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**Wnt/ β -catenin signaling mediates the antitumor activity of magnolol
in colorectal cancer cells**

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Running Title: Antitumor activity of magnolol through Wnt/ β -catenin signaling

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Abbreviations: APC, adenomatous polyposis coli; CKI α , casein kinase I α ; FBS, fetal bovine serum; Fz, frizzled; GSK3 β , glycogen synthase kinase 3 β ; LRP5/6, low-density lipoprotein-related receptors 5 and 6; MMP, matrix metalloprotease; PSF, penicillin G sodium and streptomycin and amphotericin B; TCF/Lef, T cell factor/lymphoid-enhancing factor; uPA, urokinase-type plasminogen activator

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Abstract

Abnormal activation of the canonical Wnt/ β -catenin pathway and up-regulation of the β -catenin/T-cell factor (TCF) response to transcriptional signaling play a critical role early in colorectal carcinogenesis. Therefore, Wnt/ β -catenin signaling is considered an attractive target for cancer chemotherapeutics or chemopreventive agents. Small molecules derived from the natural products were used in our cell-based reporter gene assay to identify potential inhibitors of Wnt/ β -catenin signaling. Magnolol, a neolignan from the cortex of *Magnolia obovata*, was identified as a promising candidate, as it effectively inhibited β -catenin/TCF reporter gene (TOPflash) activity. Magnolol also suppressed Wnt3a-induced β -catenin translocation and subsequent target gene expression in HEK293 cells. To further investigate the precise mechanisms of action in the regulation of Wnt/ β -catenin signaling by magnolol, we performed Western blot analysis, real-time reverse transcriptase-polymerase chain reactions, and an electrophoretic mobility shift assay in human colon cancer cells with aberrantly activated Wnt/ β -catenin signaling. Magnolol inhibited the nuclear translocation of β -catenin and significantly suppressed the binding of β -catenin/TCF complexes onto their specific DNA-binding sites in the nucleus. These events led to the down-regulation of β -catenin/TCF-targeted downstream genes such as c-myc, matrix metalloproteinase (MMP)-7, and urokinase-type plasminogen activator (uPA) in SW480 and HCT116 human colon cancer cells. In addition, magnolol inhibited the invasion and motility of tumor cells, and exhibited antitumor activity in a xenograft-nude mouse model bearing HCT116 cells. These findings suggest that the growth inhibition of magnolol against human colon cancer cells can be partly attributed to the regulation of the Wnt/ β -catenin signaling pathway.

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Introduction

Colorectal cancer is one of the leading causes of cancer-related human morbidity and mortality worldwide. Although surgery is the most effective treatment for advanced colon cancer, recurrence frequently occurs within a few years after surgery. Therefore, it is logical to develop strategies based on the carcinogenic progression of common colorectal cancers to decrease the incidence of colon cancer. The dysregulation of the Wnt/ β -catenin signaling pathway has been considered to play an important role in colon carcinogenesis.

The Wnt signaling pathway can be divided into two distinct pathways: a canonical signaling pathway mediated by β -catenin and a non-canonical signaling pathway regulated by Ca^{2+} or small G protein like Rho/Rac (Huelsen et al., 2002). The control of β -catenin levels is a key feature of the canonical Wnt/ β -catenin signaling pathway. In the absence of Wnt activation, cytosolic β -catenin is constitutively phosphorylated through casein kinase I α (CKI α) and glycogen synthase kinase 3 β (GSK3 β), a component of the β -catenin destruction complex that contains axin and adenomatous polyposis coli (APC) as scaffolding proteins. The phosphorylated β -catenin is subsequently degraded by the β -TrCP-mediated ubiquitin/proteasome pathway (Ikeda et al., 1998; Liu et al., 2005). When Wnt proteins are present, they bind to the Frizzled (Fz) receptor and to low-density lipoprotein-related receptors 5 and 6 (LRP5/6). These events lead to the dissociation of β -catenin from the APC/axin/GSK3 β destruction complex. Consequently, unphosphorylated β -catenin accumulates in the cytoplasm and translocates into the nucleus, where β -catenin interacts with T cell and lymphoid-enhancing factors (TCF/Lef) to activate the transcription of Wnt/ β -catenin-mediated target genes (Bienz, 2002; Cong and Varmus, 2004). The target genes include cell-cycle regulating genes (c-myc and cyclin D1) and genes related to metastasis and the invasion of cancer cells (MMP-7 and uPA) (He et al., 1998; Tetsu and McCormick, 1999).

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Aberrant activation of Wnt signaling has been found in a variety of cancers including colon cancers (Bright-Thomas and Hargest, 2003; Phelps et al., 2009; Shan et al., 2009). In particular, APC mutations are the most prevalent genetic alterations in colorectal cancers and lead to the accumulation of β -catenin. They are also associated with the constitutive activation of the Wnt pathway. Indeed, mutations of APC (present in over 80% of sporadic colon cancers) and/or β -catenin (present in approximately 10% of colon cancers) are found in colon cancers, suggesting that these mutations eventually activate the Wnt pathway in colorectal cancer cells. Thus, the modulation of the Wnt/ β -catenin signaling pathway is an attractive candidate for developing a targeted therapy for colorectal cancers.

In the present study, we employed a cell-based reporter gene assay to search for novel small molecule inhibitors of the Wnt signaling pathway. We identified magnolol as a potent inhibitor of the Wnt pathway. Magnolol also exhibits anti-proliferative and anti-tumor activity in colon cancer cells with the modulation of the Wnt signaling pathway.

Magnolol is one of major components of the cortex of *Magnolia obovata* Thumb. (Magnoliaceae). *M. obovata*, a medicinal plant widely distributed in Japan, has been traditionally used for gastrointestinal disorders, constipation and cough. Magnolol has also shown many biological activities, such as antimicrobial, anti-asthmatic, and anti-platelet activities (Clark et al., 1981; Teng et al., 1988; Ko et al., 2003). In addition, anti-proliferative and anti-metastatic effects against various cancer cell lines including colon cancer, hepatoma, leukemia, fibrosarcoma, melanoma, squamous carcinoma, thyroid carcinoma and prostate cancer cells, have also been reported (Zhong et al., 2003; Battle et al., 2005; Ishitsuka et al., 2005; Kong et al., 2005; Huang et al., 2007; Lee et al., 2008). However, the precise mechanism of the inhibition of colon cancer cell growth remains to be identified. Herein, we provide evidence that magnolol may represent a new class of compounds that inhibit the Wnt

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pathway by inhibiting the transcriptional activity of β -catenin/TCF.

Materials and methods

Cell culture and reagents. Human colorectal carcinoma (HCT116), colorectal adenocarcinoma (SW480), embryonic kidney (HEK293), L cells, and L cells stably transfected with Wnt3a (L-Wnt3a) were obtained from the American Type Culture Collection (Manassas, VA). L cell is used to obtain control conditioned medium for comparison to Wnt3a conditioned medium from L-Wnt3a cells. Cells were grown in the media: Dulbecco's modified Eagle medium (DMEM) for HEK293 and L cells; DMEM with 100 μ g hygromycin for HEK293-hFz1 cells; DMEM with 400 μ g G418 for L-Wnt3a cells; and RPMI 1640 for HCT116 cells. These media were supplemented with 10% FBS and antibiotics-antimycotics (PSF; 100 units/ml penicillin G sodium, 100 μ g/ml streptomycin, and 250 ng/ml amphotericin B). SW480 cells were grown in RPMI 1640 medium containing 25 mM HEPES, 10% FBS, and PSF. Dulbecco's modified Eagle medium (DMEM), RPMI 1640 medium, fetal bovine serum (FBS), antibiotics-antimycotics solution, β -catenin specific siRNA and trypsin-EDTA were purchased from Invitrogen (Grand Island, NY). Bovine serum albumin (BSA), trichloroacetic acid, TRI reagent, HEPES, mouse monoclonal anti- β -actin antibody, hygromycin B and other agents unless otherwise indicated were purchased from Sigma-Aldrich (St. Louis, MO). Mouse monoclonal anti-c-myc was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal anti-cyclin D1, anti- β -catenin, anti-PARP and anti-GSK-3 β antibodies were purchased from BD Biosciences (San Diego, CA). CompleteTM protease inhibitor cocktail was purchased from Roche Applied Science (Penzberg, Germany). Alexa Fluor 488 goat anti-mouse IgG, and SYBR Gold[®] staining solution were purchased from Molecular Probes (Eugene, OR). Gene-specific

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primers for reverse transcriptase-polymerase chain reaction (PCR), real-time PCR and scrambled siRNA were synthesized from Bioneer (Daejeon, Korea). AMV reverse transcriptase, dNTP mixture, oligo (dT)₁₅ primer, RNasin, Taq DNA polymerase, Dual luciferase assay system were purchased from Promega (Madison, MA). TCF-electrophoretic mobility shift assay kit was purchased from Panomics (Redwood City, CA).

Test compound. Magnolol (4-Allyl-2-(5-allyl-2-hydroxy-phenyl)phenol, Fig. 1), isolated from *Magnolia obovata*, was provided by Dr. KiHwan Bae (Chungnam National University, Korea) (purity: >98.5%), and dissolved in 100% dimethyl sulfoxide.

Reporter gene assay. Transient transfections were performed using Lipofectamine 2000 (Invitrogen). Briefly, cells (1.5×10^4) were seeded onto 48-well plate. TCF (T cell factor) reporter plasmid contains two sets (with the second set in the reverse orientation) of three copies of the binding site (wild type) upstream of the thymidine kinase (TK) minimal promoter and luciferase open reading frame (TOPflash, pGL3-OT). FOPflash (pGL3-OF) containing mutated TCF binding sites is used as a negative control. After 24 h, the cells were transfected with 0.1 μ g of the luciferase reporter constructs (TOPflash or FOPflash, respectively) and 0.005 μ g of the *Renilla* gene for normalization. HEK293 cells were also co-transfected with 0.02 μ g pcDNA β -catenin and 0.008 μ g of the TCF4 plasmid. After 24 h of transfection, compound was added, and the cells were incubated for 24 h, lysed in 1 \times Passive Lysis Buffer (Promega), and collected for luciferase and *Renilla* activity assays. Activity values were normalized to those for *Renilla* and expressed as the relative value compared to the control.

