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Boswellic acid exerts antitumor effects in colorectal cancer cells by modulating expression of the let-7 and miR-200 microRNA family

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Colorectal cancer (CRC) is a complex disease with genetic and epigenetic alterations in many key oncogenes and tumor suppressor genes. The active principle of a gum resin from *Boswellia serrata*, 3-acetyl-11-keto-β-boswellic acid (AKBA), has recently gained attention as a chemopreventive compound due to its ability to target key oncogenic proteins such as 5-lipoxygenase and nuclear factor-kappaB. AKBA has been shown to inhibit the growth of CRC cells; however, the precise molecular mechanisms underlying its anticancer activities in CRC remain unclear. We hypothesized that boswellic acids may achieve their chemopreventive effects by modulating specific microRNA (miRNA) pathways. We found that AKBA significantly up-regulated expression of the let-7 and miR-200 families in various CRC cell lines. Both let-7 and miR-200 are putative tumor-suppressive miRNAs. AKBA modulated the expression of several downstream targets of the let-7 and miR-200 families, such as *CDK6*, *vimentin* and *E-cadherin*. These data were further strengthened by miRNA knockdown studies, which revealed that inhibition of let-7i facilitated enhanced cancer cell proliferation, migration and invasion. In addition, AKBA also induced similar modulation of the let-7 and miR-200 downstream genes in CRC tumors orthotopically implanted in nude mice. These results indicate that AKBA-induced antitumor effects in CRC occur, at least partly through the up-regulation of specific miRNA pathways. Our data provide novel evidence that anticancer effects of boswellic acids are due in part to their ability to regulate cellular epigenetic machinery and further highlight the promise for this phytochemical in the preventative and therapeutic applications of CRC.

Introduction

Although screening modalities for early detection and therapeutic management for human colorectal cancer (CRC) has improved considerably, this disease still remains the second leading cause of cancer-related deaths in the USA and other developed countries (1,2). CRC is a complex disease that occurs as a consequence of many genetic and epigenetic alterations in key oncogenes and tumor suppressor genes. As a component of epigenetic machinery, dysregulated expression of microRNAs (miRNAs) is emerging as an important step in early processes during tumorigenesis, as well as disease progression/metastasis in various malignancies including CRC (3,4). The miRNAs function mainly through their ability to bind to the 3′ untranslated regions of messenger RNAs (mRNAs), leading to suppression of translation or degradation of the corresponding mRNAs. Each miRNA may have up to several hundred gene targets and can act as a tumor suppressor or oncogene depending upon the nature of the targeted genes.

Abbreviations: AKBA, 3-acetyl-11-keto-β-boswellic acid; CRC, colorectal cancer; EMT, epithelial-mesenchymal transition; NF-κB, nuclear factor-kappaB; miRNA, microRNA; mRNA, messenger RNA; RT–PCR, reverse transcription–PCR.

The let-7 and miR-200 families contain several well-known tumor-suppressive miRNAs that are frequently involved in the pathogenesis of human cancers. The let-7 miRNA family is well conserved across different species (5,6). These miRNAs are involved in cell differentiation and proliferation, as well as in tumorigenesis through participation in the epithelial-mesenchymal transition (EMT) process (5,6). The let-7 family consists of 12 different members (let-7a-1, -7a-2, -7a-3, -7b, -7c, -7d, -7e, -7f-1, -7f-2, -7g, -7i and miR-98), which exert their tumor-suppressive effects by targeting oncogenes such as *RAS*, *c-Myc*, *CDC34*, *CDC25A*, *CDK6*, *HMGA2, Lin28* and *Lin28B* (7–12). Previous studies analyzing clinical specimens have shown that let-7 family members are often down-regulated in various cancers including lung, ovarian and gastric cancers (13–15). In contrast to the let-7 family, the miR-200 family is composed of only five family members: miR-200a, -200b, -200c, -141 and -429. This family is known to regulate the EMT process, primarily by targeting EMT-driving transcription factors ZEB1 and ZEB2 (16,17). Members of the miR-200 family are down-regulated in human cancers such as breast, ovarian and lung cancers (16–18). In view of the potential role of these miRNA families in the evolution of carcinogenesis, an intervention targeting the let-7 and miR-200 miRNA pathways may be a promising strategy for cancer therapy (19,20).

Boswellic acids, the major constituents of a gum resin derived from the plant *Boswellia serrata,* have been traditionally used in treatments for various inflammatory diseases including arthritis and chronic colitis (21,22). Acetyl-11-keto-β-boswellic acid (AKBA), one of active principles present in boswellic acids, is known to be a non-redox and non-competitive inhibitor of 5-lipoxygenase (23,24). Boswellic acids exert antitumor effects in human cell lines established from brain tumors (25), CRC (26,27), prostate cancers (28,29) and leukemia(30). More recently, we reported that AKBA inhibits the growth of orthotopic tumors in mice with CRC (31), prostate cancer (29) and pancreatic cancer (32). AKBA suppresses nuclear factor-kappaB (NF-κB) and STAT-3-related pathways, leading to an induction of apoptosis and an inhibition of angiogenesis in cancer cells (31,33,34). The anticancer effects of boswellic acids may be due, in part, to its inhibitory effects on these intracellular signaling pathways; however, the mechanisms underlying anticancer effects of boswellic acids remain to be fully elucidated.

In this study, we hypothesized that AKBA might exert its anticancer effects by regulating specific miRNA pathways. We herein provide novel evidence that the antitumor effects of boswellic acid are in part mediated by their ability to up-regulate the expression of let-7 and miR-200 family, which in turn modulates the expression of their target genes in CRC cells *in vitro* and *in vivo*.

Materials and methods

CRC cell lines and cell cultures

Four human CRC cell lines HCT116, HT29, SW480 and SW620 were purchased from the American Type Culture Collection. All cells were routinely confirmed by morphological and cell growth characteristics, as well as verified by genotyping for various genetic and epigenetic markers. The cells were grown in Iscove's Modified Dulbecco's Medium (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum at 37°C.

