1). As shown in Figure 7A, tumor regression in response to multiple daily oral administration of SMK-17 at half MTD (200 mg/kg) was observed only in active β-catenin-mutant xenograft models, namely, models using SW48 and colo-205 cells. On the other hand, the maximum effect of SMK-17 at MTD dosing (400 mg/kg) was just growth inhibition of A375 and HT-29 cells expressing wild-type β-

catenin, without significant body weight loss. TUNEL staining revealed significant apoptosis induction in SW48 tumor tissues from SMK-17-treated but not in SW48 tumor tissues from control mice (Figure 7B). In contrast, apoptotic cells were not observed in the SMK-17-treated A375 xenograft model.

Discussion

SMK-17 is a highly selective inhibitor of MEK1 and MEK2 in a non-ATP-competitive manner, possibly because of its binding to the allosteric pocket of MEK1/2[17]. In addition, we reported that SMK-17 inhibited cell growth in colon-26 and HT-29 cell lines harboring highly phosphorylated MEK1/2 and ERK1/2[17]. In this study, we investigated the effect of SMK-17 on several types of human tumor cells in detail. We found SMK-17 to be highly effective in limiting the proliferation of tumor cells with aberrant activated MAPK pathway signaling (Figures 1B and 1C). Furthermore, tumor cells harboring BRAF mutations were found to be more sensitive to SMK-17 than were tumor cells with K-Ras mutations. This is similar to results for another MEK inhibitor[18]. These observations can be explained by the fact that BRAF mutations could directly influence MEK activity, as it is an immediate upstream effector of MEK, whereas Ras mutations activate additional signaling pathways that bypass MEK[19]. Thus, BRAF mutations predict a cell's sensitivity to MEK inhibition[19].

Interestingly, SMK-17-sensitive BRAF- and K-Ras-mutant cells could be classified into two groups based on the fate of cells after SMK-17 treatment. One contains tumor cells that undergo SMK-17-induced cell-cycle arrest (BRAF-mutant A375 and HT-29 cells and K-Ras-mutant SW620 cells), and the other consists of tumor cells that undergo apoptosis when SMK-17 inhibits the phosphorylation of ERK1/2 (BRAF-mutant colo-205 cells and K-Ras-mutant HCT 116 and LS-174T cells). Therefore, it is difficult to predict whether SMK-17 elicits cell growth inhibition or apoptosis solely on the basis of their BRAF or K-Ras mutation status.