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Preparation of Wnt3a conditioned medium. Wnt3a-conditioned medium (Wnt3a-CM) was prepared by culturing Wnt3a-secreting L cells (L-Wnt3a) in DMEM with 10% FBS for four days. The medium was harvested and sterilized using a 0.22- μ m filter. Fresh medium was added and the cells were cultured for an additional three days. The medium was collected again and combined with the previous medium.

Stable transfection. HEK293 cells (1×10^6 cells/dish in 100 mm dishes) were incubated for 24 h and transfected with the hFz1 plasmid (24 μ g, provided by Dr. S. Oh at Inje University, Korea). After 24 h, the cells were harvested and reseeded onto 100 mm dish (1×10^4 cells/dish). Medium was replaced daily with fresh medium containing 200 μ g/ml hygromycin B until cell colonies were visualized. The cell colonies were then detached and reseeded onto 24-well plate. To confirm stable transfection, reporter gene assay was performed in the presence of Wnt3a-CM.

Isolation of cytosolic and nuclear extracts. Cells (1×10^6 cells) were treated with test compounds for 24 h with or without Wnt3a-CM. Harvested cells were washed with PBS, suspended in ice-cold lysis buffer (10 mM Tris-HCl [pH 8.0], 1.5 mM $MgCl_2$, 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 2% NP-40, 50 mM sodium fluoride, 5 mM sodium orthovanadate, and protease inhibitor cocktail) on ice for 5 min. After centrifugation at 2,500 rpm for 4 min at 4°C, the supernatant was collected as a cytosolic fraction, and the pellets were washed twice with ice-cold lysis buffer without NP-40. Cells were resuspended in hypertonic nuclear extract buffer (20 mM Tris-HCl [pH 8.0], 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 50 mM sodium fluoride, and protease inhibitor cocktail) on ice for 10 min and then centrifuged at 140,000 rpm for 15 min at 4°C. This supernatant, which

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contained nuclear extracts, was collected and stored in aliquots at -70°C . The protein content of the cell lysates was determined using the Bradford assay (Bradford, 1976).

Cell proliferation assay. Cells (5×10^3 cells/well in 96 well plates) were incubated for 24 h, and treated with test compound for 24, 48, and 72 h. After incubation, cells were exposed to PreMix WST-1 solution (10 μl /well, Takara, Shiga, Japan) for 2-4 h. Absorbance was measured at 450 nm. Cell viability was determined by comparing the absorbance of vehicle-treated control group.

Western blotting. Cells were treated with test compound for 24 h. Harvested cells were disrupted and protein contents were measured by the Bradford assay. Equal amount (40-80 μg) of protein samples were subjected to 6-15% SDS-PAGE. Separated proteins were transferred to PVDF membranes (Millipore, Bedford, MA). Membranes were incubated with primary antibodies diluted in 3% non-fat dry milk in PBST (1:200-1:2000) overnight at 4°C , washed three times with PBST, and incubated with corresponding secondary antibodies. The blots were detected with an enhanced chemiluminescence detection kit (LabFrontier, Suwon, Korea) and a LAS 3000 Imager (Fuji Film Corp., Tokyo, Japan).

Reverse transcription- and real-time polymerase chain reaction. HEK293 cells (4×10^5 cells/dish in 60-mm dishes) were transfected with 2 μg pcDNA β -catenin for 24 h, and then cells were treated with test compound for 12 h. Total RNA was extracted using TRI[®] reagent and reverse transcribed with AMV reverse transcriptase and the oligo(dT)₁₅ primer. Polymerase chain reaction (PCR) was performed in a mixture containing cDNA, 0.2 mM dNTP mixture, 10 pmol of gene-specific primers, and 0.25 units of Taq DNA polymerase

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using a GeneAmp PCR System 2400 (Applied Biosystems, Foster, CA). The PCR cycling parameters used are as follows: an initial denaturation step for 4 min at 94°C; 25-30 cycles of amplification, consisting of denaturation for 30 sec at 94°C, annealing for 30 sec at 55-57°C, and elongation for 30 sec at 72°C; and a final extension step for 5 min at 72°C. The PCR products were separated by 2% agarose gel electrophoresis. The gel was stained with a SYBR Gold staining solution, and visualized under a UV transilluminator (Alpha Imager™, Alpha Innotech Corp., Santa Clara, CA). PCR primer sequences are listed (Supplemental Table 1).

Real-time PCR was conducted with a MiniOpticon system (Bio-Rad, Hercules, CA), using 5 µl of reverse transcription product, iQ™ SYBR® Green Supermix (Bio-Rad), and primers for a total volume of 20 µl. The standard thermal cycler conditions were employed: 95°C for 20 s, 40 cycles of 95°C for 20 s, 56°C for 20 s, and 72°C for 30 s, followed by 95°C for 1 min, and 55°C for 1 min. The threshold cycle (C_T), the fractional cycle number at which the amount of amplified target gene reaches a fixed threshold, was determined using by MJ Opticon Monitor software. The mean threshold cycle (C_t) value for each transcript was normalized by dividing it by the mean C_t value for the β -actin transcript for that sample. Normalized transcript levels were expressed relative to sample obtained from control. Real-time PCR primer sequences are listed (Supplemental Table 1).

Immunofluorescence. Cells were plated onto coverslips and incubated for 24 h. After incubation with test compound for 24 h, the cells were fixed with a 4% paraformaldehyde solution for 30 min at room temperature. The cells were then treated with a 0.1% Triton X-100 solution for permeabilization. After quenching with a 0.1% sodium borohydrate solution, the coverslips were blocked with blocking buffer (1% bovine serum albumin and 0.01% sodium azide in PBS) for 1 h. The coverslips were subsequently incubated with β -catenin

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antibodies diluted in blocking buffer (1:250) overnight at 4°C. The coverslips were further incubated with the corresponding fluorescence (Alexa Fluor 488)-labeled secondary antibodies for 1-2 h. The coverslips were washed three times with PBS, and then mounted with Prolong Gold[®] antifade agent (Molecular Probes, Eugene, OR). Fluorescence-labeled cells were observed under the confocal fluorescence microscope (LSM 510 META, Carl Zeiss, Germany).

Electrophoretic mobility shift assay. The binding of activated TCF and the sequence of the TCF response element were evaluated using the TCF-electrophoretic mobility shift assay kit. Activated TCF was prepared as nuclear cell extracts from SW480 cells. Binding reactions containing 8 µg extracted protein and 1.5 pmol biotin-labeled TCF binding probe were performed for 30 min at 15°C. The products were separated on 6% non-denaturing polyacrylamide gels, and visualized using an ECL detection system.

Cell invasion and motility assay. HCT116 cells (5 x 10⁴ cells/chamber) were used for invasion and motility assays. The inner and outer surface on the filter membrane of Transwell[®] (Corning) were coated with 10 µl of type I collagen (0.5 mg/ml) and 20 µl of 1:2 mixture of Matrigel:DMEM, respectively. Cells were plated on the upper chamber of the Matrigel-coated Transwell[®]. The medium of the lower chambers was also contained 0.1 mg/ml bovine serum albumin. The inserts were incubated for 24 h at 37°C. The cells that had invaded the outer surface of the membrane were fixed with methanol and stained with hematoxylin and eosin, and photographed.

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To determine the cell motility, cells were seeded into Transwell[®] on the outer surface of the filter membrane coated with type I collagen. After incubation for 24 h, the membranes were fixed, stained, and photographed as described previously.

RNA interference. RNA interference of β -catenin was performed using siRNA duplexes purchased from Bioneer (Daejeon, Korea). The sequence of the β -catenin siRNA was AUUACUAGAGCAGACAGAUAGCACC, and GGUGCUAUCUGUCUGCUCUAGUAAU for the sense and antisense strands, respectively. SW480 cells (2×10^5 cells/well in 6-well plate) were transfected with 10 nM of the siRNA duplexes using RNAiMAX (Invitrogen) according to the manufacturer's recommendations. Cells were also transfected with nonspecific control siRNA duplex for direct comparison. After 24 h of transfection, cells were collected, and the protein expression was determined using Western blotting.

In vivo tumor xenograft study. Female nude mice (five weeks old, BALB/cA-nu [nu/nu]) were purchased from Central Laboratory Animal Inc. (Seoul, Korea), and acclimated for one week at $22 \pm 2^\circ\text{C}$ with a 12 h light/dark cycle in a pathogen-free environment. All animal experiments and care were conducted in a manner conforming to the Guidelines of the Animal Care and Use Committee of Ewha Womans University (permission number: EWHA2009-2-13). HCT116 cells were injected subcutaneously into the flanks of the mice (2×10^6 cells in 200 μl medium), and tumors were allowed to develop for eight days until they reached approximately 80 mm^3 . The mice were randomized into vehicle control and treatment groups (n=5). Magnolol (5 mg/kg/body weight) was intraperitoneally injected in a solution containing 0.5% ethanol and 0.5% cremophor (ethanol : cremophor : H_2O = 0.5 :

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0.5 : 99) in a volume of 200 μ l three times a week. The control group was treated with an equal volume of vehicle. Tumor volume was measured using calipers according to the following formula: tumor volume (mm^3) = $3.14 \times L \times W \times H / 6$, where L is the length, W is the width, and H is the height.

Statistical analysis. Data are expressed as means \pm standard deviation (SD) for the indicated number of independently performed experiments. Student's t -test (SigmaStat 3.1, Systat software Inc.) was used for the determination of statistical significance. The difference was considered to be statistically significant when $P < 0.05$.