Cell growth and proliferation assay

Each cell line with different rates of confluency (HCT116, 1×10^3; HT29, 2×10^3; SW480, 2×10^3 and SW620, 8×10^3) were seeded in 96-well plates. Once adherent, cells were treated with AKBA (Sigma Chemical Company, Saint Louis, MO) at various doses. After 72 h, cell viability was measured with the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay according to standard protocols. The proliferation index in control and AKBA-treated cell lines was measured by bromodeoxyuridine (BrdU) incorporation in the cells 72 h after the treatment (Cell Proliferation ELISA, BrdU; Roche

Diagnostics, Indianapolis, IN). Each experiment was performed in triplicate and the results were obtained from three independent experiments.

Colony formation assay

The cells were seeded into six-well plates (200 cells/well) and were treated with AKBA for 24 h once cells were adherent to the plate. After 8 days, the number of colonies with >50 cells were counted, and the relative change in clonogenic survival of AKBA-treated versus untreated cells was determined. Experiments were performed in triplicate and the results were obtained from three independent experiments.

Cell cycle and apoptosis assays

Cell cycle analyses were performed using flow cytometric sorting of various cell fractions as described previously (35). Apoptosis assays were performed using the fluorescein isothiocyanate Annexin V Apoptosis Detection Kit II (BD Biosciences, Bedford, MA). The data for the sub-G_1 fraction and the apoptotic fraction of cells treated with AKBA for 48 and 36 h, respectively, were analyzed by Flowjo software (Tree Star, Ashland, OR). Results were obtained from two or three independent experiments.

Migration and invasion assays

Migration and invasion activities of the cells were determined using Boyden chambers with 8-mm-pore size membranes coated with matrigel (for invasion assays) or without matrigel (for migration assays; BD Biosciences). For each assay, 5×10^3 cells were suspended in 500 µl of serum-free medium in the upper wells. Serum-containing medium was added to the bottom wells. After 48 h of incubation, the migrated/invaded cells were stained with a standard Diff-Quik protocol. The cell numbers were counted from four representative fields of each membrane. Results were obtained from three independent experiments.

RNA extraction

Total RNA was extracted from either fresh or frozen cell pellets obtained from human CRC cell lines or from orthotropic tumor tissues by the miRNeasy Mini Kit (Qiagen, Valencia, CA). For the quantification of miRNA and mRNA expression in cell lines, RNA was extracted from control and AKBA-treated cell lines from four or more independent experiments.

Real-time reverse transcription–PCR for miRNA expression

The expression of miRNAs was quantified using Taqman real-time reverse transcription (RT)–PCR assays following the manufacturer's protocol (Applied Biosystems, Foster city, CA). In brief, 20 ng of total RNA was reverse transcribed and 6 ng of complementary DNA was used in each well for real-time RT–PCR, and each PCR reaction was performed in duplicate or triplicate.

Real-time RT–PCR for mRNA expression

RNA was reverse transcribed to complementary DNA from 1 µg of total RNA using the Advantage RT-for-PCR Kit (Clontech Laboratories, Mountain View, CA). Power SYBR Green (Applied Biosystems) real-time RT–PCR was performed with the following specific primers: *CDK6* (forward: 5′-GCCTATGGGAAGGTGTTCAA, reverse: 5′- CACTCCAGGCTCTGGAACTT), *E-cadherin* (forward: 5′-CCCACCAC GTACAAGGGTC, reverse: 5′-CTGGGGTATTGGGGGCATC), *vimentin* (forward: 5′-CGAGGAGAGCAGGATTTCTC, reverse: 5′-GGTATCAAC CAGAGGGAGTGA) and *glyceraldehyde 3-phosphate dehydrogenase* (forward: 5′-ACCCAGAAGACTGTGGATGG, reverse: 5′-CAGTGAGCTTC CCGTTCAG). Results were normalized to the expression of *glyceraldehyde 3-phosphate dehydrogenase*. The results were obtained from three or more independent experiments.

Protein immunoblotting

Western immunoblotting experiments were performed as described previously (35). The following primary antibodies were used: anti-c-Myc, cyclin D1, matrix metalloproteinase 9, intercellular adhesion molecule 1 (ICAM-1), cellular inhibitor of apoptosis protein 1 (cIAP-1) and Bcl-xl, all purchased from Santa Cruz Biotechnology (Santa Cruz, CA); antibodies against chemokine (C-X-C motif) receptor 4 from Abcam (Cambridge, MA), against vascular endothelial growth factor purchased from Thermo Fisher Scientific (Fremont, CA), against survivin from R&D systems (Minneapolis, MN), against X-linked inhibitor of apoptosis (XIAP) from BD Bioscience and against β-actin from Sigma–Aldrich as a reference. To determine if AKBA modulates the expression of the let-7 downstream targets in CRC cell lines, we used antibodies against CDK6 (Cell Signaling, Danvers, MA), E-cadherin and vimentin (both from BD Bioscience). A goat antimouse antibody and a goat antirabbit antibody obtained from Santa Cruz Biotechnology were used as secondary antibodies. The change in protein expression levels of CDK6, E-cadherin and vimentin in AKBA-treated and untreated controls were normalized to β-actin, and the band intensities were calculated by densitometry by using ImageQuant 5.2 software (GE Healthcare LifeSciences, Piscataway, NJ).

miRNA inhibitor transfection

The CRC cells were transfected with anti-miRNA inhibitors against hsa-let-7i or negative controls (AM10211 or AM17010; Applied Biosystems) at a final concentration of 100 nM using Lipofectamine2000 (Invitrogen) following the manufacturer's protocols. After 72 h of post-transfection, cell viability, proliferation and miRNA expression were analyzed by MTT, BrdU and real-time RT–PCR analyses as described previously in Materials and methods.

Orthotopic transplantation of tumors into mice

To elucidate if the expression of miRNAs and their target genes was affected by AKBA treatment *in vivo*, we utilized the CRC tumor tissues from the study as described previously (31). In brief, HCT116 cells transfected with luciferase vectors were transplanted into the cecum of male athymic nu/nu mice (4 weeks old). Ten days after implantation, mice were randomly assigned to one of the four treatment groups as follows: (i) corn-oil vehicle only (100 µl daily); (ii) AKBA 50 mg/kg/day, orally by gavage; (iii) AKBA 100 mg/kg/day and (iv) AKBA 200 mg/kg/day ($N = 6$ per each treatment group) for 28 days. The mice were killed 35 days after the randomization, and the primary cecal tumors were excised and stored at −80°C until further analysis.

