Ginkgetin induces G_2-phase arrest in HCT116 colon cancer cells through the modulation of b-Myb and miRNA34a expression

YU-JIN LEE[1], YEONG-RIM KANG[1], SO YOUNG LEE[1], YENA JIN[1,2], DONG CHO HAN[1,2] and BYOUNG-MOG KWON[1,2]

[1]Laboratory of Chemical Biology and Genomics, Korea Research Institute of Bioscience and Biotechnology; [2]University of Science and Technology, Daejeon 34141, Republic of Korea

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Abstract. Ginkgetin has been reported to display antitumor activity. However, the relevant pathway integrating cell cycle regulation and signaling pathways involved in growth inhibition in CRC cells remains to be identified. In this study, ginkgetin-treated HCT116 CRC cells exhibited significant dose-dependent growth inhibition with a GI_{50} value of 4.0 μM for 48-h treatment, together with apoptosis, via G_2-phase cell cycle arrest. When HCT116 cells were treated with 10 μM ginkgetin for 48 h, the percentage of cells in G_2/M phase increased by 2.2-fold (43.25%) versus the untreated control (19.69%). Ginkgetin regulated the expression of genes that are critically involved in G_2 phase arrest cells, such as b-Myb, CDC2 and cyclin B1. Furthermore, we found that the suppression of b-Myb expression by ginkgetin was rescued ~5.1-fold by treatment with a miR-34a inhibitor (500 nM) and b-Myb was downregulated by >80% by 100 nM miR-34a mimic. These data suggest that the miRNA34a/b-Myb/cyclin B1 cascade plays a critical role in ginkgetin-induced G_2 cell cycle arrest, as well as in the inhibition of HCT116 cell proliferation. Moreover, the administration of ginkgetin (10 mg/kg) reduced tumor volumes by 36.5% and tumor weight by 37.6% in the mice xenografted with HCT116 cells relative to their vehicle-treated counterparts. Therefore, ginkgetin is the first compound shown to regulate b-Myb by modulating miR-34a, and we suggest the use of ginkgetin as an inducer of G_2 arrest for the treatment of CRC.

Correspondence to: Dr Dong Cho Han or Dr Byoung-Mog Kwon, Laboratory of Chemical Biology and Genomics, Korea Research Institute of Bioscience and Biotechnology, 125 Gwahakro, Yoosunggu, Daejeon 34141, Republic of Korea
E-mail: dchan@kribb.re.kr
E-mail: kwonbm@kribb.re.kr

Abbreviations: CRC, colorectal cancer; CDK, cyclin-dependent kinase; PARP, poly(ADP-ribose) polymerase; FACS, fluorescence activated cell sorting; PHH3, phosphorylated histone H3 (S10)

Key words: ginkgetin, cell cycle, b-Myb, miRNA34a, colon cancer

Introduction

Cancer is a leading cause of death worldwide; 14.1 million new cancer cases were diagnosed in 2012, leading to 8.2 million deaths. Human CRC is the third most common cancer in the world, with nearly 1.3 million new cases diagnosed in 2012 (1). Despite progress in diagnosis and treatment, the incidence of CRC is increasing, and there is an urgent need to identify new agents that suppress the growth of CRC cells.

The cell cycle is controlled by a regulatory machine and is tightly regulated in an orderly manner. Three different transition points during the cell cycle, also known as cell-cycle checkpoints, namely G_1→S, G_2→M and M→G_1, as well as the regulatory networks underlying each transition, are well characterized (2). These cell cycle checkpoints are regulated by the activities of cyclin-dependent kinases (CDKs), which cooperate with different classes of cyclins. Specific cyclin-CDK complexes in turn activate different downstream targets to promote or prevent cell cycle progression (3). Proper cell cycle transitions are crucial for the control of cell proliferation, and deregulation or disruption of cell cycle modulation promotes unrestrained cell growth, which can cause tumorigenesis. Cancer cells possessing defective cell cycle checkpoints are defected due to mutations in the p53 or pRb tumor suppressor genes or through imbalances in cyclins, cyclin-dependent kinases (CDKs) and their inhibitors (4). Therefore, checkpoint-controlling molecules are potential therapeutic targets for cancer and many checkpoint-modulating agents have been developed (3,5-7).

The Myb family of transcription factors comprises three isoforms, a-, b-, and c-Myb. c-Myb was first discovered in avian myeloblastosis virus, which causes acute myeloblastic leukemia and can transform hematopoietic cells in culture; a- and b-Myb were subsequently cloned. Although b-Myb is ubiquitously expressed, a- and c-Myb show tissue-specific expression patterns. c-Myb is mainly expressed in hematopoietic stem cells, and a-Myb is abundant in testes and in B-cells (8). Interestingly, b-Myb is expressed in embryonic stem cells, and knocking out this gene results in embryonic lethality in mice (9). b-Myb is involved in cell growth, differentiation, apoptosis (10), and senescence (11). In the cell-cycle, the G_2-M transition in cancer cells is regulated through the activation of the cdc2-cyclin B1 complex, and b-Myb proteins

are involved in the regulation of cyclin B expression (12). b-Myb regulates cyclin B expression by directly binding its promoter and also upregulates cdc2 and cyclin B1 expression in conjunction E2F (13).

MicroRNAs (miRNA) are a major class of non-coding RNAs, which mediate the posttranscriptional regulation of genes that control multiple cellular processes (14). miRNA biogenesis is tightly controlled by various regulatory proteins, including DROSHA, DICER and AGO2 (15). Deregulation of these regulatory proteins is observed in human cancer cells, and many oncogenic miRNAs are significantly increased in cancer (16). The ability of miRNAs to target oncogenes provides an opportunity to discover antitumor drugs that interrupt this oncogenic network (17). Cell cycle checkpoints are precisely controlled by a number of regulatory proteins including cyclins and CDKs, and recent studies indicated that many miRNAs are also involved in cell cycle regulation (18). Specifically, miR-34, -29 and -30 family members regulate apoptosis by controlling cell cycle pathways (19,20).

Ginkgetin is a natural biflavonoid, isolated from the leaves of *Ginkgo biloba*, and we previously reported antitumor activities of ginkgetin (21,22); however, little information is available on the mechanism of the anticancer activity of the natural product.

In this study, we examined the effects of ginkgetin on cell cycle regulation and apoptosis in colorectal cancer cells, as well as *in vivo* using human colon tumor xenografts in nude mice. Our findings show that ginkgetin inhibits tumor growth through cell cycle arrest and apoptosis via the regulation of b-Myb and miR-34a expression.

Materials and methods

Reagents and antibodies. Ginkgetin was isolated from *Ginkgo biloba* leaves (23,24). Ginkgetin (>95% purity by HPLC and ^{1}H-NMR) was dissolved in dimethylsulfoxide (DMSO) as a stock solution, stored at -20˚C, and diluted with medium before each experiment. RPMI-1640, DMEM F-12, Opti-MEM reduced serum medium, FBS, PBS, trypsin-EDTA, and penicillin-streptomycin solution were purchased from Invitrogen (Grand Island, NY, USA). Propidium iodide and the chemicals used in the buffer solutions were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Primers and small interfering RNAs (siRNAs) were purchased from Bioneer (Daejeon, Korea). The primary antibodies, anti-PARP, and anti-β-actin were purchased from Cell Signaling Technology and anti-b-Myb, anti-cyclin B1, anti-CDC2, anti-pCDC2 (Y15), anti-pHisone H3 (S10) were purchased from Santa Cruz. The miRNA34a mimic (hsa-miR-34a, SMI-002), inhibitor (hsa-miR-34a-5p, SMI-001), miRNA mimic negative control (SMC-2001) and miRNA inhibitor negative control (SMC-2101) were purchased from Bioneer.