Results

Inhibitory effects of magnolol on β -catenin/TCF signaling. To search for small molecule inhibitors of β -catenin/TCF signaling, a TOPflash assay was employed. In our evaluation of approximately five hundred natural compounds magnolol was identified as one of the most potentially active compounds in this assay system (Fig. 1A). Magnolol (Fig. 1A) exhibited potent inhibitory activity against the TOPflash reporter gene in a concentration-dependent manner (Fig. 1B). However, magnolol did not affect FOPflash activity (a mutant of the TCF binding site), suggesting that the inhibitory activity by magnolol is dependent on β -catenin/TCF signaling. To further elucidate whether the inhibitory activity of β -catenin/TCF signaling by magnolol is associated with target gene expression, downstream target genes, such as cyclin D1 or c-myc, were investigated with RT-PCR or real-time PCR analysis. Under normal conditions, the β -catenin level in HEK293 cells is relatively low, and thus, the expression of target genes is maintained at a low level. However, the introduction of the β -catenin plasmid remarkably increased the expression of cyclin D1 and c-myc genes,

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target genes of β -catenin/TCF signaling. The elevated expression of the target genes by β -catenin was alleviated upon treatment with magnolol (Fig. 2A). Real-time PCR analysis also showed the downregulation of cyclin D1 and c-myc gene expressions after treatment with magnolol in a concentration-dependent manner (Fig. 2B). In addition, target protein expression, specifically, the expression of the cyclin D1 and c-myc proteins, was investigated in β -catenin-overexpressing cells. HEK293 cells were transfected with the β -catenin plasmid (1.5 μ g) for 24 h and then treated with magnolol for an additional 24 h. As shown in Fig. 2C, magnolol suppressed the expression of the cyclin D1 and c-myc proteins. These results suggest that magnolol suppressed the expression of cyclin D1 and c-myc through the inhibition of the β -catenin/TCF signaling pathway.

Inhibitory effects of magnolol on Wnt/ β -catenin signaling in HEK293-hFz1 cells.

Under normal conditions, APC/Axin/GSK3 destruction complexes phosphorylate β -catenin, and the phosphorylated β -catenin is recognized by β -TrCP and then subsequently degraded by the ubiquitin-proteasomal pathway. As a result, cytosolic β -catenin levels are maintained at a low level. In the presence of Wnt, the destruction complex is dissociated and cytosolic β -catenin is translocated into the nucleus to activate the target genes. To confirm the responsiveness of Wnt/ β -catenin signaling, various concentrations of Wnt3a-CM which were conditioned from Wnt3a-producing L cells were treated and the TOPflash reporter gene assay was performed. Treatment with 50 and 100% of Wnt3a-CM increased luciferase activity approximately three-fold compared to that of the non-treated control (Supplemental Fig. 1A). Based on these findings, we further established HEK293-hFz1 cells, which were anchored with the Wnt receptor human frizzled-1, to enhance the responsiveness of Wnt signaling. Wnt3a-CM remarkably increased the TOPflash activity in HEK293-hFz1 cells compared to

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HEK293 cells (Supplemental Fig. 1B). The activation of Wnt-mediated signaling in HEK293-hFz1 cells was also confirmed by a significant increase in β -catenin expression in the cytoplasm and nucleus (Supplemental Fig. 1C). To evaluate whether magnolol suppresses Wnt/ β -catenin-mediated transcriptional activity in HEK293-hFz1 cells the TOPflash activity and target gene expression were determined in Wnt3a-CM-treated HEK293-hFz1 cells. As shown in Fig. 3A, Wnt3a-CM markedly increased TOPflash activity, but magnolol abrogated the reporter gene activity in a concentration-dependent manner. Western blot analysis also demonstrated the suppression of the expression of target gene cyclin D1 by magnolol in Wnt3a-CM-treated HEK293-hFz1 cells (Fig. 3B).

To further elucidate the effect of magnolol on the expression level of Wnt-induced β -catenin, HEK293-hFz1 cells were treated with Wnt3a-CM in the presence or absence of magnolol for 24 h. Wnt3a-CM enhanced the expression of β -catenin in the cytosol and nucleus, and magnolol suppressed the Wnt3a-CM-induced expression of β -catenin in these cells (Fig. 3C). Because the increase of the degradation of β -catenin in the cytosolic fraction was not found by magnolol, the suppression of β -catenin in the nucleus fraction might be mainly associated with the inhibition of the nuclear translocation of β -catenin by magnolol. Immunofluorescence analysis also revealed the downregulation of β -catenin by magnolol in the Wnt3a-CM-induced HEK293-hFz1 cells (Fig. 3D).

Inhibitory effects of magnolol on the β -catenin/TCF signaling pathway in colorectal cancer cells. Based on the findings that magnolol suppressed Wnt3a-induced β -catenin and its target gene expression in HEK293-hFz1 cells, we further explored the effects of magnolol on β -catenin/TCF signaling in colon cancer cells. Colon cancer cells frequently possess mutations in APC or β -catenin and activated Wnt signaling as a result of the cellular

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accumulation of β -catenin. To investigate the inhibitory effect of magnolol on the transcriptional activity of β -catenin/TCF in colon cancer cells, the TOPflash reporter gene assay was performed in SW480 (truncated mutation of APC and wild-type β -catenin) or HCT116 (Ser45 deletion mutation of β -catenin and wild-type APC) human colon cancer cells. As shown in Fig. 4A, cells transfected with the TOPflash reporter showed the highest transcriptional activity, but magnolol suppressed the luciferase activity in human colon cancer cells.

To further clarify whether the suppressive effect of magnolol on the transcriptional activity of β -catenin/TCF is associated with the downregulation of β -catenin, β -catenin levels in the cytosol and nucleus were examined. When treated with magnolol for 24 h, magnolol suppressed the expression of β -catenin in the cytosol and nucleus in SW480 cells (Fig. 4B). Because magnolol decreased the expression of β -catenin in the nucleus of SW480 cells, it was assumed that the suppressive effect of magnolol on the transcriptional activity of β -catenin/TCF might be correlated with the decrease in the binding of β -catenin/TCF complexes to the promoter region of DNA. Therefore, the change in DNA-TCF complex binding was investigated using an electrophoretic mobility shift assay. Magnolol inhibited the binding of DNA and TCF through the TCF binding site (Fig. 4C). These findings suggest that the suppressive effects of magnolol on β -catenin/TCF transcription are associated with the downregulation of β -catenin in the cytosol and nucleus and consequently the inhibition of binding between DNA and β -catenin/TCF complexes.

Inhibitory effect of magnolol on target gene expressions, and cell invasion and migration activity in colon cancer cells. To assess the inhibitory effects of magnolol on β -catenin/TCF signaling in colon cancer cells, real-time PCR was employed to determine the

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expression of β -catenin/TCF target genes. Cyclin D1 and c-myc genes are established target genes of the β -catenin-dependent pathway and are implicated in enhancing cell proliferation (He et al., 1998; Liu et al., 2001).

As shown in Fig. 5A, treatment with magnolol suppressed the mRNA expression of cyclin D1 and c-myc. Further experiments showed that the expression of matrix metalloproteinase-7 (MMP-7) and urokinase plasminogen activator (uPA), which are associated with cancer cell invasiveness and also target genes of β -catenin/TCF transcription (Brabletz et al., 1999; Alexander et al., 2002), were also significantly suppressed by magnolol (Fig. 5B).

Magnolol also suppressed the protein expressions of cyclin D1 and c-myc, but the expression of GSK3 β was not altered. However, magnolol remarkably induced the expression of E-cadherin (Fig. 5C). In addition, magnolol dose-dependently inhibited the cancer cell invasion and motility through Transwell® (Fig. 5D).

To further confirm whether magnolol regulates cyclin D1 and c-myc through β -catenin/TCF signaling the direct effect of β -catenin siRNA was determined. SW480 cells were transfected with either β -catenin-specific siRNA or scrambled siRNA for 24 h, and then cells were incubated for an additional 24 h in serum-free media. The cells were treated with magnolol for 3 h, and then Western blotting was performed with cell lysates. Transfection with the β -catenin siRNA effectively suppressed the protein expression of β -catenin. The expression of cyclin D1 and c-myc was also decreased by β -catenin siRNA. The expression of c-myc was dramatically suppressed compared to cyclin D1. However, magnolol enhanced the suppression of cyclin D1 expression in cells transfected with β -catenin siRNA in a concentration-dependent manner (Fig. 6).

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Anti-proliferative and antitumor activity of magnolol in colon cancer cells. Because the upregulation of β -catenin plays a critical role in the proliferation of colon cancer cells (Verma et al., 2003), we assumed that the suppression of magnolol on the transcription of β -catenin/TCF might lead to growth-inhibition in colon cancer cells. The anti-proliferative effect of magnolol was investigated using the WST-1 assay in SW480 and HCT116 cells. Magnolol exhibited anti-proliferative activity in a time- and concentration-dependent manner (Fig. 7A).

In addition, the antitumor activity of magnolol *in vivo* was assessed in a nude mouse xenograft model. HCT116 cells were subcutaneously implanted into nude mice. When tumor size reached $\sim 80 \text{ mm}^3$, magnolol (5 mg/kg) was i.p. administered three times per week for 20 days. Tumor volume in the control group was approximately $1,200 \text{ mm}^3$ on day 28 after the cancer cells were inoculated. Treatment with magnolol effectively suppressed tumor growth, and the inhibitory effect in the magnolol-treated group (5 mg/kg) was approximately 54.6% compared with that of the control group (Fig. 7B), but body-weight change upon treatment with magnolol was negligible (Fig. 7C), and no overt toxicity was found under these experimental conditions.

Discussion

The Wnt/ β -catenin signaling pathway has been considered to play a crucial role in the early stages of carcinogenesis and the maintenance of colorectal cancers with either inactive mutations of the APC tumor suppressor or active mutation of the β -catenin gene (Bright-Thomas and Hargest, 2003; Liu et al., 2001; Fearnhead et al., 2001). Indeed, high levels of β -catenin accumulate in colorectal cancer cells compared to the low levels of β -catenin seen in

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normal colonic cells (Polakis, 1997; Morin, 1999). Therefore, the modulation of Wnt/ β -catenin signaling is considered to be a promising drug target in the development of cancer chemotherapeutic agents. In our program to identify potential small molecule inhibitors of Wnt signaling, over 500 compounds derived from natural products were evaluated using a cell-based TOPflash reporter gene assay in HEK293 cells. Several classes of natural products including curcuminoids, phenylpropanoids, and lignans exhibited a potential inhibition of TOPflash activity. Among the active compounds, magnolol was one of the most prominent inhibitors of Wnt reporter gene activity. Therefore, in the present study, we examined the plausible mechanisms of action for the modulation of Wnt/ β -catenin signaling and the growth inhibition of human colorectal cancer cells by magnolol.

Previous studies have demonstrated the anti-proliferation effect of magnolol on colon cancer cells by inhibiting DNA synthesis and the activation of the Ras/Raf-1/ERK pathway (Lin et al., 2002; Hsu et al., 2007). However, the detailed mechanisms of the growth inhibition of magnolol in colorectal cancer cells remain to be elucidated. Furthermore, the modulation of Wnt/ β -catenin signaling by magnolol in colon cancer cells has not been identified.