Immunohistochemistry

The orthotopic tumors were fixed with paraformaldehyde and embedded in paraffin. These tumors were stained with an anti-E-cadherin mouse monoclonal antibody (1:100 dilution, clone NCH-38; DAKO, Carpinteria, CA) using the iVIEW DAB Detection Kit (Ventana Medical Systems, Tucson, AZ) in an automated slide preparation system BenchMark XT (Ventana Medical Systems). Antigen retrieval was performed as a standard automated process at the BenchMark XT for 32 min. The primary antibody was incubated for 32 min. The images were obtained using an AxioSkop2 multichannel epifluorescence microscope equipped with the AxioVision software (Carl Zeiss, Thornwood, NY).

Statistical analysis

The statistical differences between control and treatment groups were determined by paired or non-paired two-sided Student's *t*-test. The values are represented as mean ± standard error of the mean. The differences were regarded as significant when the *P* values were <0.05.

Results

AKBA inhibits cell growth, proliferation, colony-forming ability and migration/invasion activities, and induces apoptosis in CRC cells

In order to elucidate anticancer effects of AKBA on CRC *in vitro*, we first examined the inhibitory effects of AKBA (Figure 1A) on cell growth, proliferation and colony formation efficiency in a panel of CRC cell lines. AKBA inhibited cell growth and proliferation in all CRC cell lines in a dose-dependent manner (Figure 1B). The MTT assay showed that 20 µM AKBA exerted somewhat weaker inhibition of cell viability (0–34%), whereas 40 µM strongly inhibited cell viability (57–89%; Figure 1B, left panel). Similarly, the BrdU assay revealed that treatment with 20 µM AKBA resulted in a weak inhibition of proliferation (0–18%), whereas 40 µM considerably inhibited cell proliferation (41–85%; Figure 1B, right panel). Consistent with these observations, in the colony formation assay, AKBA treatment caused 18% and 92% inhibition of clonogenic survival in cells treated with 20 and 40 µM AKBA, respectively (Figure 1C).

To determine whether AKBA-induced inhibition of growth and proliferation might be due to induction of cell death, we performed apoptosis assays. AKBA-induced apoptosis in CRC cells in a dose-dependent manner, compared with non-treated cells, as evidenced by 6.8% and 18% increase in sub-G_1 fractions (Figure 1D). Furthermore, treatment of cells with AKBA for 48 h drastically inhibited migration (95% and 99% inhibition) and invasion (96% and 99% inhibition) of CRC cells at 10 and 20 µM, which are considerably lower doses than those required to inhibit cell growth and induce apoptosis (Figure 1E).

AKBA inhibited NF-κB-related pathways in CRC cells

Previously, we reported that boswellic acids inhibit NF-κB activation and in turn inhibit several of the NF-κB downstream proteins in other cell lines (33). Therefore, we used western blot analysis to elucidate if AKBA inhibited any of the NF-κB-related proteins in our panel of CRC cells. We observed that the expression of proteins involved in