Cell culture and cell viability assay. HCT116, SW620, and HCA7 were purchased from ATCC and maintained as monolayer cultures in media recommended by the ATCC. To investigate cell proliferation, cells were seeded at a density of 7,000 cells per well in 96-well plates in RPMI-1640 medium containing 10% FBS. The medium was replaced with fresh complete medium that contained the test compounds or 0.1% DMSO. After incubation for 24 or 48 h, the cell proliferation reagent WST-1 (Dojindo Laboratories) was added to each well. WST-1 formazan was quantitatively measured at 450 nm using an enzyme-linked immunosorbent assay reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Synchronization and fluorescence-activated cell sorting (FACS) analyses. Cells were synchronized at metaphase by exposure to nocodazole (500 ng/ml) for 16 h. After treatment, metaphase cells were collected by a gentle shake-off method, centrifuged for 5 min at room temperature, and washed twice with fresh medium. To relieve cells from the M phase arrest, cells were replated in 60-mm cell culture dishes ($4x10^5$/T25 flask) and incubated in fresh medium for various time periods with or without ginkgetin. To analyze the DNA content by flow cytometry, cells were trypsinized from the culture flask. After centrifugation at 300 x g for 5 min at room temperature, the supernatant was removed. The cells were then washed twice with PBS solution and fixed overnight with 3 ml of ice-cold 70% EtOH. Fixed cells were harvested by centrifugation at 300 x g for 3 min at room temperature and washed twice with PBS. Collected cells were resuspended in PBS (100 μl/$1x10^5$ cells) and treated with 100 μg/ml of RNase A at 37˚C for 30 min. Propidium iodide was then added to a final concentration of 50 μg/ml for DNA staining and 20,000 fixed cells were analyzed on a FACSCalibur (Becton-Dickinson, San Jose, CA, USA). Cell cycle distribution was analyzed using ModFit's program (Becton-Dickinson).

Quantitation of mRNA using quantitative real-time PCR. RNA was isolated using the RNeasy kit (Qiagen, Hilden, Germany). Then, 1 μg of total RNA was transcribed into cDNA with maxime PCR premix (iNTRON, Kyunggi-do, Korea). Real-time PCR was performed using IQ™ SYBR Green supermix (Bio-Rad) according to the manufacturer's instructions using an iQ5 real-time PCR detection system. Triplicate 20 μl PCR reactions were carried out with polymerase activation at real-time PCR cycles. The following primers were used for real-time PCR: cyclin B1 forward primer (5'-ATGGAACTAACTATGTTGGACTATG-3'), reverse primer (5'-AGATTCTTCAGTATATGACAGGTA ATG-3'), CDC2 primer (P149402), b-Myb (P174647), and GAPDH (P267613). Primers were purchased from Bioneer. Relative amounts of CDC2, cyclin B1 and b-Myb were normalized against GAPDH mRNA.

Knockdown of cyclin B1 and B-Myb using small interfering RNA in HCT116 cells. The sequences of each siRNA are as follows: b-Myb (5'-GAA ACG AGC CUG CCU UAC ATT-3'), cyclin B1 (5'-GAA UUC UGC ACU AGU UCA A-3') and the negative control (5'-CCU ACG CCACCAAUU UCU U-3'). Cells were plated at a density of $3x10^5$ cells/well in 6-well plates and transfected with 50 nM b-Myb, cyclin B1 and negative control siRNAs after pre-incubation for 20 min with RNAi max (Invitrogen, Carlsbad, CA, USA) in serum-free Opti-MEM medium. RPMI-1640 medium containing 10% FBS was added 5 h after the beginning of the incubation. After transfection for 48 h, cells were collected and used for preparation of whole cell lysates.

Western blot analysis. Cell lysates were prepared as reported previously. Protein concentrations were determined using a Bio-Rad protein assay kit (Bio-Rad) with BSA as a standard. Then, 30 μg of protein lysates were subjected to 7.5 or 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (Roche). Membranes were blocked with TBST (50 mM Tris-HCl pH 7.6, 150 mM NaCl, and 0.05% Tween-20) containing blocking reagents (Roche) for 1 h. Antibodies were used at dilutions recommended by the manufacturers. Horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG from Jackson ImmunoResearch was used as the secondary antibody. Blots were incubated in primary antibodies for 2 h at room temperature and washed three times in TBST. The proteins were visualized with chemiluminescence peroxidase reagents (Roche Applied Science). Western blot analyses were repeated 2 or 3 times.

Quantitation of microRNA. miR-29a, 29c, 30b, and miR-34a expression levels were evaluated by using real-time PCR. miRNA expression values were normalized by using specific primers to U6 as an endogenous reference RNA. Real-time PCR was performed using IQ SYBR Green supermix (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions using an iQ5 real-time PCR detection system. The following primers were used for real-time PCR; miR-29a (RT, GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCAC TGGATACGACTAACCG; FP, TCCACACGCATAGCACC ATCTG), miR-29c (RT, GTCGTATCCAGTGCAGGGTCCG AGGTATTCGCACTGGATACGACTAACCG; FP, ACACTC CACGCATAGCACCATT), miR-30b (RT, GTCGTATCCAG TGCAGGGTCCGAGGTATTCGCACTGGATACGACAGC; FP, ATCACACTCCACGCATGTAAACATCCTA), miR-34a (RT, GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCA CTGGATACGACACAAC; FP, CTCCACGCATGGCAGTG TCTT), U6 (RT, AACGCTT CACGAATTTGCGT; FP, CTCG CTTCGGCAGCACA), URP (CCAGTGCAGGGTCCGAG GTA).

Knockdown and overexpression of miRNA-34a. The miRNA34a mimic (hsa-miR-34a, SMI-002), inhibitor (hsa-miR-34a-5p, SMI-001), miRNA mimic negative control (SMC-2001) and miRNA inhibitor negative control (SMC-2101) were purchased from Bioneer. HCT116 cells were plated at a density of $3x10^5$ cells/well in 6-well plates and transfected with 20, 50 and 100 nM negative control mimic, miR-34a mimic and 500 nM miRNA inhibitor negative control, has-miR-34a-5p after pre-incubation for 20 min with RNAi max (Invitrogen, Carlsbad, CA, USA) in serum-free Opti-MEM medium. RPMI-1640 medium containing 10% FBS was added 5 h after the beginning of the incubation. After transfection for 24 h, cells were collected and used for preparation of RNAs for real-time PCR and western blotting.

In vivo xenograft model. All animals were maintained in a specific pathogen-free facility under protocols approved by the Institutional Animal Care and Use Committee. Five- to six-week-old female BALB/c nude mice (Oriental Bio., Korea) were used for animal studies. Xenograft experiments with HCT116 cells were performed in accordance with protocols approved by the Korea Research Institute of Bioscience and Biotechnology (KRIBB) Animal Experimentation Ethics Committee. To establish human colon tumors in mice, $1.2x10^7$ HCT116 cells were subcutaneously implanted in the right flank region of 6-week-old female S.P.F BALB/c nude mice. Vehicle control and ginkgetin (10 mg/kg, intraperitoneal injection) were administered five times per week for 23 days (8 mice/group). Tumor sizes were calculated using the formula V = length x width x height x 0.5 mm^3. Tumor volumes and weight at the end of treatment were compared using Student's t-test.

Statistical analysis. The data are expressed as the means ± standard deviations (SDs), and the degree of significance was analyzed using Student's t-test. P-values <0.05 were considered statistically significant.

Results

Ginkgetin inhibits tumor cell growth and induces apoptosis. We previously reported that ginkgetin (Fig. 1A) has antitumor activities against prostate cancer cells via the activation of the caspase pathway or the inhibition of STAT3 activity (21,22). To further understand the biological activity of ginkgetin on cancer cells, we first investigated the inhibitory effect of ginkgetin of tumor cells by a cell proliferation assay. A variety of colon cancer cells (HCT116, HCA-7 and SW620) was treated with different concentrations of ginkgetin for 48 h. As shown in Fig. 1B, ginkgetin inhibited the growth of HCT116, SW620, and HCA7 cells with GI_{50} values of 4.0, 3.5 and 10 μM, respectively. Because ginkgetin strongly inhibited the growth of HCT116 (colon tumor cells) in a dose- and time-dependent manner, with a GI_{50} value of 4.0 μM for 48 h treatment (Fig. 1C), and the incidence of CRC is sharply increasing, we focused on the biological activity of ginkgetin on HCT116 cells.