Our cell-based reporter assays showed that magnolol effectively inhibited the Wnt/ β -catenin signaling pathway stimulated by β -catenin/TCF and Wnt3a-CM, suggesting that magnolol might target β -catenin or its downstream effectors. It was reported that the expression of cyclin D1 and c-myc, target genes of β -catenin/TCF transcription, was induced by the introduction of exogenous β -catenin cDNA in HEK293 cells (He et al., 1998; Tetsu and McCormick, 1999; Shtutman et al., 1999). To confirm whether magnolol affects the activation of Wnt signaling mediated by β -catenin overexpression, HEK293 cells were transfected with β -catenin cDNA, and the subsequent expression of cyclin D1 and c-myc was

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estimated by real-time PCR and RT-PCR. The elevated expression of cyclin D1 and c-myc genes mediated by β -catenin was suppressed by magnolol, indicating that magnolol might inhibit β -catenin/TCF-mediated target gene expressions. Employed the human frizzled receptor type 1 (hFz1)-encoded established cells HEK293-hFz1, we also confirmed the direct modulation of the Wnt signaling pathway by magnolol. Magnolol potentially suppressed Wnt-mediated downstream signaling with the inhibition of TOPflash activity, downregulation of cyclin D1 expression, a decrease in β -catenin expression, and translocation of β -catenin into the nucleus in Wnt3a-CM-treated HEK293-hFz1 cells.

Based on our results of the effects of magnolol on β -catenin/TCF and Wnt3a-mediated signaling in the HEK293 and HEK293-hFz1 cell systems, we further evaluated the effects of magnolol on Wnt/ β -catenin signaling in human colorectal cancer cells, which possess intrinsically activated Wnt signaling through mutations in the APC or β -catenin genes as well as increased levels of both cytosolic and nuclear β -catenin (Weinberge, 2005; Sievers et al., 2006). The present study showed that magnolol inhibited the endogenously activated transcriptional activity of β -catenin/TCF in both SW480 cells (APC mutants) and HCT116 cells (β -catenin mutants). The inhibition of β -catenin-mediated transcription by magnolol was also confirmed by the suppression of β -catenin/TCF target gene expressions, including the expression of MMP-7, uPA, cyclin D1, and c-myc, in colorectal cancer cells. These results suggest that magnolol suppresses cancer cell invasiveness and proliferation in the Wnt-activated colorectal cancer cells.

Similar results were seen for the Wnt3a-CM-stimulated expression of β -catenin in HEK293-hFz1 cells; magnolol also suppressed the overexpression of β -catenin in both the cytosol and nucleus in SW480 cells. The suppression of β -catenin expression led to a decrease in DNA-TCF binding upon magnolol treatment of SW480 cells. These data suggest

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that the blocking effect of magnolol on β -catenin accumulation in the cytoplasm leads to a decrease in the level of β -catenin in the nucleus, thus suppressing the expression of target genes. To further clarify the involvement of β -catenin in the modulation of the Wnt signaling pathway by magnolol, the target gene expression of β -catenin/TCF-mediated transcription was confirmed by transfection of SW480 cells with β -catenin-specific siRNA. The expression of cyclin D1 and c-myc was markedly suppressed by the transfection of β -catenin siRNA, suggesting that β -catenin regulates the expression of these target genes. Co-treatment of magnolol with β -catenin siRNA enhanced the suppression of cyclin D1 expression, indicating the involvement of β -catenin in the modulation of the Wnt signaling pathway by magnolol.

To further elucidate the regulation of β -catenin by magnolol we determined the expression of one plausible modulator E-cadherin. E-cadherin is a transmembrane glycoprotein that mediates tight adhesion between cells, and the cytoplasmic domain of E-cadherin interacts with α -, β -, and γ -catenin. In a previous study, a signal to the nucleus via β -catenin was hypothesized to be controlled by E-cadherin because of the dual roles of β -catenin: induction of the nuclear signal and cadherin-mediated adhesion at the plasma membrane (Cox et al., 1996; Gottardi et al., 2001). In the present study, we found that magnolol enhanced the expression of E-cadherin in Wnt3a-CM-stimulated HEK293-hFz1 cells, and the level of E-cadherin was also increased by magnolol in SW480 and HCT116 cells. These data suggest that magnolol might increase the expression of E-cadherin, and thus, the level of cytosolic β -catenin should be downregulated. The upregulation of E-cadherin and suppression of MMP-7 and uPA by magnolol were also somewhat correlated with the inhibition of colon cancer cell invasion and motility. However, further studies are needed to

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elucidate whether the magnolol-induced E-cadherin binds and sequesters β -catenin in the cytosol. Recent reports by Thorne et al., (2010) show that the inhibition of Wnt signaling by the small molecule inhibitor pyrvinium is associated with the activation of casein kinase 1α , a component of the β -catenin destruction complex in the cytosol. Thus, the precise mechanisms of the upstream regulation of β -catenin in the cytosol by magnolol still remain to be clarified.

Because magnolol suppressed the expressions of cyclin D1 and c-myc, major factors in cancer cell proliferation, further experiments were designed to investigate whether magnolol affects the proliferation of cancer cells by altering Wnt signaling in human colorectal cancer cells. Magnolol effectively inhibited the proliferation of both SW480 and HCT116 cells in a concentration-dependent manner *in vitro*. Tumor growth was also suppressed by magnolol in nude mouse xenograft model bearing HCT116 cells (Fig. 7). These data suggest that the anti-proliferative effects of magnolol in colorectal cancer cells might partly be associated with the downregulation of Wnt signaling.

In summary, the present study demonstrates that magnolol might be a potential candidate in the development of small molecule inhibitors of Wnt signaling. Therefore, magnolol might be useful for treating sporadic colon cancer cells either alone or in combination with other chemotherapeutic agents.

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Authorship Contributions

Participated in research design: Kang, Park HJ, Chung, Min, Park EJ, and Lee SK.

Conducted experiments: Kang, Park HJ, Chung, Lee MA and Shin

Contributed new reagents or analytic tools: Kang, Park HJ, and Chung

Performed data analysis: Kang, Park HJ, Chung, and Lee SK.

Wrote or contributed to the writing of the manuscript: Kang, Park HJ, Chung, and Lee
SK.

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Footnotes

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YJ Kang and HJ Park contributed equally to this work

No potential conflicts of interest were disclosed.

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Figure legends

Fig. 1. Inhibition of β -catenin/TCF transcriptional activity by magnolol. (A) The chemical structure of magnolol. (B) Inhibitory effect of magnolol on the TOPflash/FOPflash activity in HEK293 cells. HEK293 cells were transiently transfected with β -catenin and TCF4, TOPflash or FOPflash and *Renilla* in the presence of magnolol. Results indicate mean \pm SD (n = 3), and are representative of the findings from three or more separate experiments. * $P < 0.05$ compared to DMSO control

Fig. 2. Effect of magnolol on target gene expressions of β -catenin/TCF signaling. (A) HEK293 cells were transfected with 2 μ g of either pcDNA or β -catenin plasmid, and then cells were treated by 25 μ M magnolol for 12 h. Total RNA was extracted and reverse transcribed with AMV reverse transcriptase and the oligo(dT)₁₅ primer. Polymerase chain reaction (PCR) was performed as described in Materials and methods. The PCR products were separated by gel electrophoresis. The gel was stained with a SYBR Gold staining solution, and visualized under a UV transilluminator. β -Actin gene was served as an internal standard. (B) Inhibitory effects of magnolol on the cyclin D1 and c-myc expressions elevated by β -catenin in HEK293 cells. HEK293 cells were transfected with 3 μ g of either pcDNA or β -catenin plasmid, and then treated with indicated concentrations (25 and 50 μ M) of magnolol for 12 h. The expression level of cyclin D1 and c-myc were examined using real-time PCR. * $P < 0.05$ compared to pcDNA alone-transfected cells, # $P < 0.05$ compared to DMSO control. (C) Inhibitory effects of magnolol on the target protein expressions of β -

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catenin/TCF signaling in HEK293 cells. Cells were transfected with 1.5 μ g of either pcDNA or β -catenin plasmid. β -Actin gene was used as an internal standard. Data are representative of three independent experiments.

Fig. 3. Effects of magnolol on the Wnt3a-induced β -catenin/TCF signaling in HEK293-hFz1 cells. (A) HEK293-hFz1 cells were treated with various concentrations of magnolol (20, 30 and 40 μ M) in the presence of Wnt3a-CM. After 24 h incubation, the TOPflash activity was determined. * P <0.05, ** P <0.01 compared to DMSO control. (B) Inhibition of the cyclin D1 expression by magnolol in the Wnt3a-CM-treated HEK293-hFz1 cells. HEK293-hFz1 cells were treated with indicated concentrations of magnolol for 24 h. The protein expression of cyclin D1 was measured by Western blot analysis. β -Actin was used as an internal standard. (C) Suppressive effect of magnolol on the expression of β -catenin induced by treatment with Wnt3a-CM in HEK293-hFz1 cells. HEK293-hFz1 cells were treated with magnolol in the presence of Wnt3a-CM, and then cytosolic, and nuclear fractions of the cells lysates were separated and analyzed the expression of β -catenin by Western blot. Anti-PARP was used for a marker of nuclear fraction. (D) Suppressive effect of magnolol on the expression of β -catenin in the Wnt3a-CM-stimulated HEK293-hFz1. The cells were treated with magnolol for 24 h in the presence of Wnt3a-CM, and then incubated with the anti- β -catenin antibody. The expression of β -catenin was investigated under confocal fluorescence microscopy (x600).

Fig. 4. Inhibitory effect of magnolol on the transcriptional activity of β -catenin/TCF in colon cancer cells. (A) Colon cancer (SW480 and HCT116) cells were co-transfected with TOPflash (or FOPflash) and *Renilla* for 24 h, and then magnolol was treated for an additional

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24 h. The TOPflash or FOPflash activity was determined. * $P < 0.05$ compared to DMSO control. (B) Suppression of magnolol on the expression of β -catenin both in cytosolic and nuclear fractions in SW480 cells. SW480 cells were treated with magnolol for 24 h, and then analyzed the expression of β -catenin by Western blot. β -Actin was used as a loading control, and PARP was used as a nuclear fraction marker. (C) The inhibitory effects of magnolol on the binding of TCF complexes to DNA. SW480 cells were treated with various concentrations of magnolol (25, 50 and 75 μ M) for 24 h, and then nuclear extracts were isolated. EMSA was performed with nuclear extracts from treated or untreated cells.