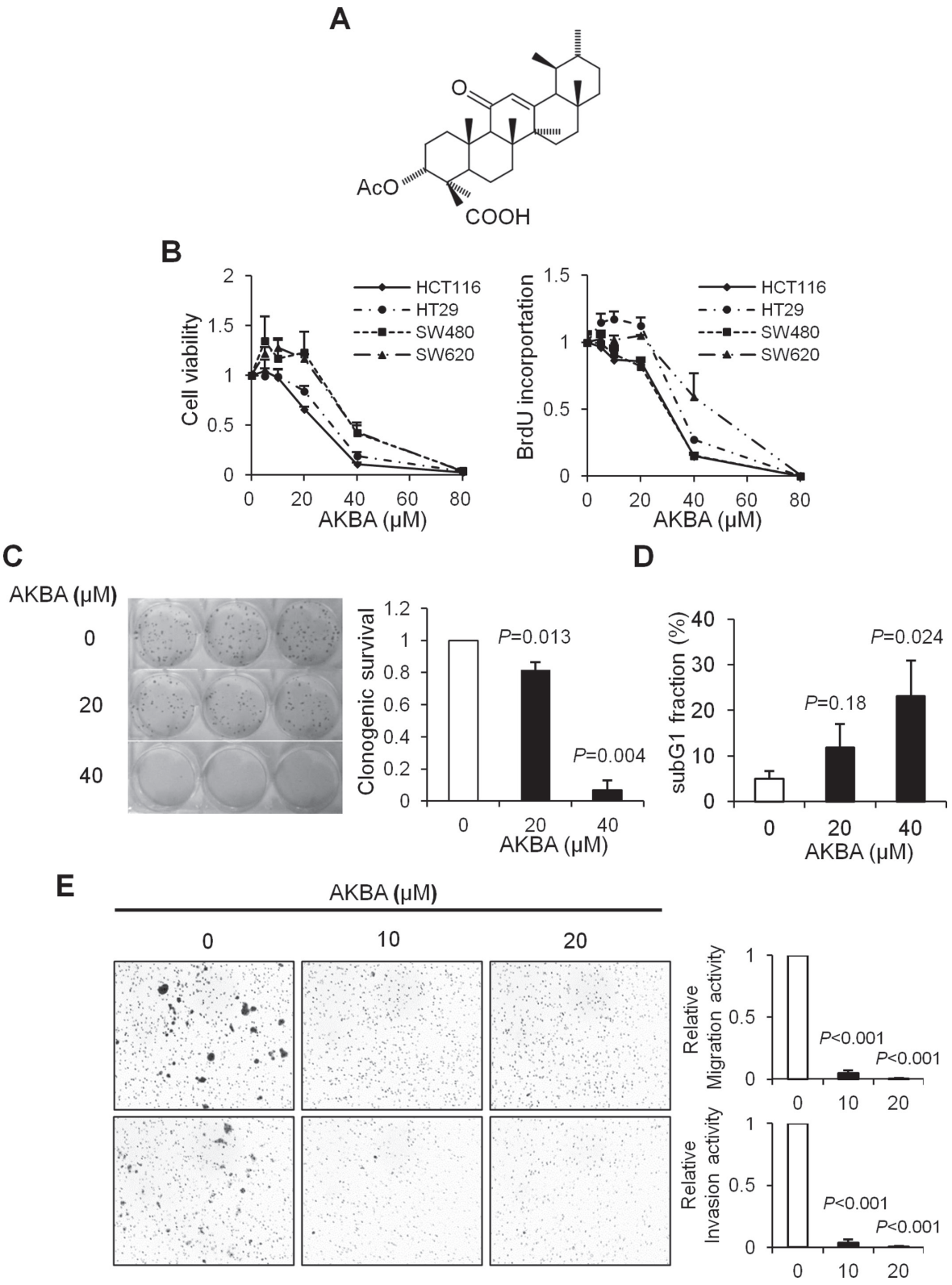

Fig. 1. AKBA inhibits cell viability, proliferation, colony formation, migration/invasion and induces apoptosis in CRC cells. (**A**) Molecular structure of AKBA. (**B**) Cell viability of four CRC cell lines treated with dimethyl sulfoxide alone or AKBA in a MTT assay (left panel) and a BrdU assay (right panel). Clonogenic survival (**C**) and apoptotic fractions (**D**) of HCT116 cells treated with AKBA. (**E**) Migration (upper panel) and invasion activities (lower panel) of HCT116 cells treated with AKBA. Data are obtained from three or more independent experiments. The *P* value is calculated by paired or non-paired two-sided Student's *t*-test.

proliferation (c-Myc and cyclin D1), invasion and metastasis (matrix metalloproteinase 9, intercellular adhesion molecule 1 and C-X-C motif receptor 4), angiogenesis (vascular endothelial growth factor) and apoptosis (Bcl-xL, survivin, cellular inhibitor of apoptosis protein 1 and X-linked inhibitor of apoptosis) were down-regulated after AKBA treatment in a dose-dependent manner (Figure 2).

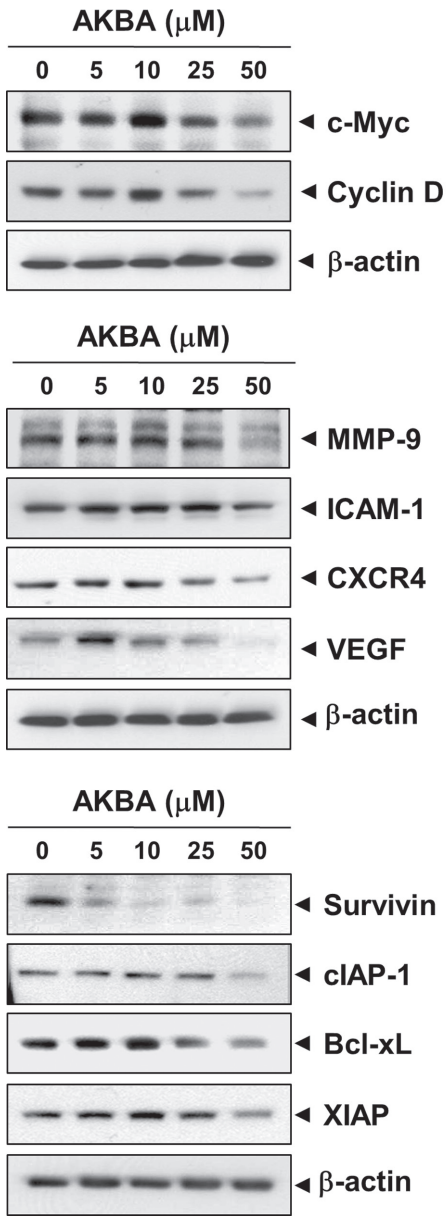

Fig. 2. AKBA inhibits NF-κB-related pathways in CRC cells. Western blot analyses for proteins involved in the NF-κB-related pathway in HCT116 cells. AKBA treatment for 24 h resulted in inhibition of these proteins in a dose-dependent manner.

AKBA up-regulates let-7 and miR-200 family of miRNAs and modulates the expression of their downstream targets

A part of the anticancer effects of AKBA may be due to its ability to inhibit NF-κB-related pathways. However, we also noticed that the concentration of AKBA needed to inhibit migration and invasion activities of CRC cells (10 μM) was much lower than the concentration of AKBA (50 μM) necessary to inhibit expression of proteins known to be involved in cell invasion and/or metastasis-related pathways such as matrix metalloproteinase 9, intercellular adhesion molecule 1 and C-X-C motif receptor 4. The let-7 and miR-200 families have been shown to be involved in the EMT process, which is one of the crucial steps that regulates metastasis in human cancers (16,17,36). In light of these observations, we hypothesized that AKBA might exert anticancer abilities through up-regulation of the let-7 and miR-200 families of miRNA. To address this hypothesis, we analyzed miRNA expression levels of let-7b, let-7i, miR-200b and miR-200c in our four CRC cell lines treated with or without AKBA (30 μM). The real-time RT–PCR analysis showed that these four miRNAs were significantly up-regulated by AKBA treatment in all cell lines (1.4–1.9-fold for let-7b, 1.4–1.8-fold for let-7i, 1.2–1.9-fold for miR-200b, 1.1–1.7-fold for miR-200c; Figure 3A) confirming our hypothesis.