In order to determine whether the growth-inhibitory effects of ginkgetin were caused by apoptotic death in HCT116 cells, the cleavage of poly(ADP-ribose) polymerase (PARP) were carried out. Ginkgetin led to the cleavage of PARP (Fig. 1D). These results clearly suggest that ginkgetin inhibits the growth of HCT116 cells by inducing apoptosis.

Ginkgetin induces G_2/M phase arrest in HCT116 cells. Because the primary cellular response to chemotherapy-induced DNA damage includes cell cycle arrest and apoptosis, we performed fluorescence activated cell sorting (FACS) analysis, which is a standard tool to analyze the cell cycle distribution in cell populations. Cells were harvested at different times (24 and 48 h) after treatment 5 or 10 μM of ginkgetin, and the cell cycle distribution of treated cells was examined by flow cytometry. As shown in Fig. 2, the G_2/M fraction of ginkgetin-treated cells was increased and the fraction of cells in G_0/G_1 phase was decreased in a dose- and time-dependent manner in HCT116 cells (Fig. 2). A 24 h treatment of HCT116 cells with ginkgetin (5 or 10 μM) resulted in G_2/M distribution rates of 27.05 and 40.85%, respectively. The G_2/M distribution of HCT116 cells was 24.02 and 43.25% at 48 h after treatment with ginkgetin (5 or 10 μM), respectively. In particular, when HCT116 cells were treated with 10 μM ginkgetin for 48 h, the percentage of cells in G_2/M phase

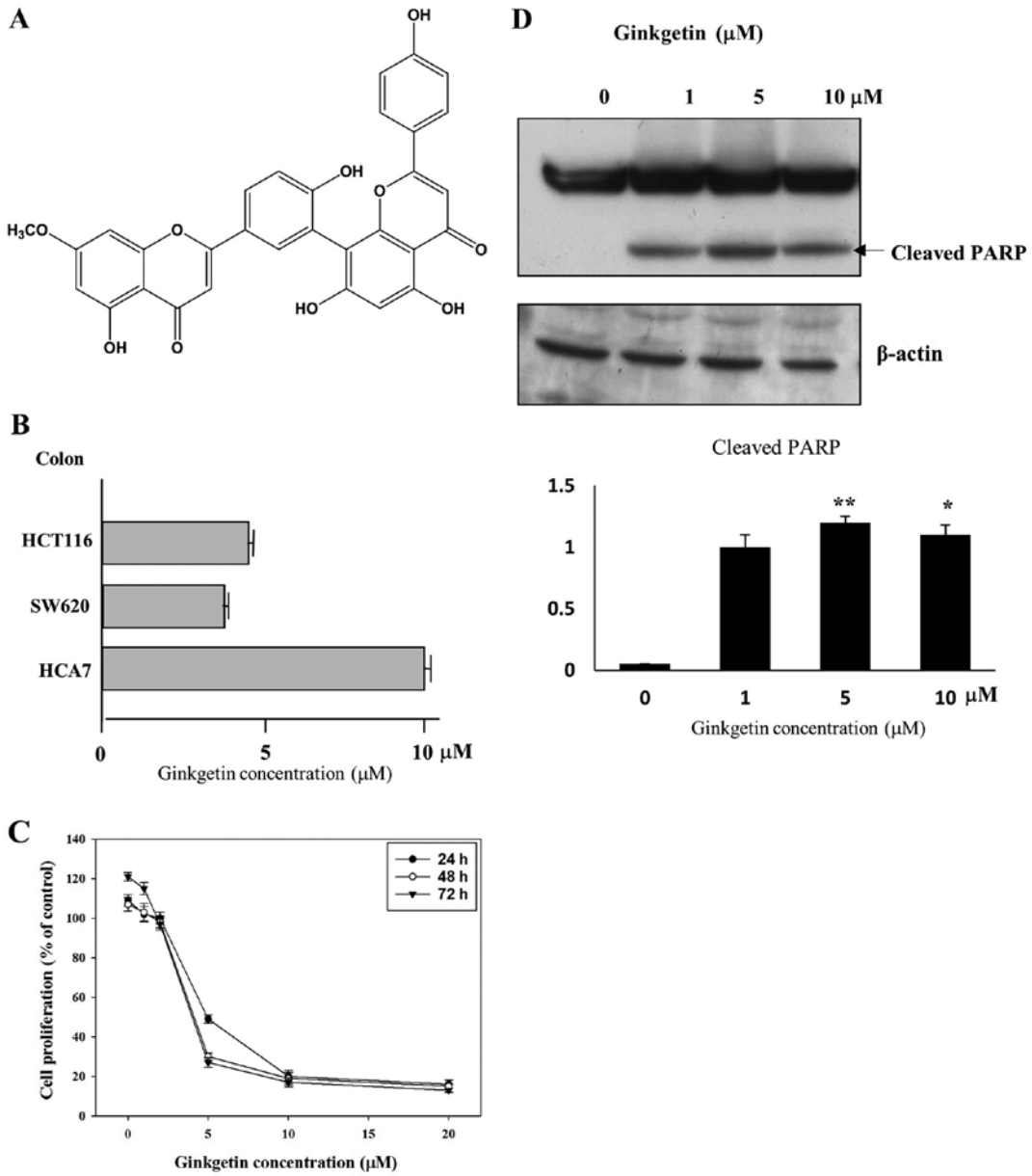


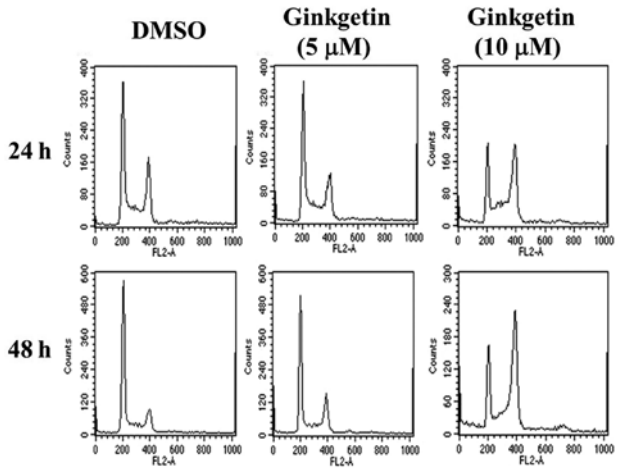
Figure 1. Ginkgetin inhibits growth of various cancer cell lines. (A) Structure of ginkgetin. (B) Ginkgetin was treated in a variety of colon cancer cells (HCT116, HCA7 and SW620) for 48 h and a GI_{50} value of ginkgetin in colon cancer cells is shown. (C) Ginkgetin inhibits growth of HCT116 in a time- and dose-dependent manner. (D) Effect of ginkgetin on PARP degradation. HCT116 cells were treated with ginkgetin (1, 5 and 10 μM) or vehicle solvent (0.1% DMSO), and equal proteins were resolved on 7.5% SDS-polyacrylamide gels. Proteins were visualized by western blotting using specific anti-PARP. All data represented the mean from three independent experiments (mean ± SD). *P<0.05 and **P<0.01 indicate statistically significant differences versus untreated group.

increased by 2.2-fold (43.25%) versus the untreated control (19.69%).

Previously, it was reported that ginkgetin induced the accumulation of cells at the G_0/G_1 phase through the inhibition of STAT3 activity in DU145 (prostate cancer) cells, whereas, ginkgetin efficiently induced G_2/M phase arrest in Daoy (medulloblastoma) cells by inhibiting Wnt target gene expression (21,24). It is interest that the different types of cell cycle progression were observed in ginkgetin-treated different tumor cells. Therefore, we examined whether ginkgetin modulates cell cycle progression in HCT116 cells.