However, we also found that their sensitivity to the MEK inhibitor was closely related to the status of mutations in canonical Wnt/β-catenin signaling (Figure 1C). Indeed, we found that a common feature of tumor cells that undergo apoptosis following SMK-17 treatment is the presence of an active mutation in β-catenin. Mutations in β-catenin in these cells involve the deletion or the exchange of serine and threonine residues at positions 45 and 33 (HCT 116 cells: S45 deletion, SW48 cells: S33Y, LS-174T cells: S45F, and SK-MEL-1 cells: S33C), thus interfering with its efficient
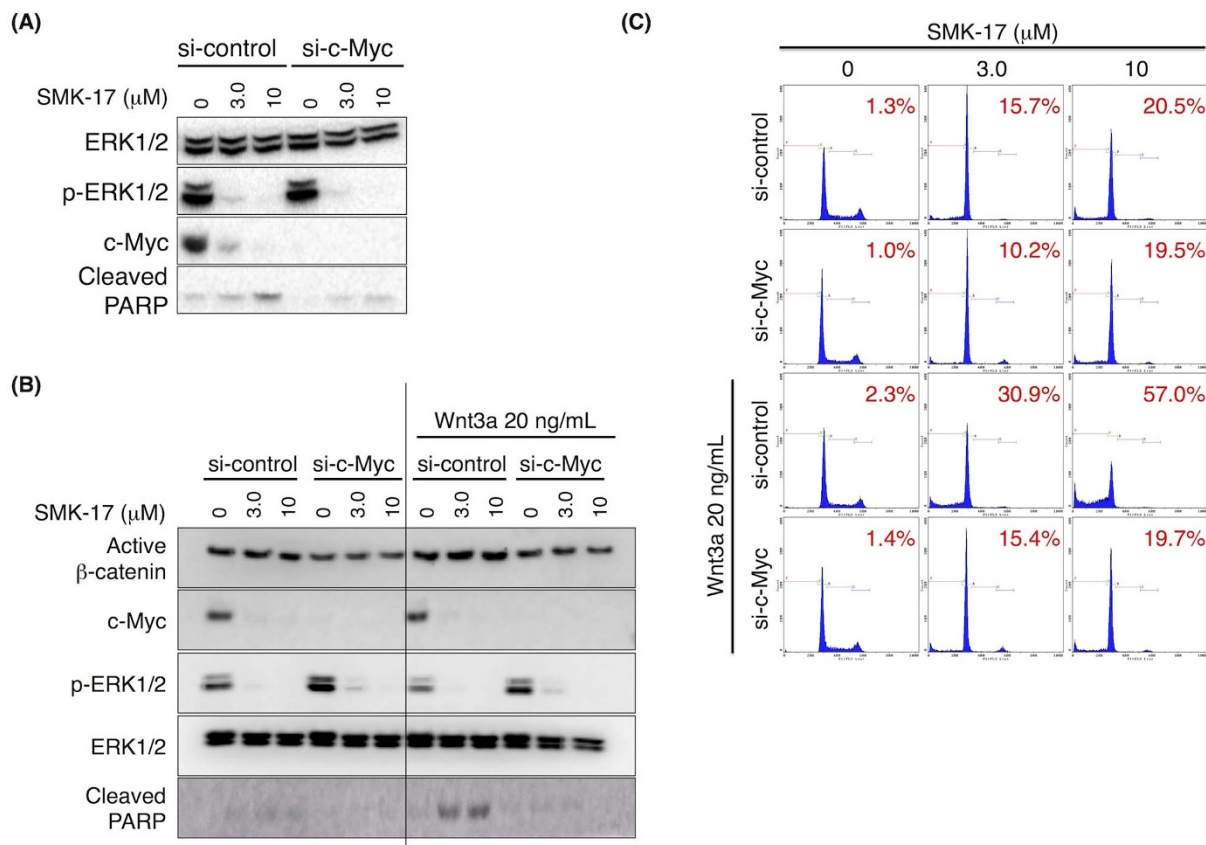
Figure 6 | Involvement of c-Myc in SMK-17-induced apoptosis. (A) HCT 116 cells were transfected with control or *c-myc* siRNA. Transfected cells were treated with the indicated concentrations of SMK-17 for 24 h. Western blotting of cell lysates showed reduced expression of phospho-ERK1/2 (p-ERK1/2) and c-Myc protein and increased expression of cleaved PARP. (B) A375 cells were transfected with control or *c-myc* siRNA. Transfected cells were treated with the indicated concentrations of SMK-17 in the presence or absence of 20 ng/mL Wnt3a for 24 h. The expression of ERK1/2, p-ERK1/2, c-Myc, active β-catenin, and cleaved PARP was detected by western blotting. (C) A375 cells were transfected with control or *c-myc* siRNA. Transfected cells were treated with the indicated concentrations of SMK-17 in the presence or absence of 20 ng/mL Wnt3a for 48 h. The apoptosis induction was detected by flow cytometry analysis after cytological staining using PI.

ubiquitination and degradation by the proteasome[20]. Furthermore, colo-201 and colo-205 cells showed a homozygous A-to-G missense transition mutation at codon 287 in exon 6, resulting in the substitution of serine for asparagine (N287S). Therefore, among the tumor cells tested, only active β-catenin mutants, irrespective of the status of BRAF or K-Ras mutation, underwent apoptosis after treatment with the MEK inhibitor at minimum effective concentrations for the inhibition of ERK1/2 phosphorylation.

A requirement for an active β-catenin mutation for SMK-17-induced apoptosis was confirmed by the following findings: 1) SMK-17 induced apoptosis in A375 cells expressing the active form of β-catenin and harboring wild-type β-catenin (Figure 3A and 3B). 2) Stimulation of Wnt/β-catenin signaling with Wnt3a enhanced the ability of SMK-17 to induce apoptosis in A375 cells (Figures 4A and 4B). 3) Inhibition of β-catenin signaling by the expression of DN-TCF7L2 suppressed SMK-17-induced apoptosis in HCT 116 cells harboring an active β-catenin mutation (Figures 5A and 5B). These results are consistent with the findings of another study showing that apoptosis mediated by targeted BRAF inhibition in melanoma is dependent on β-catenin[21].