Based on these results, we wanted to determine what effect AKBA treatment in these CRC cell lines had on direct or downstream targets of the let-7 and miR-200 family of miRNAs. We chose to measure the effect of AKBA on CDK6, E-cadherin and vimentin, all downstream targets of the let-7 and miR-200 families of miRNAs. Levels of *CDK6* mRNA expression were significantly reduced in all cell lines by treatment with AKBA, whereas levels of *E-cadherin* were significantly increased in HCT116 and HT29 cells (and a similar trend was seen in SW620 cells; $P = 0.08$; Figure 3B). The results for AKBA's effect on protein levels showed the same trends as those observed for mRNA. Levels of CDK6 declined precipitously after AKBA treatment in all three cell lines in a time-dependent manner (Figure 3C). Likewise, E-cadherin expression was markedly up-regulated in HCT116 cells, whereas there was a relatively modest increase after AKBA treatment in HT29 cells (Figure 3C). E-cadherin protein levels were not detectable in SW620 cells. On the other hand, protein expression of vimentin was clearly down-regulated by treatment with AKBA in SW620 cells, but vimentin protein levels were not detectable in HCT116 and HT29 cell lines (Figure 3C). Taken together, we interpret these results to indicate that AKBA induced up-regulation of the let-7 and miR-200 miRNAs, as manifested by subsequent modulation of the expression of their target genes, to impact CRC cell growth and metastasis.

Let-7i inhibition causes an increase in cell growth, proliferation and migration/invasion potential

Next, we questioned whether forced inhibition of the expression of these specific miRNAs affects the malignant behavior of AKBA-treated CRC cells. For this experiment, we chose to measure let-7i from the panel of our four miRNAs analyzed because this miRNA has previously been shown to possess a tumor-suppressive role in ovarian cancer (37), but its functional significance in CRC carcinogenesis remains unknown. First, we transfected an let-7i inhibitor or negative control into the CRC cell lines and compared the differences between these cells in terms of cell growth and proliferation. We observed that transfection with the let-7i inhibitor caused a significant increase in both cell growth (1.2–1.7-fold) and proliferation (1.1–1.3-fold) in all four cell lines (Figure 4A), in spite of only modest inhibition of let-7i expression (19–37% inhibition; Figure 4B). Second, we transfected a let-7i inhibitor or the negative control into CRC cells and then treated them with AKBA and compared the effects on cell growth and proliferation. Use of the let-7i inhibitor significantly attenuated the inhibitory effects of AKBA on cell growth in all cell lines and proliferation in three of four cell lines (Figure 4A). Next, we determined whether the let-7i inhibition affected migration/invasion activity of CRC cells. Transfection with the let-7i inhibitor significantly increased both the migratory and invasive potential of HCT116 cells (1.2-fold increase in migration and 1.8-fold increase in invasion; Figure 4C). These results suggest that let-7i plays an important role as a tumor suppressor in CRC and that the antitumor effects of AKBA are in part achieved through the up-regulation of the let-7i miRNA.

AKBA up-regulates let-7 and miR-200 family in vivo

Our finding that AKBA up-regulates the let-7 and miR-200 miRNA families and then modulates the expression of their targets genes *in vitro* prompted us to further validate these observations *in vivo*. For this purpose, we used the orthotopic CRC model, in which HCT116 cells were injected into cecum of nude mice and AKBA was subsequently administered by gavage in different doses (0, 50, 100 and 200 mg/kg/day for 28 days; Figure 5A), as reported previously (31). The administration of AKBA markedly inhibited growth of transplanted tumors in animals in a dose-dependent manner (Figure 5B) (31). Moreover, we observed the inhibition of distant metastases in mice treated with AKBA (31). To further ascertain the miRNA-modulatory activity of AKBA in this *in vivo* model, we first examined the expression of let-7 and miR-200 in these orthotopic tumors by real-time RT–PCR and