Ginkgetin specifically induces G_2 arrest in HCT116 cells. Cell cycle synchronization was used for observing the cell cycle progression and many methods have been established to synchronize cells at specific phases of the cell cycle (25). To identify whether the growth inhibition by ginkgetin is due to the arrest of cells at G_2 or M phase, synchronize experiment was performed with nocodazole, because the microtubule-depolymerizing agent specifically blocks the cell cycle at the metaphase-anaphase transition and allows for the collection of a cell population that is in M phase (26).

To synchronize cells in M phase, HCT116 cells were treated with nocodazole. M phase-synchronized cells were isolated by selective detachment, washed to remove the nocodazole, and then replated in medium with or without ginkgetin (10 μM). The cells were harvested at the indicated times and the cell cycle distribution was analyzed by FACS.



		DMSO (%)	5μM (%)	10μM (%)
24 h	Apoptosis	2.02 ± 0.28	4.28 ± 0.31	6.95 ± 0.58
	G0/G1	44.99 ± 3.39	44.62 ± 4.21	23.61 ± 2.23
	S	23.65 ± 3.12	24.06 ± 3.28	28.58 ± 2.92
	G2/M	29.33 ± 2.28	27.05 ± 1.68	40.85 ± 3.83
48 h	Apoptosis	4.08 ± 0.58	6.54 ± 0.88	12.28 ± 1.28
	G0/G1	59.64 ± 3.28	49.49 ± 3.45	20.10 ± 2.01
	S	16.5 9± 2.12	19.95 ± 2.08	24.36 ± 2.81
	G2/M	19.69 ± 2.71	24.02 ± 3.08	43.25 ± 4.19

Figure 2. Cell cycle profiles of HCT116 cells treated with ginkgetin. HCT116 colon cancer cells were treated with indicated concentrations of ginkgetin or vehicle solvent (0.1% DMSO) for 24 and 48 h. After incubation, cells were harvested and fixed with 70% ethanol. Fixed cells were incubated with RNase A and propidium iodide. Fixed cells (20,000) were subjected to FACSCalibur analysis to determine the distribution of cells throughout the G_1, S and G_2/M phases. Data are represented as mean ± SD; n=3.

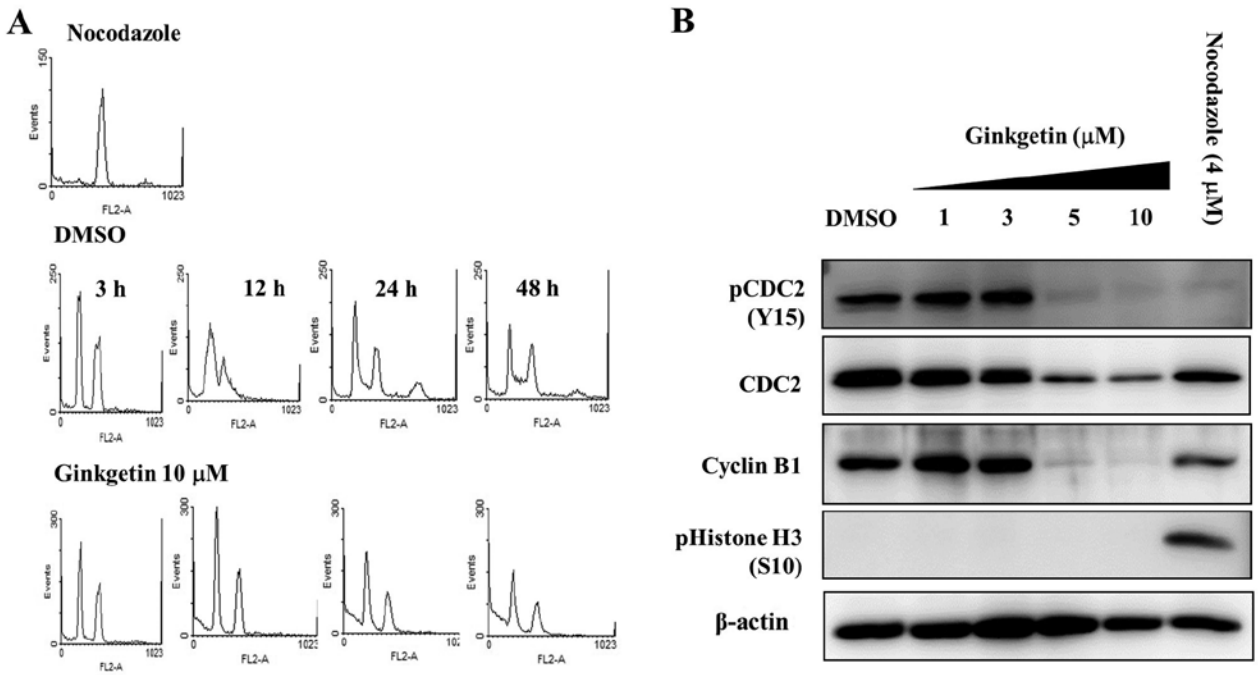
If ginkgetin induces M phase arrest, replating cells with ginkgetin should maintain the M phase arrest. However, the synchronized HCT116 cells were completely released from M phase arrest 3 h after replating cells both in the absence and the presence of ginkgetin (Fig. 3A). As shown in Fig. 3A, after 3 h of release, HCT116 cells without and with ginkgetin (10 μM) exhibited G_0/G_1 distribution rates of 42.40 and 39.12%, respectively. The G_2/M or G_2 distribution of the HCT116 cells was 43.74 and 41.81% after 3 h of release without and with ginkgetin, respectively. The apoptotic cell population was slightly increased, from 10.79 to 18.37%, in HCT116 cells released from nocodazole-induced arrest in the absence of ginkgetin, however, cells released with ginkgetin showed an increased population of apoptotic cells from 14.9 to 43.65% in a time-dependent manner (Fig. 3A).

To investigate the mode of action of ginkgetin on G_2 cell cycle arrest, the protein levels of pCDC2 (Y15) (also known as CDK1 kinase), CDC2 and cyclin B, which is important components for arresting in G_2 phase, were measured in ginkgetin-treated HCT116 cells. Immunoblotting demonstrated that the levels of pCDC2 (Y15), CDC2 and cyclin B1 were decreased at the concentrations of ginkgetin (5 and 10 μM) that induced G_2 arrest (Fig. 3B). Phosphorylated histone H3 (S10) (PHH3) correlates closely with M phase of the cell cycle (27). For this reason, PHH3 is used as a mitosis-specific marker. After 24 h of ginkgetin treatment, western blotting with a PHH3 antibody showed that PHH3 was undetectable in ginkgetin-treated cells; however, PHH3 was strongly detected in the nocodazole-treated cells. In addition, CDC2 and cyclin B1 were also detected in nocodazole-treated cells. These results indicate that ginkgetin inhibits proliferation and induces apoptosis in HCT116 cells via G_2 phase arrest.

Ginkgetin induces G phase arrest by modulation of b-Myb expression. As shown Fig. 3B, ginkgetin markedly downregulated the key regulators of the transition through G_2 phase and entry into mitosis such as cyclin B1 and CDC2. To confirm the G_2 arrest by ginkgetin, the relative mRNA expression levels of G_2 arrest-related genes such as b-Myb, CDC2, cyclin G_1, and cyclin B1 were analyzed by quantitative real-time PCR in HCT116 cells treated with 7 μM ginkgetin. Untreated control cells served as a reference (ratio = 1). Ginkgetin induced the downregulation of b-Myb, CDC2, cyclin G1, and cyclin B1 mRNA levels in a time-dependent manner (Fig. 4A). We also confirmed that the levels of G_2 arrest-related proteins such as b-Myb, CDC2 and cyclin B1 were downregulated by ginkgetin (Fig. 4B). As mentioned previously, b-Myb regulates the expression of S and G_2/M genes such as cyclin B and CDC2 (CDK1) (28), and b-Myb is required for the transcription of cyclin B1 (29). Furthermore, when b-Myb siRNA was transfected into HCT116 cells, the cell growth rate was decreased (Fig. 4C). Therefore, we focused on the expression of b-Myb, CDC2 and cyclin B1 to investigate the effects of ginkgetin on cell cycle progression.

