Ginkgetin induces G2-phase arrest in HCT116 colon cancer cells through the modulation of b-Myb and miRNA34a expression

YU-JIN LEE1, YEONG-RIM KANG1, SO YOUNG LEE1, YENA JIN1,2, DONG CHO HAN1,2 and BYOUNG-MOG KWON1,2

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

Received March 21, 2017; Accepted July 26, 2017

DOI: 10.3892/ijo.2017.4116

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 GI50 value of 4.0 µM for 48-h treatment, together with apoptosis, via G2-phase cell cycle arrest. When HCT116 cells were treated with 10 µM ginkgetin for 48 h, the percentage of cells in G2/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 G2 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 G2 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 G2 arrest for the treatment of CRC.

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 G1→S, G2→M and M→G1, 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 defective 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 G2→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 oncogetic 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 1H-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). Primers and small interfering RNAs (siRNAs) were purchased from Bioneer (Daejeon, Korea). GAPDH (P267613). Primers were purchased from Bioneer.

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 premix (Bio-Rad) according to the manufacturer's instructions using an iQ5 real-time PCR detection system. The following primers were used for real-time PCR: cyclin B1 forward primer (5'-ATGGGACTACTAGTCTGACTG-3'), reverse primer (5'-AGATTCTCTAGTATAGACAGGTAATG-3'), CDC2 primer (PI49402), b-Myb (PI74647), and GAPDH (P267613). Primers were purchased from Bioneer. Relative amounts of CDC2, cyclin B1 and b-Myb were normalized against GAPDH mRNA.

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 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 3 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/1x10^6 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).

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).

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 AGG AGC CUG CUU UAG ATT-3'), cyclin B1 (5'-GAA UUC UGC AGC AGU ACU UCA UCA A-3') and the negative control (5'-CCU ACG CCAAAAU 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 fluoride 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) and 10 µL of cDNA as a template. The thermal profile consisted of an initial denaturation step of 95 C for 10 min, followed by 40 cycles of denaturation at 95 C for 15 s, annealing at 55 C for 30 s, and extension at 72 C for 30 s. The following primers were used for real-time PCR; miR-29a (RT, GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCA; FP, ACACTCGTCTT), URP (CCAGTGCAGGGTCCGAGGTATTCGCA), U6 (RT, AACGCTTCAACGAATTTGCGT; FP, CTCGATCGACTAACC). The following primers were used for real-time PCR; miR-29b (RT, GTCTATCCAGTCCGAGGTATTCGCA; FP, TGGATACGACTAACC), miR-29c (RT, GTCGTATCCAGTCCGAGGTATTCGCA; FP, TGGATACGACTAACC), miR-30b (RT, GTCGTATCCAGTCCGAGGTATTCGCA; FP, TGGATACGACTAACC), miR-34a (RT, GTCGTATCCAGTCCGAGGTATTCGCA; FP, TGGATACGACTAACC), miR-34b (RT, GTCGTATCCAGTCCGAGGTATTCGCA; FP, TGGATACGACTAACC). Real-time miRNA expression values were normalized by using specific expression levels were evaluated by using real-time PCR. (SMC-2101) were purchased from Bioneer. HCT116 cells were plated at a density of 3x10⁵ cells/well in 6-well plates in RPMI-1640 medium containing 10% FBS. Treatment was started 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.

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 3x10⁵ cells/well in 6-well plates and transfected with 20, 50 and 100 nM negative control, has-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 (KIRIBB) Animal Experimentation Ethics Committee. To establish human colon tumors in mice, 1.2x10⁶ 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 mm³. 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 on tumor cells by a cell proliferation assay. A variety of colon cancer cells (HCT116, HCA-7 and SW620) were 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 GI50 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 GI50 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 G2/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 G2/M fraction of ginkgetin-treated cells was increased and the fraction of cells in G0/G1 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 G2/M distribution rates of 27.05 and 40.85%, respectively. The G2/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 G2/M phase
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 G0/G1 phase through the inhibition of STAT3 activity in DU145 (prostate cancer) cells, whereas ginkgetin efficiently induced G2/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 G2 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 G2 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.
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 G0/G1 distribution rates of 42.40 and 39.12%, respectively. The G2/M or G2 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 G2 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 G2 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 G2 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 G2 phase arrest.

Ginkgetin induces G2 phase arrest by modulation of b-Myb expression. As shown Fig. 3B, ginkgetin markedly downregulated the key regulators of the transition through G2 phase and entry into mitosis such as cyclin B1 and CDC2. To confirm the G2 arrest by ginkgetin, the relative mRNA expression levels of G2 arrest-related genes such as b-Myb, CDC2, cyclin G1, 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 G2 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 G2/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 G2, 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 G2 arrest in HCT116 cells.

Ginkgetin regulates b-Myb expression by modulating miR-34a expression. MicroRNAs can act as potent oncogenes
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...
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 in Figure 4, ginkgetin downregulated the expression of these miRNAs in a time-dependent manner. The expression of each miRNA was normalized against the GAPDH gene. The expression of b-Myb was also measured by western blotting using specific antibodies.

Figure 4. Expression of G2 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.
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 by ginkgetin.

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.
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 mm³ in the...
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$_2$/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$_2$ 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 B1/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$_2$ 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...
miR-34a inhibitor (Fig. 5C and D). Recent progress in cancer miR-34a/b -Myb/cyclin B1 pathway. To our knowledge, this report is the first systematic study to demonstrate selective G epigenetic silencing of miR-34a by aberrant CpG methylation; however, this is therefore needed and we are investigating the epigenetic modification by ginkgetin.

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 G2 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 G2 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-induced G2 cell cycle arrest and found that ginkgetin induces G2 arrest and leads to apoptosis in HCT116 cells (CRDC) 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 G2 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

This study was supported by the KRIIBB Research Initiative Program, the Bio-Synergy Research Project (2012M3A 9C4048777), the Bio and Medical Technology Development Program (2015M3A9B5030311), funded by the Ministry of Science, ICT and Future Planning.

References