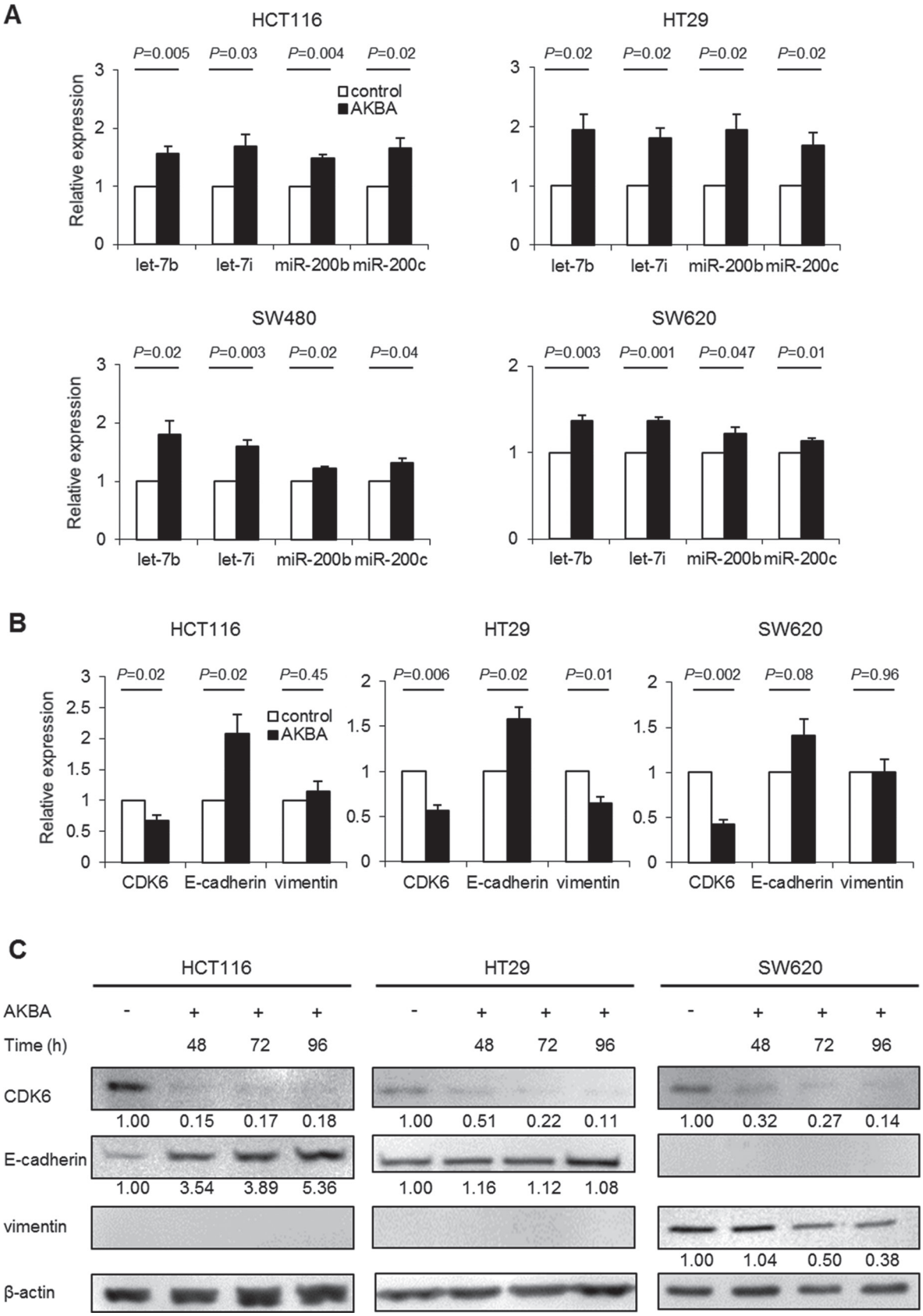

Fig. 3. AKBA up-regulates let-7 and miR-200 expression and regulates their downstream targets in CRC cells. (**A**) The expression of let-7 and miR-200 in four CRC cell lines treated with dimethyl sulfoxide alone or 30 μM of AKBA for 72 h. The mRNA (**B**) and protein expression (**C**) of the direct or downstream target genes in the cell lines treated with dimethyl sulfoxide alone or 30 μM of AKBA. Data are obtained from three or more independent experiments. The *P* value is calculated by paired two-sided Student's *t*-test. Numbers below each band represent the relative levels of each protein normalized to β-actin, as determined by densitometry.

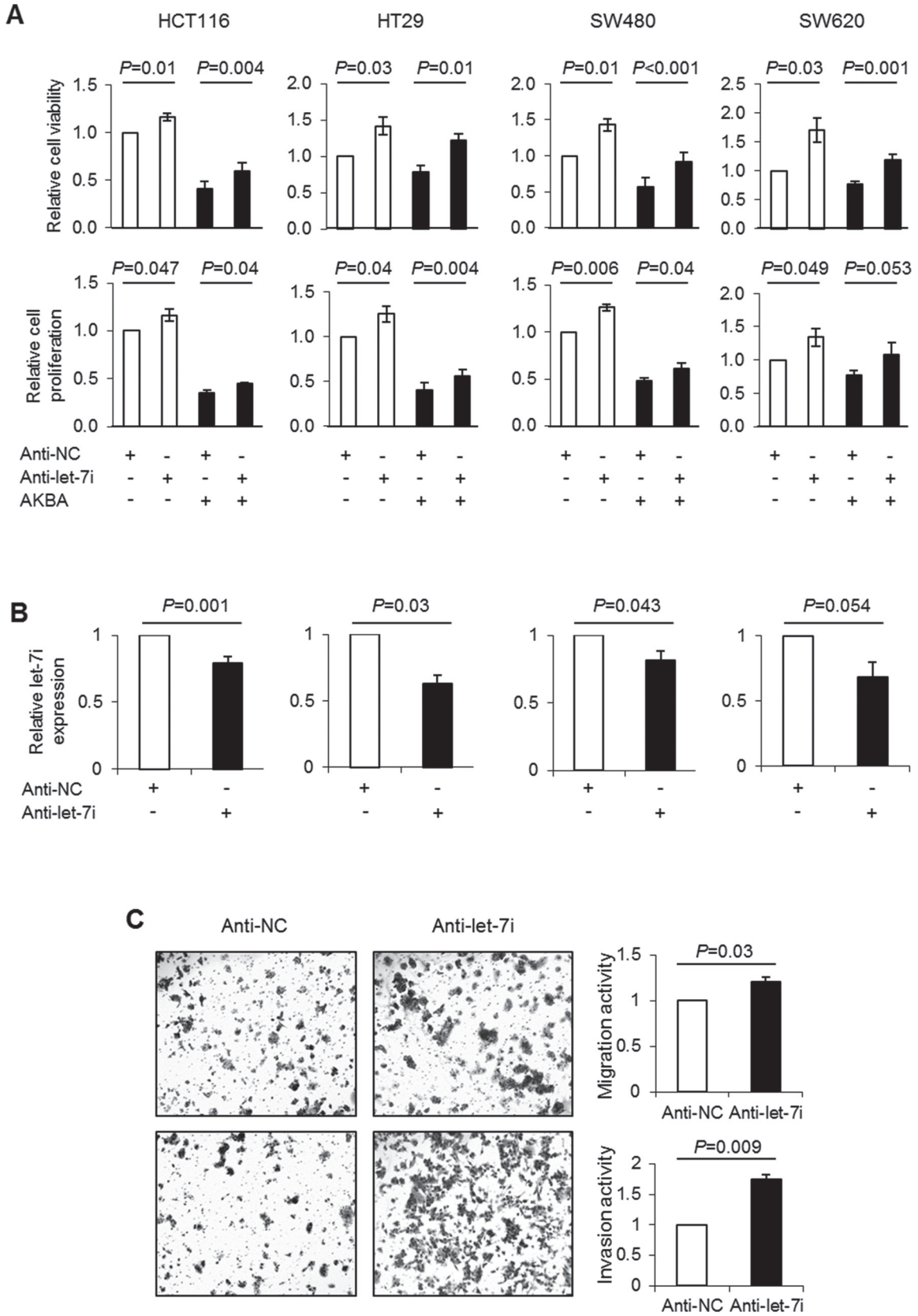

Fig. 4. let-7i down-regulation causes an increase in cell viability and proliferation, and migration/invasion. (**A**) Cell viability determined using a MTT assay (upper panel) and cell proliferation using the BrdU incorporation index (lower panel) of the four CRC cell lines treated with negative control inhibitor (anti-NC) or let-7i inhibitor, and subsequently treated with dimethyl sulfoxide alone or 30 μM of AKBA. (**B**) The expression levels of let-7i in the four cell lines treated with antinegative control inhibitor or let-7i inhibitor. (**C**) Migration (upper panel) and invasion activity (lower panel) of HCT116 cells transfected with negative control inhibitor or let-7i inhibitor. Data are obtained from three or more independent experiments. The *P* value is calculated by paired two-sided Student's *t*-test.