We explored the effect of ginkgetin on G_2 arrest-related genes, including b-Myb, CDC2 and cyclin B1 in HCT116 cells using siRNA gene knock-down experiments. Because b-Myb regulates the transcription of cyclin B1 and CDC2, we tested whether repression of b-Myb has reverse effect on the expression of CDC2 and cyclin B1 by transfecting HCT116 cells with siRNA against b-Myb. As shown in Fig. 4D, b-Myb protein expression was completely abolished by b-Myb siRNA, and cyclin B1 protein levels were also strongly decreased in repressed cells. We also observed an approximately 40% reduction in the amount of CDC2 in b-Myb-depleted cells. These results are consistent with those in ginkgetin-treated cells (Fig. 4B). Next we repeated the experiments using siRNA-mediated repression of cyclin B1 and detected no effects on CDC2 protein levels, however, b-Myb protein levels were decreased by ~50% compared with the negative-control cells (Fig. 4D). Thus, b-Myb is pivotal for ginkgetin-induced G_2 arrest in HCT116 cells.

Ginkgetin regulates b-Myb expression by modulating miR-34a expression. MicroRNAs can act as potent oncogenes



		3 h (%)	12 h (%)	24 h (%)	48 h (%)
DMSO	Apoptosis	10.79 ± 1.13	13.64 ± 1.88	13.91 ± 1.56	18.37 ± 1.79
	G0/G1	42.40 ± 4.28	51.16 ± 7.53	45.39 ± 4.67	29.98 ± 3.68
	S	3.06 ± 0.78	11.58 ± 3.23	5.00 ± 0.92	14.84 ± 2.28
	G2/M	43.74 ± 4.12	23.62 ± 3.71	35.69 ± 3.28	36.82 ± 2.97
Ginkgetin	Apoptosis	14.09 ± 0.95	20.88 ± 1.28	33.08 ± 3.20	43.65 ± 3.75
	G0/G1	39.12 ± 2.76	43.62 ± 2.91	34.66 ± 3.01	27.70 ± 2.54
	S	4.99 ± 0.31	4.03 ± 0.91	6.95 ± 1.10	8.11 ± 1.28
	G2/M	41.81 ± 3.09	31.48 ± 2.78	25.31 ± 3.10	20.55 ± 1.65

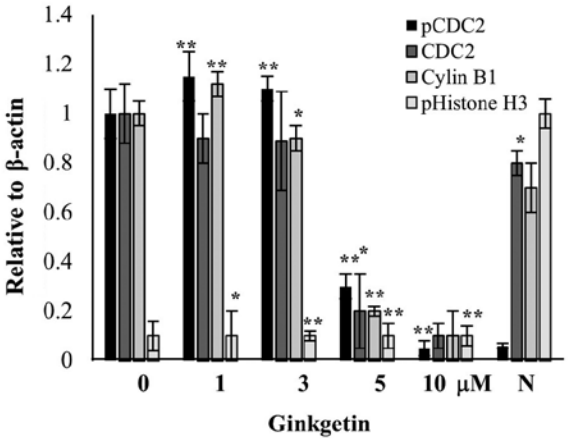


Figure 3. Ginkgetin induces G phase arrest in HCT116 cells. (A) Cell cycle profiles of M-phase synchronized HCT116 cells in the presence of ginkgetin. HCT116 cells were treated with 1 μg/ml nocodazol for synchronizing in mitosis. The mitotic cells were collected by shaking off, washed twice in PBS, and replated in T25 flask in media containing 0.1% DMSO or ginkgetin 10 μM. At the indicated time-points after nocodazole release, cells were harvested and fixed with 70% ethanol. Fixed cells were incubated with RNase A and propidium iodide. Fixed cells (20,000) were subjected to FACSCalibur analysis to determine the distribution of cells. (B) Ginkgetin induces G_2 phase arrest via CDC2 and cyclin B1. Cyclin B1, CDC2, pCDC2 (Y15), proteins decreased in a dose-dependent manner. HCT116 cells were treated with ginkgetin 1, 3, 5 and 10 μM or nocodazole 4 μM at 37˚C. Proteins were visualized by western blotting using specific antibodies. Cell lysates were probed for the mitotic marker phospho-histone H3 (S10) to determine the effects of Chk1 on the ability of paclitaxel to confer M-phase arrest. Data are presented as mean ± SD; n=3. *P<0.05 and **P<0.01 indicate statistically significant differences versus DMSO-treated group.

or tumor suppressors and can regulate the progression of cancer via cell cycle control (18). Martinez *et al* reported that overexpression of miR-29 and miR-30 inhibits b-Myb expression and reduces DNA synthesis (30) and an inverse correlation between overexpression and downregulation of b-Myb has also been reported (31). Therefore, we hypothesized