What remains unclear is the mechanism by which SMK-17 selectively induced apoptosis in cell lines harboring active, mutated β-catenin. Through interaction with TCF/LEF transcription factors, β-catenin promoted the expression of Wnt target genes such as those for *c-Myc, cyclin D2*, and *CD44*[22]. There is now strong evidence that increased c-Myc expression is a key component of Wnt signaling, which regulates the expression of genes involved in diverse cellular

processes such as apoptosis, cell cycle progression, cell growth, and DNA replication[23,24]. Furthermore, c-Myc protein is known to be phosphorylated and stabilized by ERK1/2[25]; indeed, inhibition of ERK phosphorylation by SMK-17 led to decreased c-Myc expression (Figure 6A). Nevertheless, we confirmed that c-Myc leads to SMK-17-induced apoptosis in tumor cells harboring β-catenin mutations; c-Myc knockdown inhibited SMK-17-induced apoptosis not only in β-catenin-mutant HCT 116 cells, but also in Wnt3a-stimulated A375 cells.

We also determined which c-Myc-mediated, apoptosis-related proteins contributed to SMK-17-induced apoptosis in tumor cells harboring β-catenin mutations. Several studies demonstrated the involvement of BIM in apoptosis induced by RAF or MEK inhibitor[21,26–31]. BIM, the Bcl-2 homology domain 3-only (BH3-only) protein, is known to bind and inhibit prosurvival Bcl-2 family members. Expression of BIM is regulated by c-Myc[26,32] and is inhibited by BRAF-MEK-ERK signaling in mouse and human melanocytes, as well as in human melanoma cells[33]. Therefore, inhibition of BRAF-MEK-ERK signaling increases the levels of BIM expression, inducing cells to undergo apoptosis. We also found that SMK-17 induced an increase in BIM expression levels upon inhibition of ERK phosphorylation in β-catenin-mutant HCT 116, SW48, and colo-205 cells (Supplemental Figure S2). However, knockdown of BIM failed to suppress SMK-17-induced apoptosis in all cell lines tested (data not shown). Therefore, proteins related to c-Myc-mediated apoptosis other than BIM might be responsible for the active β-catenin-mediated apoptosis induced by SMK-17. Furthermore, consistent
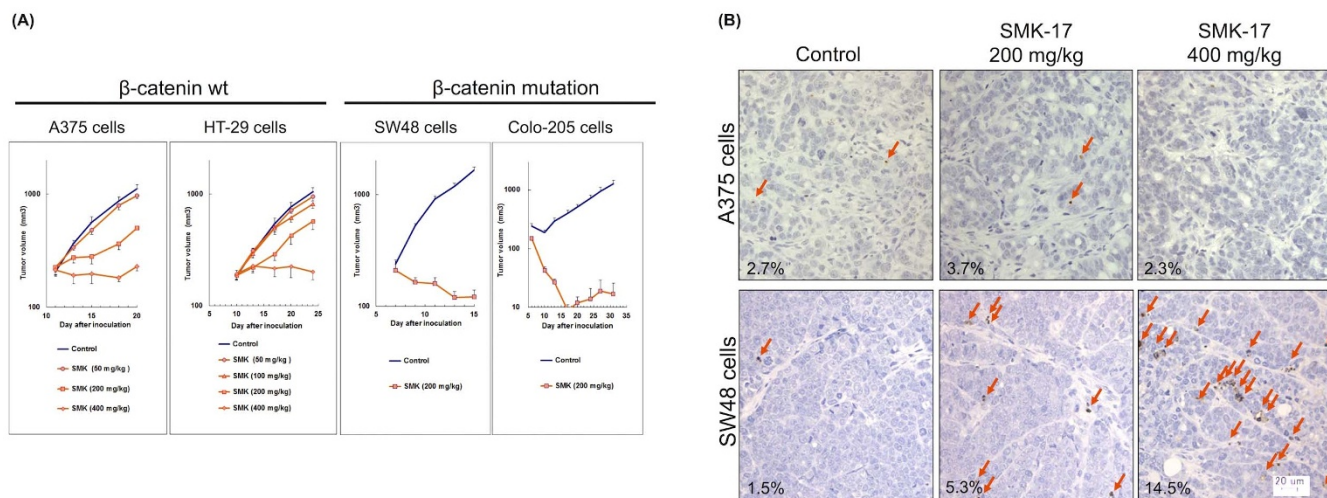
Figure 7 | Antitumor activities of SMK-17 *in vivo*. (A) SMK-17 induced tumor regression and apoptosis in β-catenin-mutated cell lines *in vivo*. SMK-17 suspensions and the vehicle control (0.5% MC) were orally (po) administered at 10 mL/kg once daily. (B) Apoptosis induced by SMK-17 in tumors. TUNEL staining results were obtained from tumors from xenograft mice 24 h after the final oral administration of SMK-17 (q.d. × 2 days). Each indicated score (%) was the mean ratio of the apoptotic cells in the tissue slices from 4 independent tumor-bearing mice.