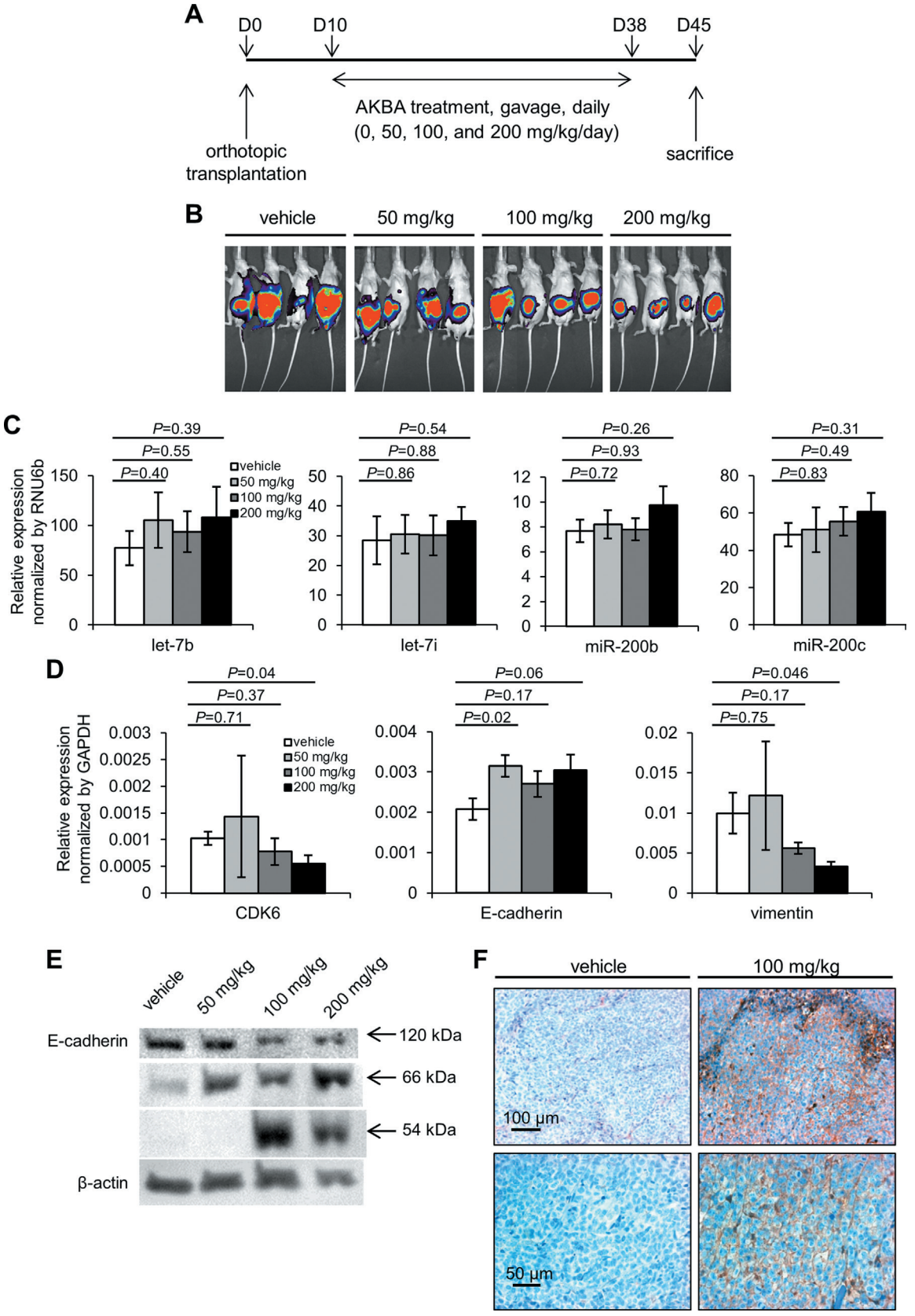

Fig. 5. AKBA modulates the expression of let-7 and miR-200 and downstream target genes in orthotopic mice tumors. (**A**) The schematic diagram of the AKBA treatment protocol in mice. (**B**) Bioluminescent images of luciferase vector-containing tumors in anesthetized mice. (**C**) The expression of let-7 and miR-200 in orthotopically implanted tumors treated with vehicle ($N = 6$) or AKBA with indicated doses ($N = 5$ per each treatment group). (**D**) The expression of *let-7* and *miR-200* target genes (CDK6, E-cadherin and vimentin) in the orthotopic tumors. (**E**) Western blot analysis of E-cadherin expression in the tumors. (**F**) A representative of immunohistochemical photomicrograph for E-cadherin staining in vehicle and AKBA-treated (100 mg/kg/day) tumors from animals. The *P* value is calculated by non-paired two-sided Student's *t*-test.

observed a trend toward up-regulation of let-7b, let-7i, miR-200b and miR-200c in AKBA-treated tumors (Figure 5C). Second, we wanted to determine if AKBA affected the target gene and protein expression in these tumors. Using RT–PCR analyses, we observed that *CDK6* and *vimentin* expression was significantly down-regulated in tumors treated with 200 mg/kg/day of AKBA, and *E-cadherin* expression was significantly up-regulated in tumors treated with 50 mg/kg/day (Figure 5D). These results were confirmed by western blot analysis for E-cadherin protein expression (Figure 5E); protein levels of CDK6 and vimentin were undetectable in these tumors (data not shown). Therefore, we focused on the results of AKBA treatment on E-cadherin expression in the tumors. To further confirm these results, we performed immunohistochemistry using an anti-E-cadherin antibody and noticed that E-cadherin was strongly expressed in tumors treated with 100 mg/kg/day of AKBA compared with control tumors (Figure 5F). The fact that treatment with AKBA affects not only the levels of miRNA *in vivo*, but the amounts of their target genes, lend further support to our hypothesis that AKBA exerts antitumor effects partly through modulation of specific miRNA pathways in human CRC.