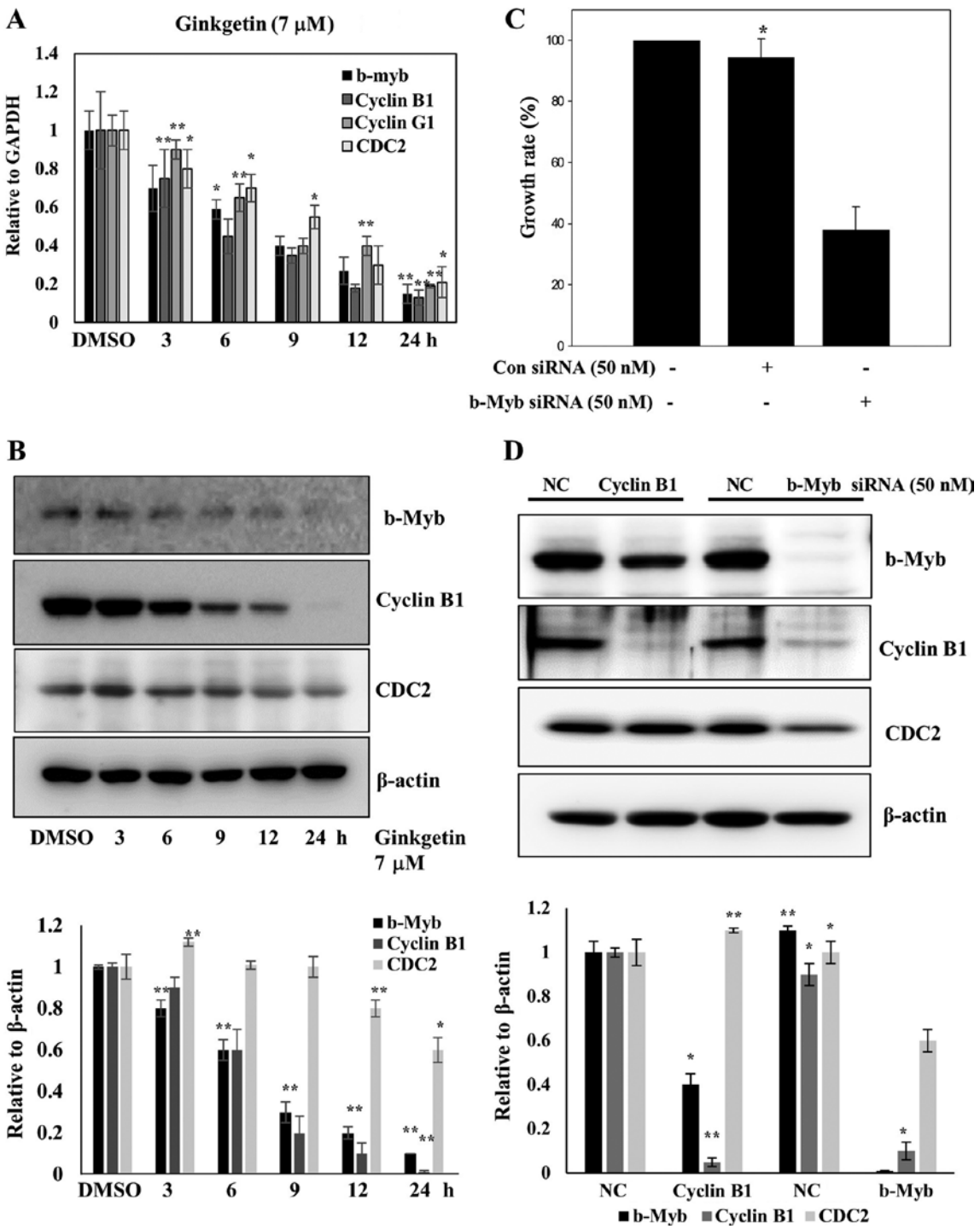


Figure 4. Expression of G_2 arrest genes in HCT116 cells by ginkgetin. (A) HCT116 cells were treated with 7 μM ginkgetin for various times (3, 6, 9, 12 and 24 h) at 37˚C. Ginkgetin induces downregulation of cell cycle genes in a time-dependent manner such as cyclin G1, b-Myb, CDC2 and cyclin B1. HCT116 cells were treated with ginkgetin 7 μM for various time at 37˚C, exposed to real-time PCR. The expression of each mRNA was normalized against the GAPDH gene. (B) Ginkgetin induces downregulation of cell cycle proteins such as b-Myb, CDC2 and cyclin B1 in a time-dependent manner. HCT116 cells were treated with ginkgetin 7 μM for various time at 37˚C. Proteins were visualized by western blotting using specific antibodies. (C) Knockdown of b-Myb induced the growth inhibition of HCT116 cells. HCT116 cells were transfected with negative control siRNA and b-Myb siRNA (50 nM). Forty-eight hours post-transfection, the growth rate was measured by WST-1 assay. (D) Knockdown of cyclin B1 and b-Myb using siRNAs. HCT116 cells were transfected with siRNA (50 nM) of the genes for 48 h. Bar graph represents the average number of viable cells. Negative control siRNA were included in the assay as a reference. The siRNA-transfected cells were lysed, and equal amounts of cell lysates were resolved on 7.5% SDS-acrylamide gels. Proteins were determined by western blot analysis using specific antibodies. Data are presented as mean ± SD; n=3. *P<0.05 and **P<0.01 indicate statistically significant differences versus control group.

that ginkgetin downregulates b-Myb expression through the induction of microRNAs and set out to characterize the mechanism of b-Myb transcriptional repression by ginkgetin. Based on our hypothesis, we measured the expression of several miRNAs, including miR-29a, -29c, -30b and-34a, which are reported to regulate b-Myb expression. As shown

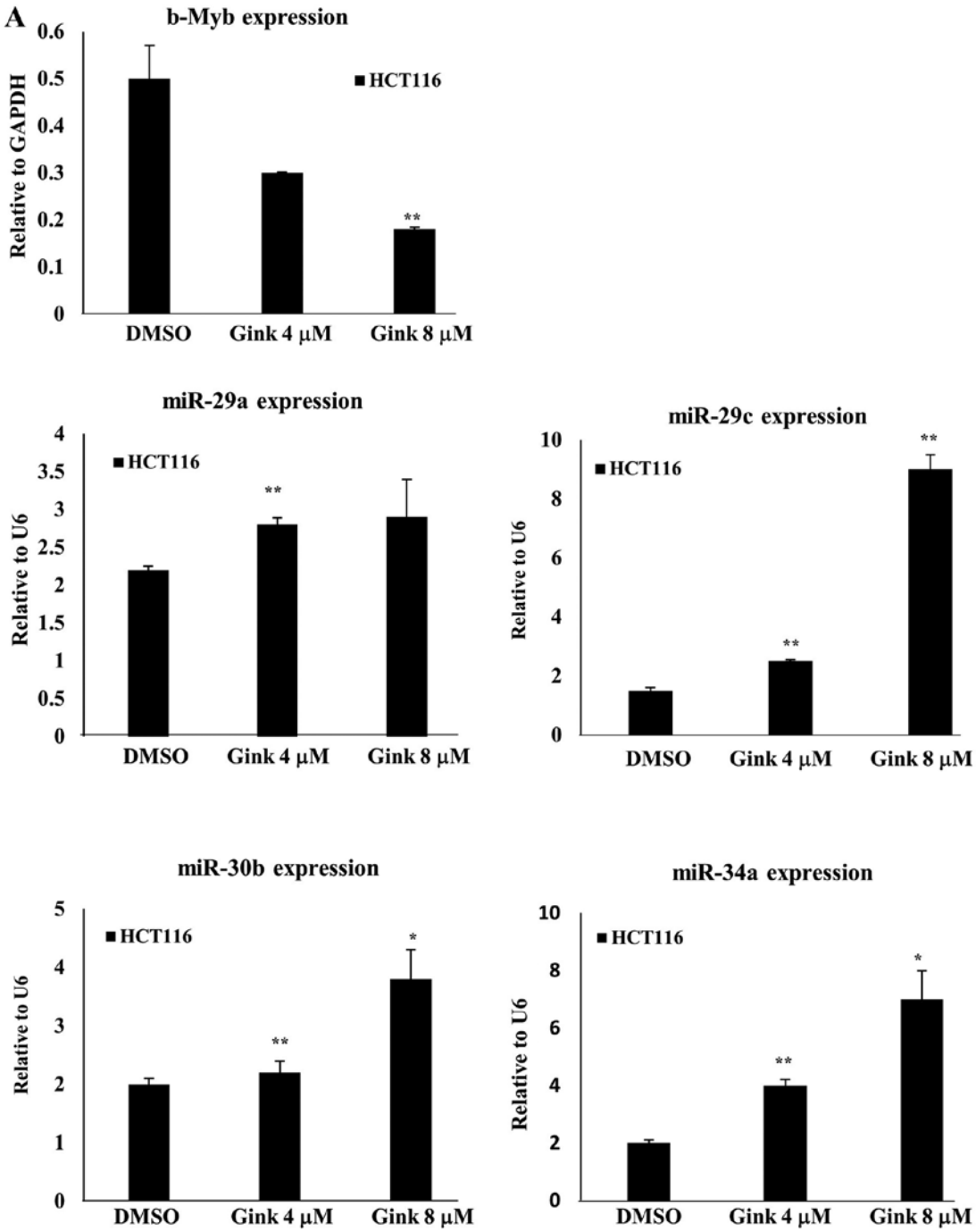


Figure 5A. Ginkgetin suppresses b-Myb via upregulation of miRNAs. (A) Expression of b-Myb mRNA and b-Myb-targeting miRNAs (miR-29a, -29c, -30b and-34a) in CRC cells treated with ginkgetin.

in Fig. 5A, ginkgetin increased the expression levels of miR-29a, -29c, -30b and-34a in HCT116 cells. In particular, we observed a significant induction of miR-29c and -34a in response to ginkgetin treatment in a dose- and time-dependent manner (Fig. 5A and B). To test the effect of the miRNA on b-Myb expression in HCT116 cells, we transfected HCT116 cells with the precursor to miR-29c and-34a (designated miR-29c and -34a mimic), and as control, the cells were transfected with scrambled miRNA. We found that only the miR-34a mimic downregulated b-Myb protein expression (data not shown). In addition, miR-34a was upregulated in ginkgetin-treated cells a time-dependent manner. Therefore, we selected miR-34a as a modulator of b-Myb in ginkgetin-treated HCT116 cells. miR-34a mimic significantly decreased b-Myb expression in a dose-dependent manner and b-Myb was downregulated by >80% at 100 nM miR-34a mimic. An ~40% reduction in cyclin B1 expression was also observed in 100 nM miR-34a mimic-treated cells (Fig. 5C), consistent with a previous report in which miR-34a overexpression downregulated b-Myb expression (31). To confirm these results, we treated HCT116 cells with a specific miR-34a inhibitor and measured the expression levels of b-Myb and cyclin B1. miR-34a inhibitor strongly induced the expression of endogenous b-Myb and also markedly rescued the reduced b-Myb expression caused