with the previous report[34], c-Myc protein expression in β-catenin mutated tumor cells seems to be higher than in β-catenin/APC wild-type tumor cells (Supplemental Figures S3A & B). On the other hand, the amount of c-Myc mRNA seemed to not correlate with the transcriptional activity of TCF7L2 (Supplemental Figure S3C). Moreover, c-Myc protein expression had no increase in level in Wnt3a-stimulated A375 cells (Figure 6B). Therefore, the link between TCF7L2 and c-Myc is still controversial, and although knockdown of c-Myc inhibited SMK-17-induced apoptosis, we cannot exclude the possibility that another target protein of the canonical Wnt pathway apart from c-Myc is responsible for apoptosis induced by the MEK inhibitor.

In this study, we used three APC-mutant colorectal tumor cells, namely, SW480, SW620, and DLD-1 cells. SW480 and SW620 cells have a mutant version of the APC tumor suppressor gene, which causes premature termination of the protein at amino acid 133[35,36]. Codon 1416 of the *APC* gene is mutated, and the other allele is lost in DLD-1 cells, resulting in constitutively active TCF/LEF[37]. β-catenin and APC mutations appear to be mutually exclusive, possibly reflecting the fact that both components act in the same pathway.

Nevertheless, SMK-17 induced cell death in SW480 cells and in β-catenin-mutant cells, but not in SW620 or DLD-1 cells (Supplemental Figure S4). SW480 and SW620 cells are two colon tumor cell lines established from the same patient although they have different metastatic potential[38]. They are well-characterized and represent the differing features of primary and metastatic sites[39]. Indeed, we found that expression levels of survivin, an anti-apoptotic protein, was significantly higher in SW620 than in SW480 cells (Supplemental Figure S4); this difference in survivin expression levels may explain why SW620 but not SW480 cells are resistant to SMK-17-induced apoptosis. On the other hand, SMK-17 did not inhibit cell growth or apoptosis in DLD-1 cells. DLD-1 cells expressed high amounts of ERK1/2 protein, although its phosphorylation level was quite low (Supplemental Figure S4). Therefore, it is possible that proliferation of DLD-1 cells is independent of the MEK/MAPK pathway. Thus, although we do not know at present why β-catenin mutations, but not APC mutations, could predict a cell line's sensitivity to MEK inhibitors, we found that TCF7L2 transcriptional activity was higher in APC mutants than in β-catenin mutants (Supplemental Figure S5). This difference may affect the balance of c-Myc-induced expression of pro-apoptotic and anti-apoptotic protein, thus regulating tumor cell survival. Therefore, moderately enhanced TCF7L2 activity that is regulated by β-catenin mutations might be required for apoptosis induced by MEK inhibitor. Another possible explanation is that although tumor initiation by either loss of APC function or oncogenic β-catenin mutations is functionally equivalent, the APC gene may have other functions, namely the ability to capture the kinetochore in the establishment of the mitotic spindle, a loss which underlies malignant progression, therefore, mutations in APC are responsible for chromosomal instability, and are independent of their roles in Wnt signal transduction[40]. Chromosome instability will then confer a mutant phenotype, allowing further malignant progression through numerical and structural chromosomal rearrangements. Thus APC mutation will accumulate mutations in other genes including pro- and anti-apoptotic genes, allowing cells to be resistant to MEK inhibitors. Thus, because of the diverse phenotypes produced by gene instability, it appears challenging to predict the sensitivity of APC mutant cells to MEK inhibitors.

To link these findings to a clinically relevant model, we conducted *in vivo* studies using active β-catenin-mutant xenograft models. Significant tumor regression without any severe toxicity was observed in β-catenin-mutant SW48 and colo-205 cells in response to daily oral administration of SMK-17 (Figures 7A and 7B).