Fig. 5. Inhibitory effect of magnolol on the expression of target genes in human colon cancer cells. (A) Cells were treated with indicated concentrations of magnolol for 24 h, and then the mRNA level of c-myc or cyclin D1 were examined using real-time PCR. * $P < 0.05$, ** $P < 0.01$ compared to DMSO control. (B) Inhibitory effect of magnolol on the gene expression of MMP-7 or uPA in human colon cancer cells. Cells were treated with indicated concentrations of magnolol for 24 h, and then the mRNA level of MMP-7 or uPA was examined using real-time PCR. * $P < 0.05$, ** $P < 0.01$ compared to DMSO control. (C) Inhibition of magnolol on the protein expressions related with Wnt signaling pathway. SW480 cells or HCT116 cells were treated with indicated concentrations of magnolol for 24 h, and then the expressions of c-myc, cyclin D1, E-cadherin, and GSK3 β were determined by Western blot. β -Actin was used as an internal standard. (D) Effect of magnolol on invasion and motility of colon cancer cells. HCT116 cells were treated with magnolol (25, 50, or 75 μ M) for 24 h in the collagen/Matrigel-coated Transwell® (invasion; the upper panel) and in the collagen-coated membrane on the outer surface of Transwell® (motility; the lower panel).

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Fig. 6. Effect of magnolol on the expression of cyclin D1 and c-myc in SW480 cells transfected with β -catenin specific siRNA. SW480 cells were transfected with either siRNA directed against β -catenin or scrambled siRNA using RNAiMAX for 24 h, and then cells were serum starved for an additional 24 h. Subsequently, magnolol (25, 50 or 75 μ M) were treated for 3 h, and β -catenin, c-myc, or cyclin D1 protein expressions were determined by Western blot.

Fig. 7. Anti-proliferation of magnolol in human colon cancer cells. (A) SW480 or HCT116 colon cancer cells were treated with various concentrations of magnolol for 24, 48 or 72 h. Cell proliferation was measured using WST-1 assay. % of proliferation was determined by comparison with vehicle-treated control cells (n=3). * P <0.05, ** P <0.01 compared to DMSO control. (B) Inhibition of tumor growth by magnolol and (C) body weight change in HCT116 xenograft nude mouse model. HCT116 cells (2×10^6 cells) were injected s.c. into the flanks of nude mice and tumors were allowed to develop for 8 days until they reached ~ 80 mm³, then treatment was initiated (n=5). The mice were intraperitoneally administered three times a week with magnolol (5 mg/kg) in a solution containing 0.5% ethanol and 0.5% cremophor (ethanol:cremophor:H₂O = 0.5:0.5:99) in a volume 200 μ l for 20 days. Control group was treated with an equal volume of vehicle. Tumor volume and body weight were monitored every 2-3 days. * P <0.05 compared to vehicle alone-injection.