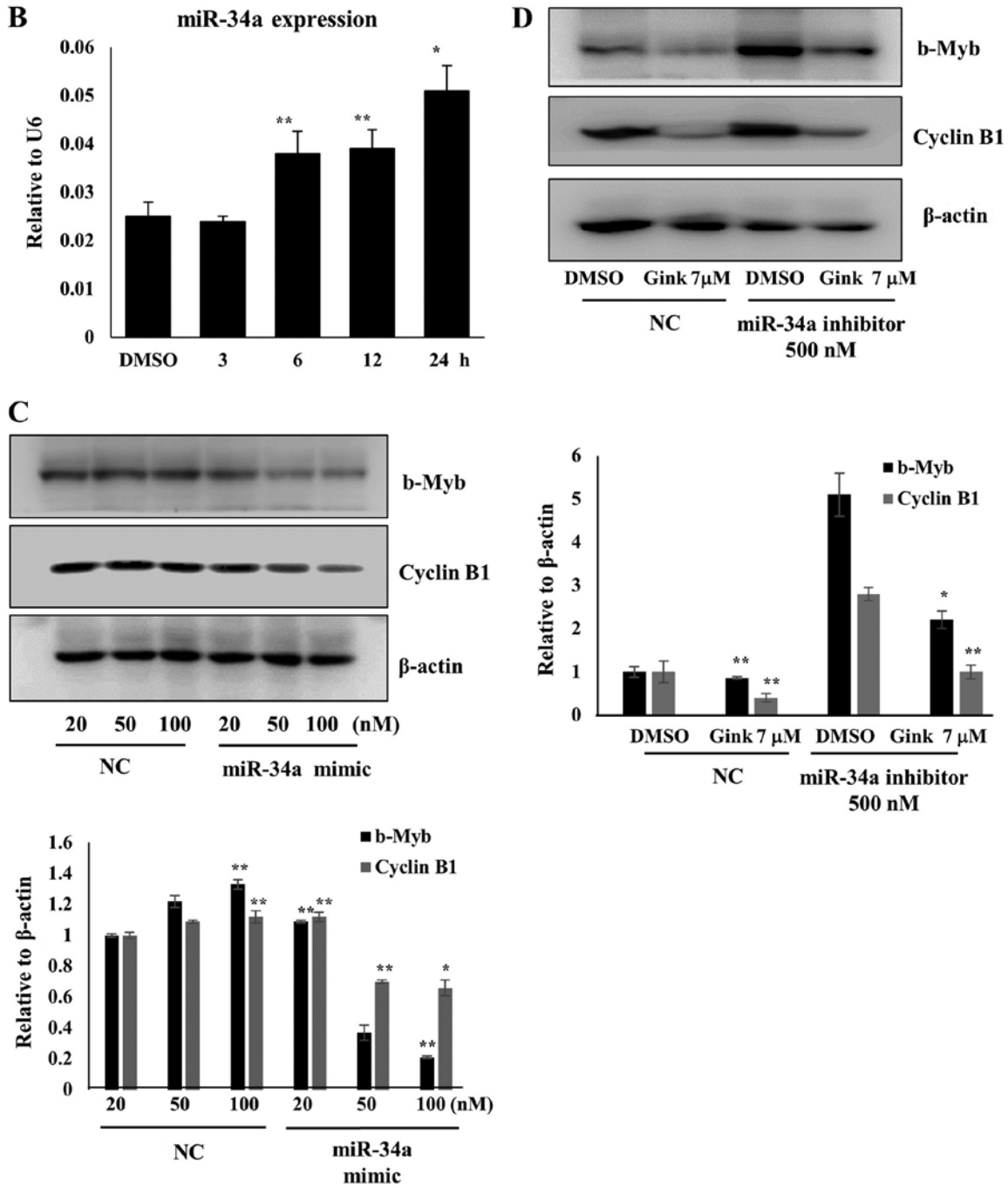


Figure 5B. Continued. (B) Time-dependent (3, 6, 9, 12 and 24 h) miR-34a expression change in HCT116 cells treated with 7 μM of ginkgetin. (C) Western blot analyses for b-Myb and cyclin B1 proteins in HCT116 cells transfected with negative control or miR-34a mimic. (D) Expression change of b-Myb and cyclin B1 proteins by ginkgetin treatment in HCT116 cells transfected with negative control or miR-34a inhibitor. Gink, Ginkgetin. NC, negative control. b-Myb mRNA normalized to GAPDH and miRNAs normalized to U6. Each protein normalized to β-actin. Data were represented as mean ± SD; n=3. *P<0.05 and **P<0.01 indicate statistically significant differences versus control group.

by ginkgetin (Fig. 5D). miR-34a inhibitor also increased the expression of endogenous cyclin B1 and rescued the reduced cyclin B1 expression caused by ginkgetin. These results suggest that ginkgetin exerts its anticancer activity in part through a newly defined mechanism, i.e., the miR-34a/b-Myb cascade.

Ginkgetin inhibits tumor growth in a mouse xenograft model of HCT116 cells. To measure the ginkgetin efficacy *in vivo*, a HCT116 tumor xenograft model of nude mice was used. HCT116 cells were subcutaneously implanted into the right flank of a nude mouse on day 0, and ginkgetin feeding was started at day 1 after HCT116 xenograft implantation. Vehicle (0.5% Tween-80) and ginkgetin in 0.5% Tween-80 were intraperitoneally administered five times per week to tumor-bearing nude mice at a concentration of 10 mg/kg/day for 20 days (eight mice/group). Compared with control (vehicle), ginkgetin treatment suppressed tumor xenograft growth throughout the study. On day 21, the mice were sacrificed, and tumor volume and weight were measured. At the end of the experiment, tumor volume per mouse was 456.7±54 mm^3 in the

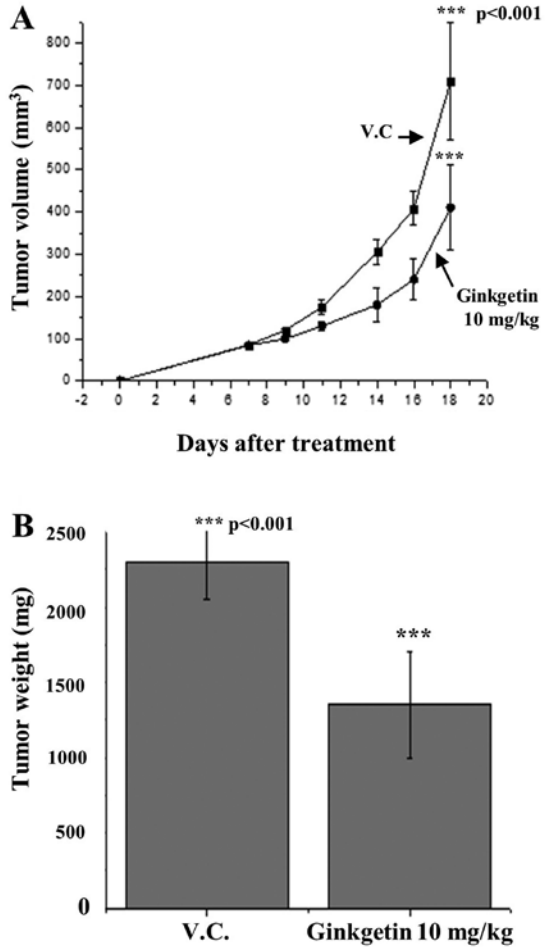


Figure 6. Ginkgetin inhibits the growth of the HCT116 cells in a nude mouse model. For the evaluation of the *in vivo* antitumor activity of ginkgetin, HCT116 cells were implanted subcutaneously into the right flank of nude mice. The mice received intraperitoneal injections of vehicle control or 10 mg/kg (days 3-20, 15 times) for ginkgetin. The results shown were obtained from one assay using 20 mice (8 mice for each compound). Tumor size (A) and tumor weight (B) are presented (***P<0.001). Statistical significance (P-value) was determined with an unpaired t-test.

ginkgetin-treatment group compared with 716.4±63 mm^3 in the control group, reflecting a 36.5% decrease in tumor volume (Fig. 6A). Consistent with this observation, ginkgetin caused a 37.6% decrease in tumor weight (P=0.01) compared with control (Fig. 6B). Ginkgetin did not affect body weight gain and diet consumption profiles, which were almost identical to those of the control group.