At present, MEK inhibitors have been evaluated in both preclinical and clinical studies as a potential therapeutic option for patients with melanomas carrying the BRAF[V600]-mutation; however, single-agent efficacy has been limited. Therefore, rational combination strategies using MEK inhibitors with other molecularly targeted drugs are expected to lead to greater efficacy. For example, several studies have reported crosstalk between Wnt/β-catenin and MAPK signaling[41,42]. Accordingly, the combination of MEK blockade and Wnt pathway modulation has shown synergistic anti-proliferative effects in both *in vitro* and *in vivo* preclinical colorectal tumor models[43]. However, we report in this study that a MEK inhibitor alone could induce significant tumor regression in β-catenin-mutant xenograft models. As β-catenin is mutated in up to 10% of all sporadic colon carcinomas resulting from point mutations or in-frame deletions of serine and threonine residues phosphorylated by GSK3β[44], our findings support the clinical use of MEK inhibitors as single agents for patients with colorectal carcinoma carrying active β-catenin mutations.

On the other hand, studies with patient cohorts have been reported to determine whether active Wnt signals correlate with clinical responses to the BRAF inhibitor, and it was reported that increased nuclear β-catenin in biopsies is associated with decreased

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survival in patients treated with the BRAF inhibitor[45]. However, apoptosis induction by a MEK inhibitor was found to simply not correlate to nuclear accumulation of β-catenin (Supplemental Figure S6), therefore, studies with larger patient cohorts are required to determine whether β-catenin mutation can predict a better clinical response to MEK inhibitors.

Methods

Compounds and reagents. SMK-17 and PD184352 were synthesized by Daiichi Sankyo Co. Ltd. SMK-17 synthesis was performed according to the procedure described in the patent application WO2004083167. U0126 was purchased from Sigma-Aldrich (St. Louis, MO). Wnt3a was purchased from Wako Pure Chemical Industries (Osaka, Japan).

Cell lines and cell culture. JIMT-1 cells were provided by Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany). OVCAR-5 cells were obtained from the National Cancer Institute (NCI, Bethesda, MD). EC109 cells were provided by Columbia University (New York, NY). MS-1 cells were by Dr. M. Takada at the Rinku General Medical Center (Osaka). The other cell lines used were purchased from the American Type Culture Collection (ATCC, Rockville, MD), and were maintained in the recommended media supplemented with 10% heat-inactivated fetal bovine serum (HyClone Laboratories, Thermo Fisher Scientific, Waltham, MA). Cells were passaged every 2–3 days to maintain exponential growth.

Cell growth inhibition assays. For growth inhibition experiments, cells were plated in black 96-well plates (Corning Inc., Corning, NY) at 1,000–2,000 cells/100 μL/well. After 24 h culture, compounds were added and the cells were incubated for another 72 h. Cell numbers were measured by using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI). Nonlinear curve fitting was performed from triplicate sets of data.

Western blot analysis. Anti-phospho-ERK1/2 (T202/Y204), anti-phospho-MEK (S217/221), anti-ERK1/2, anti-cleaved PARP, anti-c-Myc, anti-BIM, anti-survivin, HRP-linked anti-mouse IgG, and HRP-linked anti-rabbit IgG were from Cell Signaling Technology (Danvers, MA). Anti-unphosphorylated β-catenin was from Promega. Anti-β-actin was from Sigma-Aldrich. PhosphoSTOP phosphatase inhibitor cocktail tablets and Complete Mini protease inhibitor cocktail tablets were from Roche Diagnostics (Indianapolis, IN). Cells were seeded in 6-well plates (Corning) and incubated for 1 day before compound treatment. The cells were then treated with the compounds for the indicated time. Cells were harvested and lysed immediately with RIPA buffer (50 mM Tris HCl pH 7.5, 150 mM NaCl, 1 mM Na₃VO₄, 0.l% SDS, 0.5% deoxycholic acid, 1% IGEPAL CA-630, 1 PhosphoSTOP tablet, and 1 Complete Mini tablet). Tumors were harvested from mice, stored at −80°C, and disrupted by grinding for 30 sec at 2,500 rpm twice with a Multi-Beads Shocker (Yasui Kikai, Shizuoka, Japan) in RIPA buffer. After incubation on ice for 30 min, lysates were centrifuged at 14,000 g for 15 min to remove the insoluble fragments. The supernatants were analyzed by western blotting. Equal amounts of total protein were resolved on SDS-PAGE gels and blotted with the indicated antibodies. The chemiluminescent signal was generated with Western Lightning Plus (PerkinElmer, Waltham, MA) and detected with an LAS-1000 imager (Fujifilm, Tokyo, Japan). The densitometric quantitation of specific bands was performed by using Multi Gauge Software (Fujifilm).