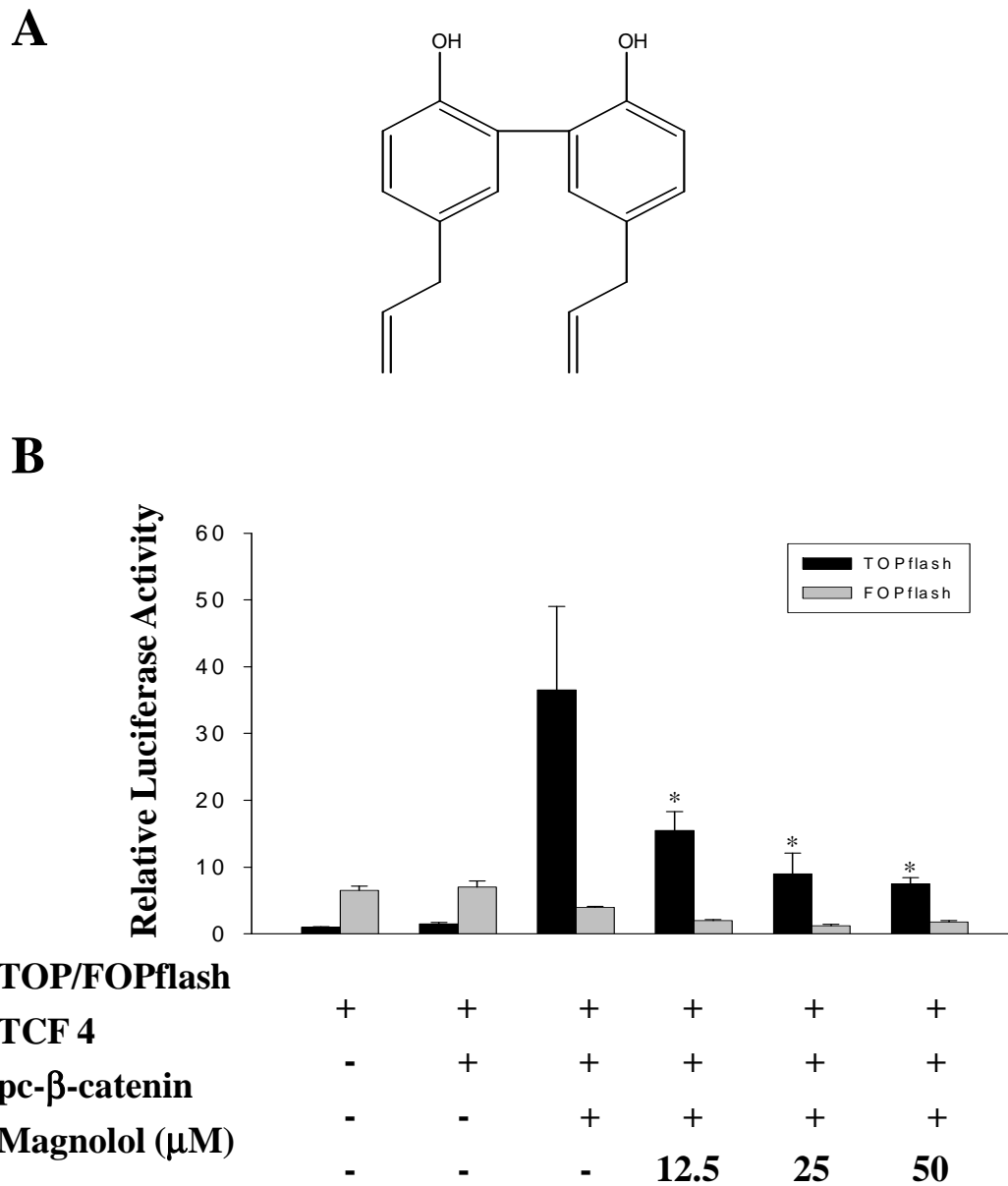


Fig. 1

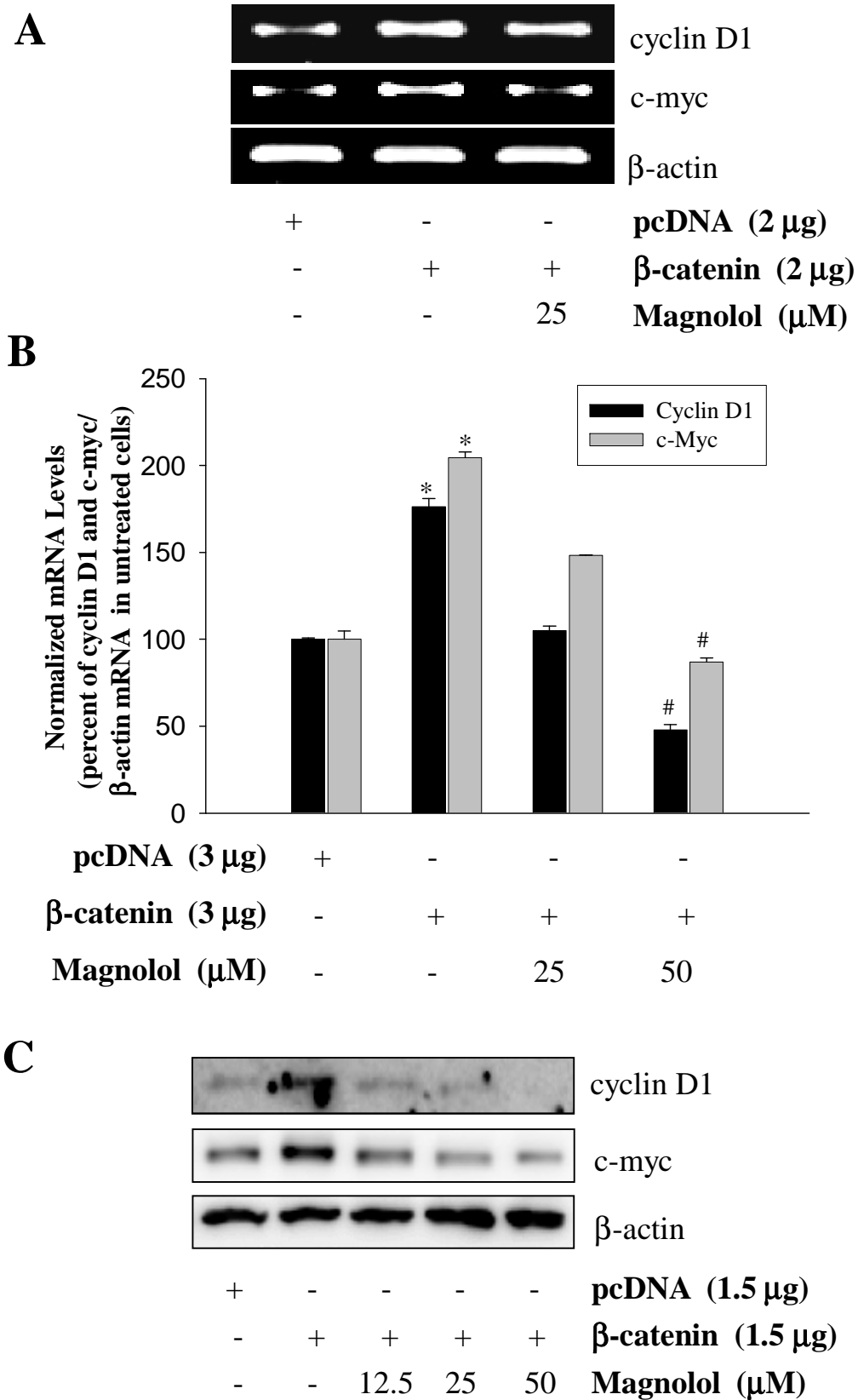


Fig. 2

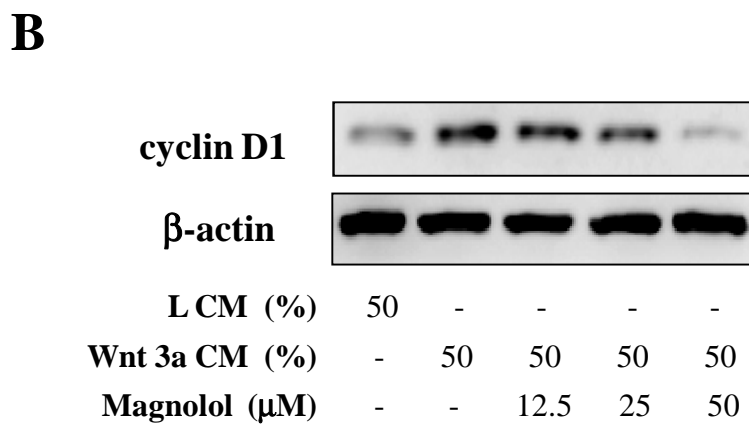
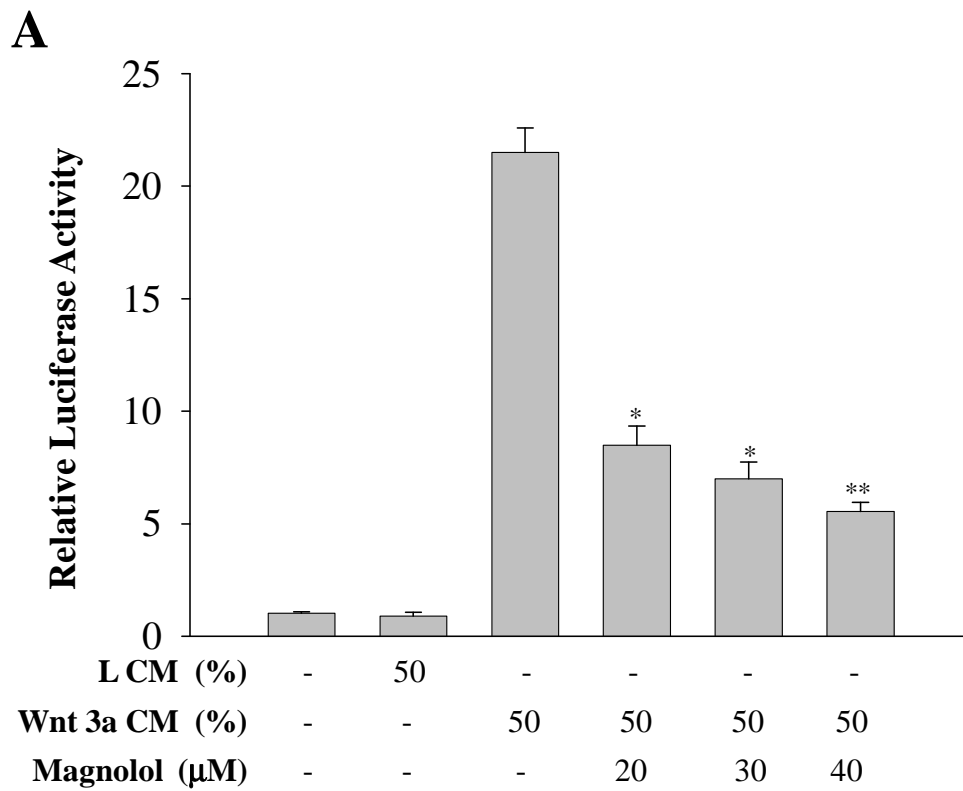


Fig. 3

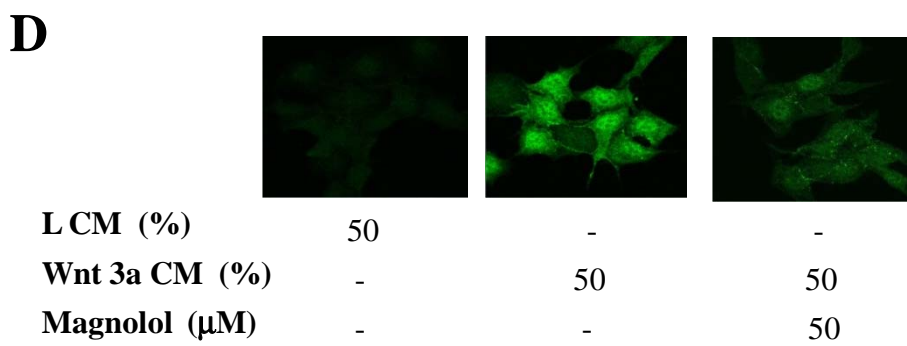
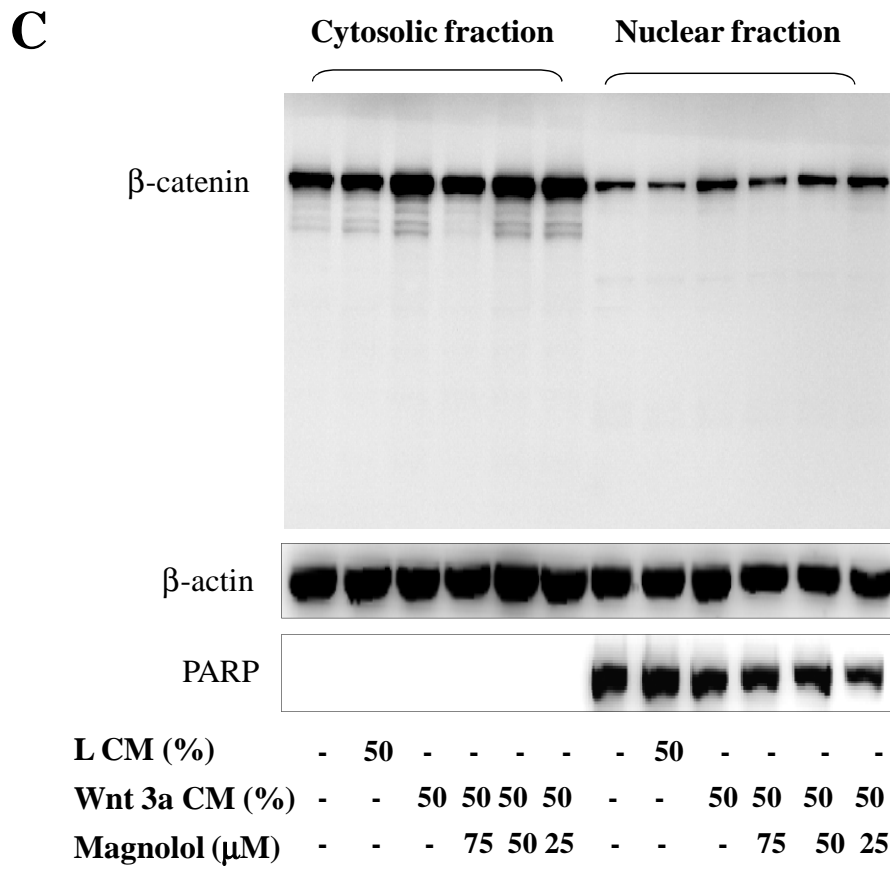


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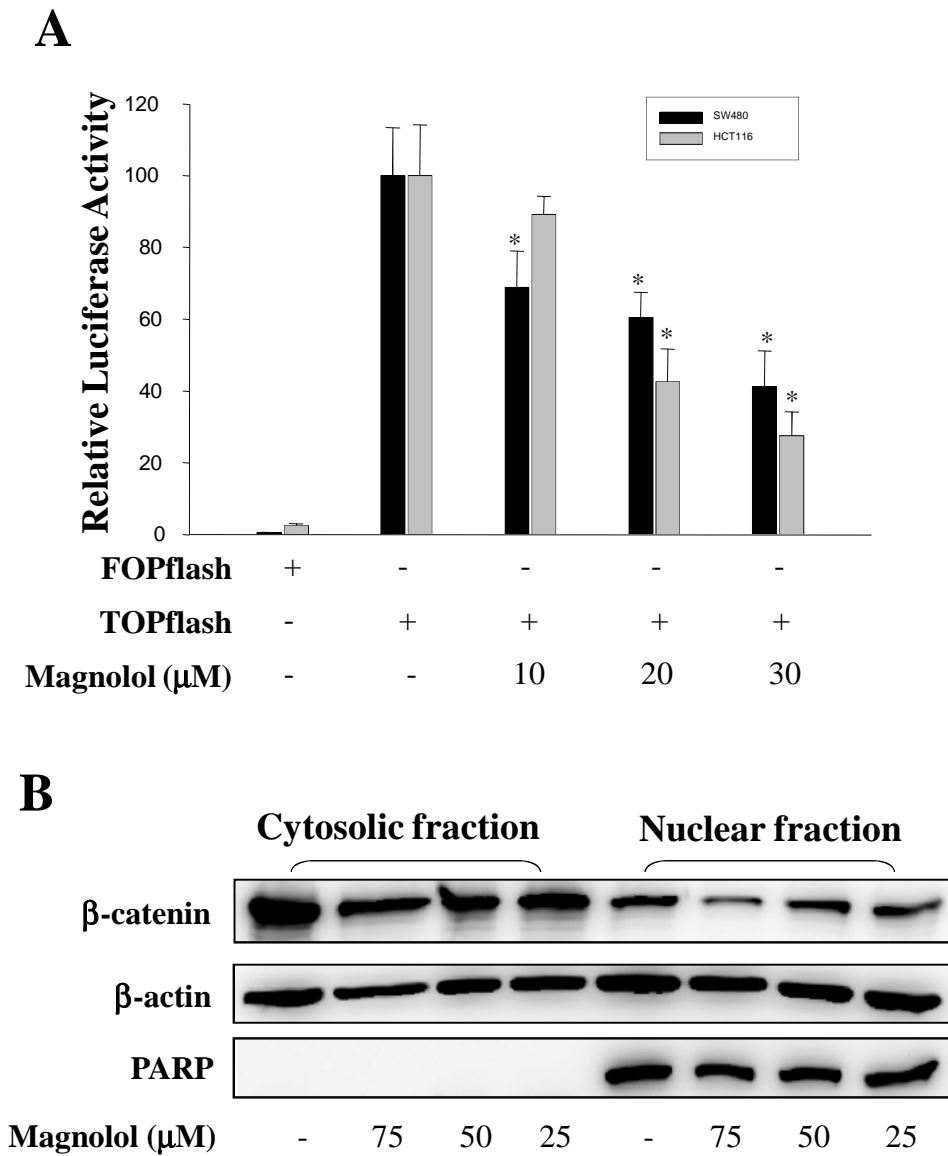