Discussion

We consume flavonoid-rich foods, which are widely associated with the reduced risk of chronic diseases based on evidence from epidemiological and human intervention studies (32). However, whether isolated flavonoids can confer the same benefits in preventing and controlling chronic diseases is unclear; therefore, extensive pre-clinical studies are needed to understand the mechanisms of natural flavonoids against different types of human diseases (33). Ginkgetin is a natural biflavonoid isolated from the leaves of *Ginkgo biloba*, and we previously reported on the antitumor activities of ginkgetin (21,22); however, less is known about the mechanisms underlying the anticancer activities of this biflavonoid. In this study, we examined the effects of ginkgetin on various tumor cells and found that ginkgetin inhibited the growth of human tumor cells (Fig. 1A). Based on these results, we investigated the effect of ginkgetin on cell cycle regulation (Fig. 2), inducing apoptosis (Fig. 1C and D), as well as its *in vivo* antitumor activities against human colon tumor (HCT116 cells) xenografts in nude mice (Fig. 6), because colon cancer is the third-most prevalent cancer worldwide.

Many natural products including flavonoids, act on the G_2 checkpoint and induced G_2/M cell cycle arrest in human cancer cells (34), however, a few natural product selectively induced G_2 arrest in human cancer cells. Recently, Liberio *et al* reported that eusynstyelamide B, marine natural product, induced G_2 cell cycle arrest and increased the G_2/M cell population without elevating the levels of the mitotic marker PHH3 in MDA-MB-231 (breast cancer) and LNCap (prostate cancer) cells (35). Although ginkgetin induced G_0/G_1 phase arrest in DU145 cells (STAT3-dependent prostate cancer) by inhibiting STAT3 activity and downregulating cyclin D1 expression (21), we found that ginkgetin selectively induced G_2 cell cycle arrest, as confirmed by M phase-synchronized experiments and using the mitotic marker PHH3 in HCT116 colon cancer cells (Fig. 3A and B). As shown Fig. 3, the microtubule depolymerizing agent nocodazole, which arrests cells in mitosis, increased the level of a mitotic marker PHH3; however, the protein was not detected in ginkgetin-treated HCT116 cells by western blotting. The mitosis-promoting activity of the cyclin B/CDC2 kinase is a critical target for inducing G phase arrest, and this activity is regulated by multiple G_2 checkpoint mediators, including the CDK inhibitor $p21^{CIP1/WAF1}$, CHK1, ATM, ATR, Polo-like kinases, and the CDC25 family (4). However, ginkgetin did not inhibit kinase activity and has no effect on the expression of p21 (data not shown). Interestingly, ginkgetin downregulated the expression of cyclin B1 and CDC2 instead of inhibiting the activity of cyclin B/CDC2 complexes (Fig. 4B). Cyclin B1 expression levels began to decrease after 3 h of treatment, followed by the loss of CDC2 and cyclin B1 protein levels that were dramatically reduced after 24 h of treatment.

Therefore, we postulated the involvement of a transcriptional regulator of cyclin B1 in the G phase arrest by ginkgetin. At early stages of the cell cycle, a very small amount of b-Myb binds to the cyclin B1 promoter; however, binding is significantly increased at the end of S phase (28). b-Myb shRNA significantly decreased the activity of the cyclin B1 promoter compared with control shRNA-transfected cells (29). To gain insight into the mechanism by which ginkgetin regulates b-Myb expression, real-time PCR, siRNA, and western blotting were performed to examine whether ginkgetin directly regulates the expression of cyclin B1. Our results showed that ginkgetin downregulated b-Myb expression both at the mRNA and protein level in a time-dependent manner; b-Myb expression levels were significantly decreased after 12 h of ginkgetin treatment (Fig. 4A and B). When b-Myb was depleted by siRNA, HCT116 cell growth was inhibited, and cyclin B1 expression was dramatically decreased. We did not detect any changes in b-Myb and CDC2 expression in cyclin B1 siRNA-transfected HCT116 cells. Our results suggest that ginkgetin regulates cell cycle progression through b-Myb-mediated cyclin B1 and

CDC2 expression. This finding is consistent with previous studies, which suggest that Myb is important for regulating the G_2/M transition and is a key modulator for cyclin B1 expression in certain human tumor cells (28,29,36).

In ginkgetin-treated HCT116 cells, b-Myb and cyclin B1 expression was downregulated. In cells in which b-Myb was silenced by siRNA, cyclin B1 expression was completely inhibited, and cell growth was also inhibited; therefore, identifying the transcriptional regulator of b-Myb is central to elucidating the mechanism of ginkgetin-induced G_2 arrest in HCT116 cells. To characterize the mechanism of b-Myb transcription repression by ginkgetin, we have searched published reports and found that miR-29a, miR-29c, miR-30b, and miR-34a are associated with b-Myb expression in human cancer cells (30,31). MicroRNAs regulate various cellular processes, including cancer cell proliferation and tumor progression (37). In particular, miR-34a has been identified as a therapeutic agent against human cancer, because miR-34a is an important tumor suppressor whose expression is epigenetically silenced in various human cancers; miR-34a induces cell cycle arrest, apoptosis, senescence, suppresses the epithelial-mesenchymal transition and inhibits the proliferation of cancer stem cells (38). Our study showed that ginkgetin markedly increased the expression of miR-29a, miR-29c, miR-30b, and miR-34a, but only miR-34a affected b-Myb expression in HCT116 cells. Upregulation of miR-34a by ginkgetin contributed to the downregulation of b-Myb, leading to G_2 arrest and apoptosis in HCT116 cells (Figs. 2, 4A and 5B). These results were confirmed by overexpression using a miR-34a mimic and miR-34a inhibitor (Fig. 5C and D). Recent progress in cancer research has demonstrated that changes in DNA methylation, histone modifications, and miRNAs are intricately linked to the initiation, promotion and progression of cancer. The epigenetic silencing of miR-34a by aberrant CpG methylation frequently occurs in various types of human cancer (39). Dihydroxyflavone has been reported to demethylate miR-34a and increases its expression in breast cancer cells (40). Therefore, we hypothesize that ginkgetin controls the expression of miR-34a through its epigenetic modulation; however, ginkgetin did not affect the demethylation of miR-34a (data not shown). A deeper investigation of the underlying mechanism is therefore needed and we are investigating the epigenetic modification by ginkgetin.

In this study, we focused on investigating the mechanism of ginkgetin-inducing G_2 cell cycle arrest and found that ginkgetin induces G_2 arrest and leads to apoptosis in HCT116 cells (CRC) through a newly defined mechanism, i.e., the miR-34a/b-Myb/cyclin B1 pathway. To our knowledge, this report is the first systematic study to demonstrate selective G_2 cell cycle arrest in CRC through the miR-34a/b-Myb/cyclin B1 cascade using a natural flavonoid. These findings suggest the usefulness of therapeutic approaches aimed at modulating the levels of miR-34a by a natural product and are useful to understand the mechanisms of natural flavonoids against human cancer cells (41).

Acknowledgements

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