Discussion

In this study, we provide important evidence in the support of epigenetic mechanisms contributing to the anticancer effects of boswellic acids in CRC. First, we found that AKBA up-regulated the expression of let-7 and miR-200 families of miRNAs in a panel of CRC cell lines. Second, we established that not only did AKBA induce changes in the expression of the let-7 and miR-200 families but also AKBA caused alterations in expression of their known target genes (*CDK6*, *E-cadherin* and *vimentin*) in CRC cells. Third, through forced inhibition of let-7i, we present evidence that let-7i plays a tumor-suppressive role in CRC cells, and that AKBA exerts its anticancer effects in part by modulating the expression of specific miRNA pathways, including let-7i and its downstream target genes. Finally, we validated our findings *in vitro* in an orthotopic tumor model. We were able to see the AKBA-induced modulation of the expression of various let-7 and miR-200 target genes *in vivo*.

Li *et al.* reported that 3,3′-diindolylmethane and isoflavone, which are similar natural agents, can cause up-regulation of let-7 and miR-200 family members, leading to reversal of the EMT process in gemcitabine-resistant pancreatic cancer cells *in vitro* (38). Our findings in CRC cells demonstrating an increase in the same subset of miRNA families in response to treatment with boswellic acid are consistent with this previous report. In addition, we extended our work by evaluating the potent miRNA-modulatory ability of AKBA in an *in vivo* model of CRC. We observed a similar trend for AKBA-induced change in miRNA expression in the xenograft animal model as well, although these differences did not reach significant levels perhaps because of the limited number of mice analyzed. Another possible explanation for the nonsignificant differences is that the miRNA modulation might have conceivably occurred in animals at an earlier time point than the one we analyzed (5 weeks after the beginning of treatment) in this *in vivo* model. Importantly, we found that the downstream targets of miRNAs were markedly modulated by AKBA even in the animal model. These findings suggest that this botanical has the ability to regulate the expression of specific miRNAs that target a subgroup of genes, including the let-7 and miR-200 family members, which orchestrate EMT processes in cancer cells.

The let-7 family is generally decreased in various cancers and is considered a tumor suppressor; however, some let-7 family members may be oncogenic in certain cell lines (5,39,40). This evidence raises the possibility that each let-7 family member may not only have overlapping functions but also have distinct functions in a cell-type or tissue-specific manner. It will be important to identify the role of each let-7 family member in cancers of various organs. To date, the functional significance of let-7i in CRC has remained largely unknown; however, in our study, we found let-7i functioned as a tumor suppressor in CRC. In normal colonic mucosa, expression of let-7i is less abundant compared with other let-7i family members such as let-7a and let-7b (41). Consistent with these reports, we observed that the expression of let-7i was 2–4-fold lower than that of let-7b in our cell lines (data not shown) and was 2-fold lower than that of let-7b in orthotopic tumors obtained from mice. Nonetheless, even the modest levels of let-7i inhibition in our study facilitated enhanced malignant behaviors in CRC cells, suggesting that let-7i acts as a tumor suppressor in CRC. Yang *et al.* have reported that let-7i expression was significantly reduced in chemotherapy-resistant ovarian cancers (37). The investigators showed that suppression of let-7i led to increased cisplatin resistance, whereas its over-expression led to decreased cisplatin resistance in ovarian and breast cancer cells (37). In addition, Blower *et al.* demonstrated that let-7i expression might be associated with chemosensitivity to various cytotoxic drugs in lung cancer cells (42). Our data demonstrating that inhibition of let-7i miRNA attenuated the anticancer effects of AKBA concur with these previous studies and suggest that these anticancer effects are achieved partly through the up-regulation of the let-7 family, specifically let-7i.

The miR-200 family is another well-studied tumor suppressor miRNA (16–18). Yet, the consequences of miR-200 dysregulation in CRC, including the metastatic process, have been poorly understood. However, Davalos *et al.* have recently reported that promoter hypermethylation of miR-200c/141 leads to their reduced expression in CRC tissues, and this process in turn permits acquisition of mesenchymal characteristics (43). Consistent with this suggestion, in an independent study using a large number of clinical specimens, we have recently confirmed the observation of frequent hypermethylation of the miR-200c/141 locus in primary CRC tissues, which eventually allowed metastasis of primary CRC cells to the liver (data not shown). In conjunction with the data from this study regarding the functional role of let-7i, it is plausible to speculate that AKBA-induced inhibition of the metastatic potential of CRC cells may be due in part to its ability to modulate both let-7 and miR-200 miRNAs.

In an orthotopic tumor model, we found that the downstream targets of miRNAs were significantly affected by treatment with AKBA. This result supports our hypothesis that these target genes can be affected through modulation of the miRNA pathways in CRC tumors *in vivo*. However, the precise mechanisms underlying AKBA-mediated increases in levels of miRNAs are still unclear. Previous studies reported that let-7i expression is regulated through transcriptional repression mediated by an NF-κB p50-CEBPB complex (44,45). This increase in let-7i expression may be due, in part, to the decreased expression of NF-κB induced by AKBA; however, the precise mechanisms by which the let-7 and miR-200 family members are regulated by boswellic acids and other botanicals need to be elucidated in future studies.

In light of accumulating experimental evidence and our results demonstrating that AKBA inhibits metastatic activity through up-regulation of the let-7 and miR-200 families, boswellic acids may serve as promising therapeutic agents to prevent or block metastasis of CRC. Furthermore, because the EMT phenotype itself may be associated with chemoresistance (46), it would be interesting to determine if boswellic acids in combination with other conventional chemotherapeutic drugs would exert synergistic effects in the treatment of CRC.

In conclusion, we provide novel evidence that AKBA exerts its anticancer effect, at least in part, by modulating specific miRNAs within the let-7 and miR-200 miRNA families. We also propose that let-7i acts as a tumor suppressor in CRC and some of the antitumor effects of AKBA are achieved by increasing levels of this miRNA. Our findings could provide a rationale for further investigation of this botanical-derived compound in chemoprevention and/or treatments in human CRC.

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