Fig. 4

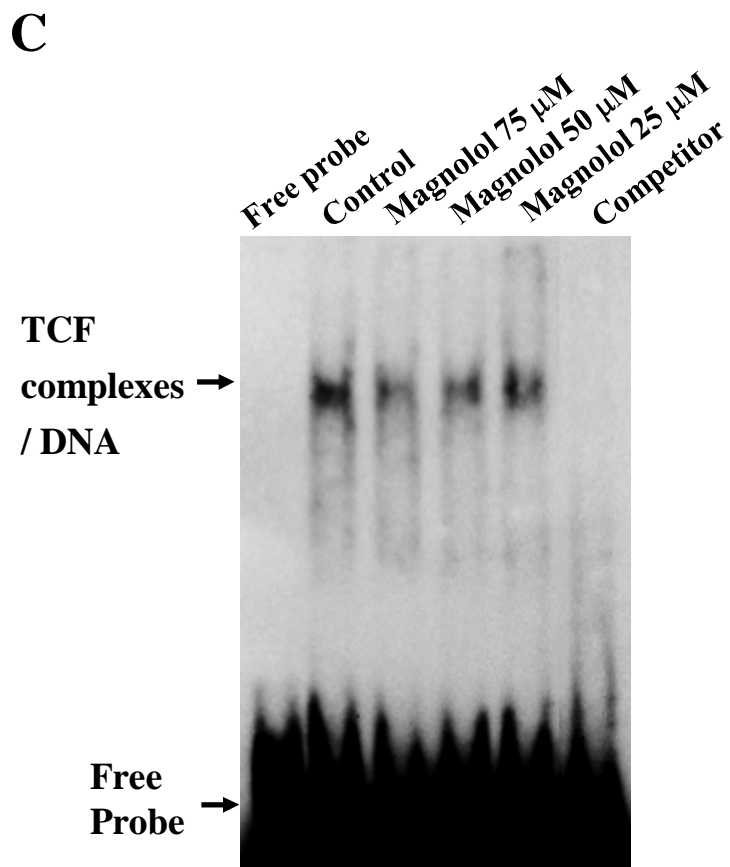
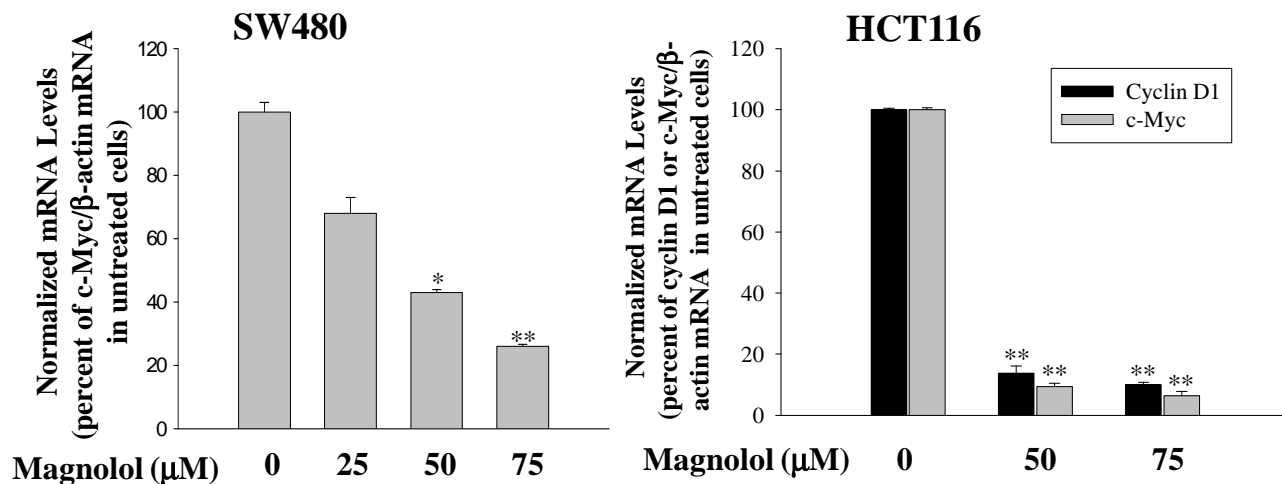


Fig. 4

A



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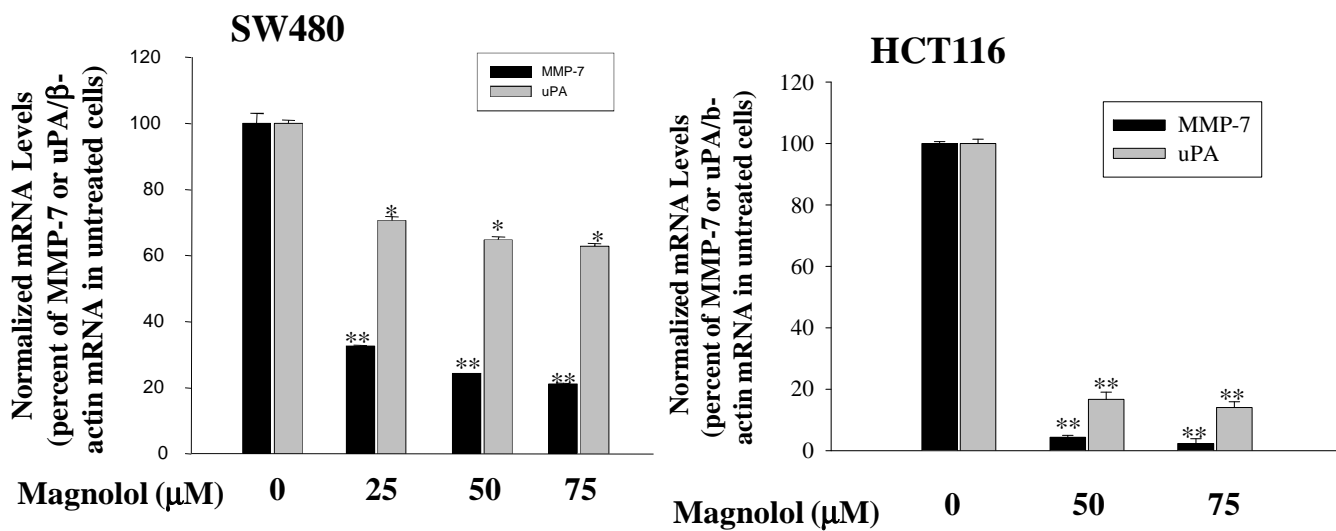


Fig. 5

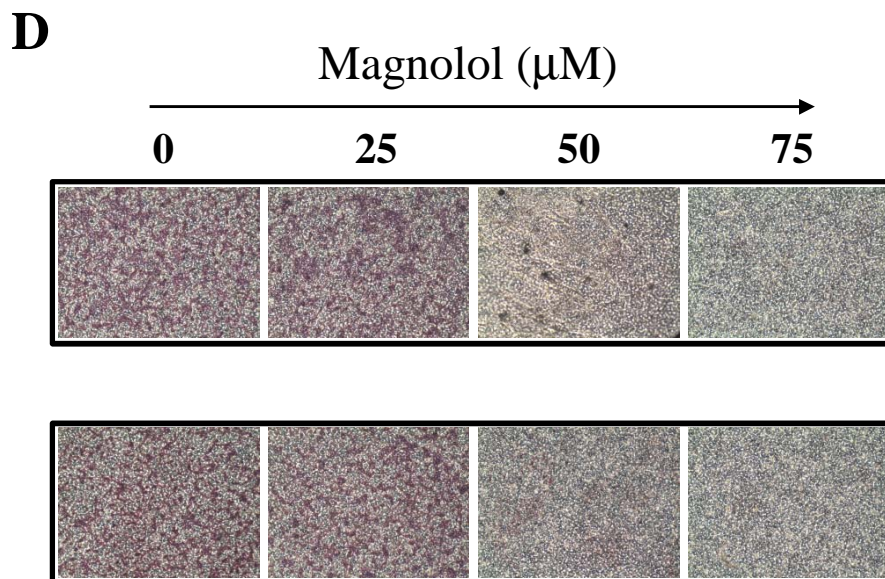
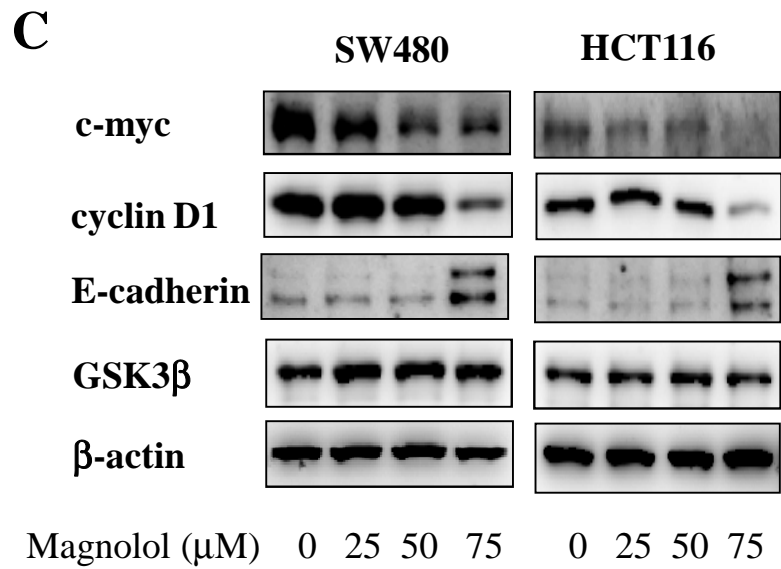