Cell cycle and apoptosis measurement. The percentage of cells in different phases of the cell cycle, including the sub-G1 population, was measured by flow cytometry using propidium iodide (PI, Wako) staining[46]. In brief, cells were treated with each compound for the indicated time, fixed with 70% ethanol at 4°C, and then stained with 50 μg/mL PI and 10 μg/mL RNase A (Wako) for 20 min at 37°C. PI fluorescence was measured on an EPICS ALTRA (Beckman Coulter, Brea, CA).

Detection of early apoptotic cells was determined by using annexin V/APC and 7AAD (BD Biosciences, San Jose, CA) according to the manufacturer's protocol. Briefly, 1 × 10⁵ cells were exposed to each compound and washed with PBS twice. They were then incubated at room temperature with annexin V/APC and 7-AAD for 15 min. Annexin V/APC- and 7-AAD-stained cells were enumerated with a FACSCanto (BD Biosciences). Annexin V/APC-positive or -negative cells were regarded as apoptotic and non-apoptotic cells, respectively.

In the vector-transfection experiments, we co-transfected the EGFP expression vector as a transfectant marker with DN-TCF7L2 (Merck Millipore, Billerica, MA) or an active, mutated human β-catenin (S37A and S45A) expression vector (Daiichi Sankyo Co. Ltd.). EGFP-negative group were gated out by the cutoff results of non-transfectant during FCM analysis.

TCF7L2 reporter and expression vector. We used commercially available TOPFlash and *Renilla* luciferase reporters (Invitrogen Life Technologies, Carlsbad, CA) together with the DN-TCF7L2 vector. Transfection of plasmid vectors was performed by using Lipofectamine LTX (Invitrogen Life Technologies) according to the manufacturer's protocol. Reporter activity was measured by the Dual-Glo Luciferase Assay System (Promega).

RNA interference. Double-stranded siRNA oligonucleotides against *c-myc* (Dharmacon, Lafayette, CO) and non-coding siRNA (negative control; Invitrogen Life Technologies) were used for RNA interference assays. Reverse transfection was performed by using RNAiMAX (Invitrogen Life Technologies) according to the manufacturer's protocol.

Anti-tumor testing *in vivo*. Specific pathogen-free female nude mice (BALB/cA Jcl-nu) were purchased from CLEA Japan. NOD-SCID mice were from Charles River Laboratories International Inc. SMK-17 was suspended in 0.5% methyl cellulose solution (Wako) and given daily to the animals by gavage at a volume of 10 mL/10 kg body weight. Control animals received 0.5% methyl cellulose solution (vehicle control). For the SW48 xenograft study, a suspension of 1 × 10⁷ cells was injected subcutaneously into the axillar region of the mice at day 0. For studies involving other tumor cell lines, 2 × 10⁶ cells were injected subcutaneously into female Balb/c-nu/nu mice at day 0. Tumor-bearing mice were grouped according to tumor volume, and drug administration was performed. The administration volume for each animal was calculated based on their recent body weight. Tumor volumes were calculated according to the following equations: tumor volume (mm³) = 1/2 × (tumor length) × (tumor width)². Animal care and experiments were conducted under the standard operational protocol of the Daiichi Sankyo's Institutional Animal Care and Use Committee.

TUNEL staining of xenograft tumor tissue was based on the protocol using the TdT-Fragel DNA Fragmentation detection kit (Merck Millipore). Tissue sections were viewed at 100× magnification, and images were captured with a digital camera (Olympus, Tokyo, Japan). Four fields per section were analyzed, with the peripheral connective tissue and necrotic regions excluded. The total tissue area analyzed in each section was 2.0 mm² (n = 4/group). The numbers of tumor cell indicating TUNEL-positive were quantified manually.

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Acknowledgments

Author contributions

M.K. and M.I. were responsible for project planning and experimental design. M.K., A.N. and Y.SH. performed most of the experiments on the effects of SMK-17 with culture cells and analyzed the data. M.K. also performed animal experiments. R.M. conducted design of plasmid vectors. Y.SA., T.N. and E.T. helped with experimental design. All authors contributed to discussion and manuscript preparation.

Additional information

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