Fig. 5

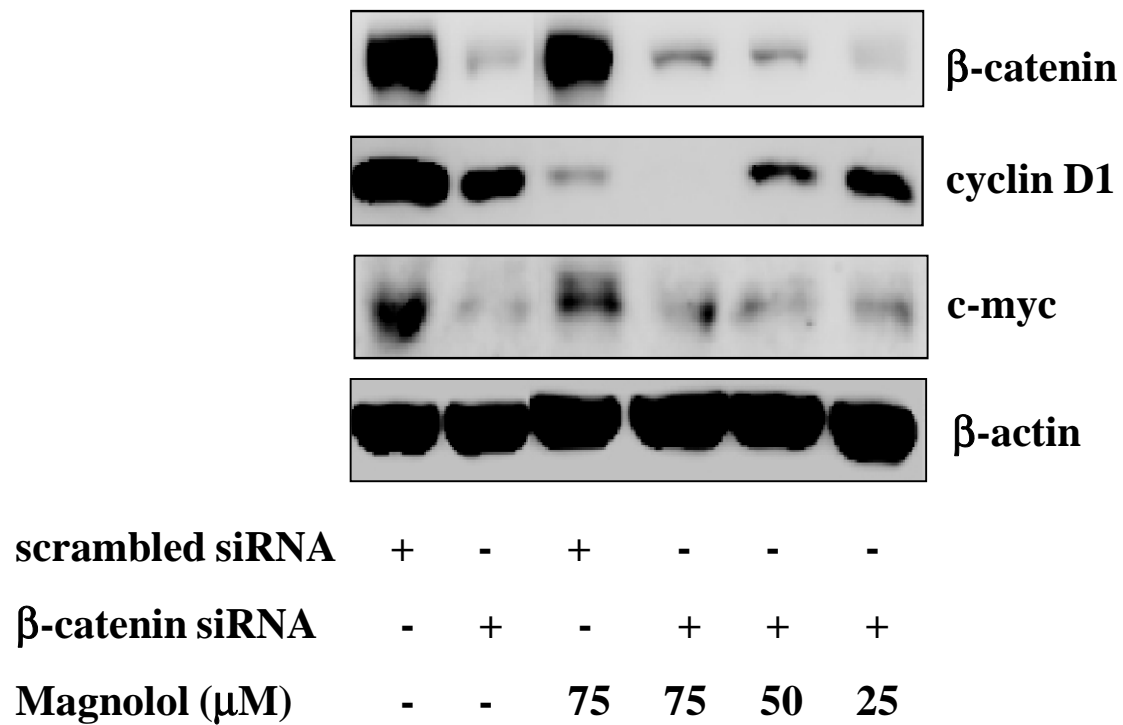


Fig. 6

A

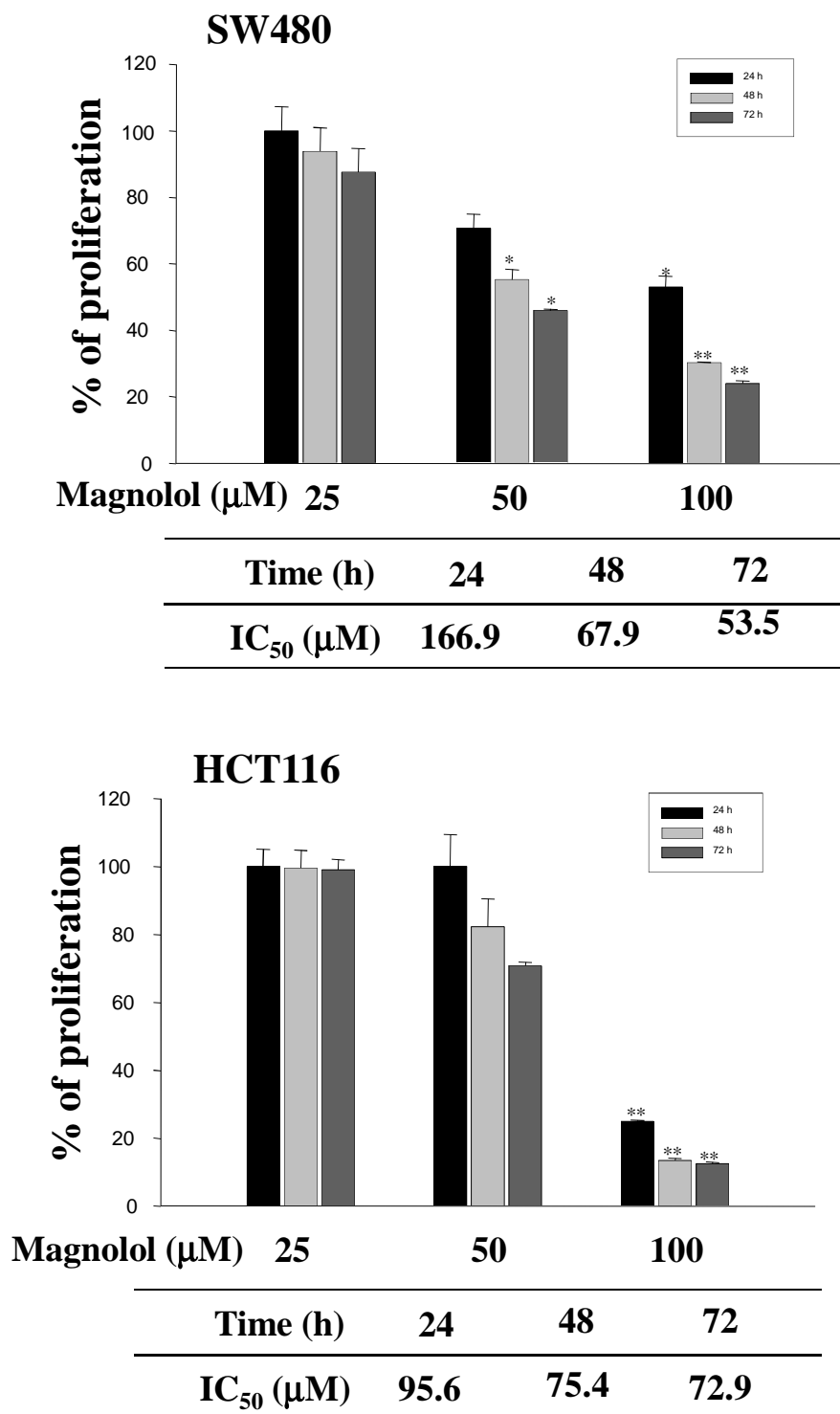
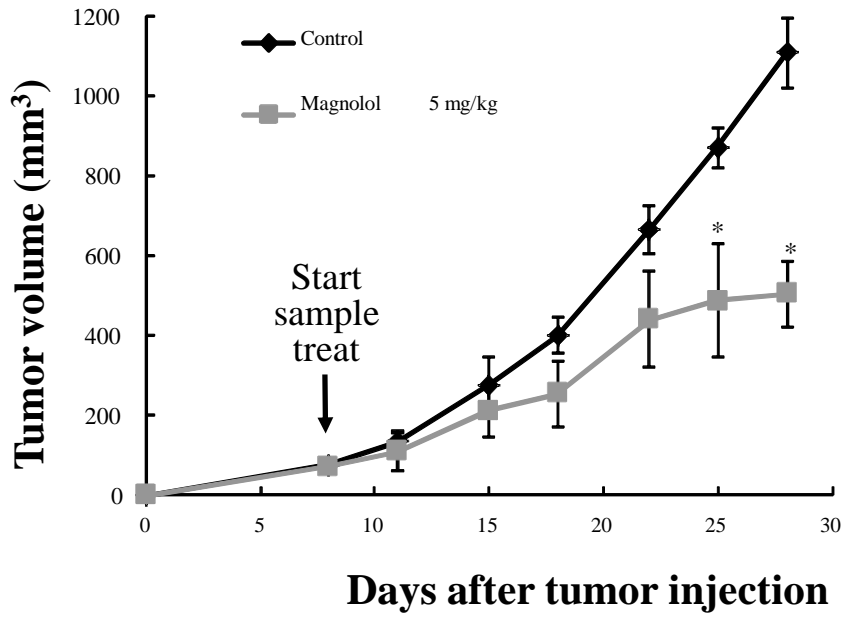


Fig. 7

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C

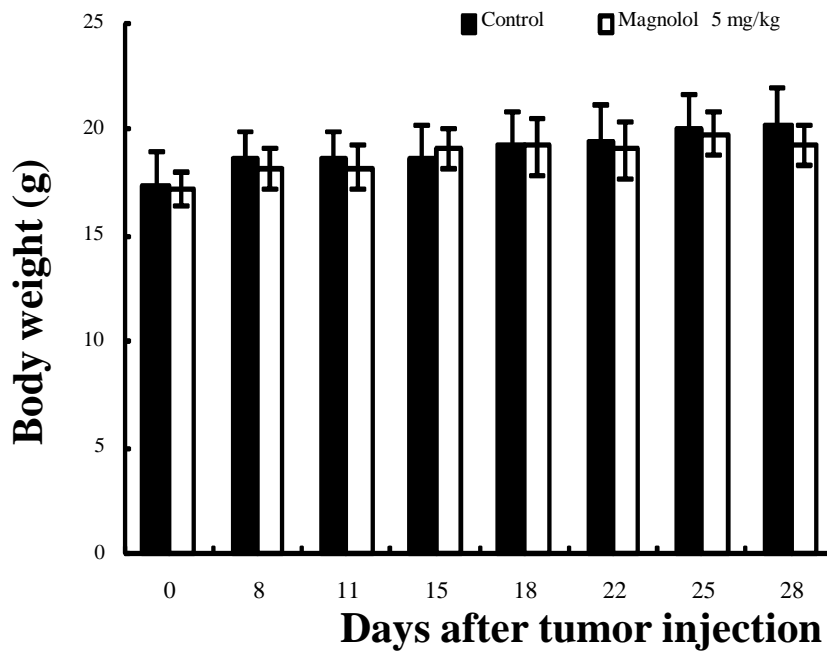


Fig. 7