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Original Research Article

Silymarin-Mediated Degradation of c-Myc Contributes to the Inhibition of Cell Proliferation in Human Colorectal Cancer Cells

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Abstract - In this study, we elucidated the molecular mechanism of silymarin by which silymarin may inhibits cell proliferation in human colorectal cancer cells in order to search the new potential anti-cancer target associated with the cell growth arrest. Silymarin reduced the level of c-Myc protein but not mRNA level indicating that silymarin-mediated downregulation of c-Myc may result from the proteasomal degradation. In the confirmation of silymarin-mediated c-Myc degradation, MG132 as a proteasome inhibitor attenuated c-Myc degradation by silymarin. In addition, silymarin phosphorylated the threonine-58 (Thr58) of c-Myc and the point mutation of Thr58 to alanine blocked its degradation by silymarin, which indicates that Thr58 phosphorylation may be an important modification for silymarin-mediated c-Myc degradation. We observed that the inhibition of ERK1/2, p38 and GSK3β blocked the Thr58 phosphorylation and subsequent c-Myc degradation by silymarin. Finally, the point mutation of Thr58 to alanine attenuated silymarin-mediated inhibition of the cell growth. The results suggest that silymarin induces the cell growth arrest through c-Myc proteasomal degradation via ERK1/2, p38 and GSK3β-dependent Thr58 phosphorylation.

Key words – Cancer chemoprevention, Cell growth arrest, c-Myc, Human colorectal cancer, Silymarin

Introduction

Silymarin from the *Silybum marianum* (milk thistle) has received a tremendous attention over the last decade (Abenavoli *et al*., 2010). It has been reported that silymarin has antioxidant activity (Draz *et al*., 2015), anti-diabetes (Kazazis *et al*., 2014), anti-obesity (Gu *et al*., 2016), anti-inflammatory activity (Guo *et al*., 2016) and hepatoprotective effect (Mereish *et al*., 1991). In previous study, we reported that silymarin suppressed the growth of human colorectal cancer cells through cyclin D1 proteasomal degradation (Eo *et al*., 2015) and induced apoptosis via activating ATF3 (Eo *et al*., 2016). In addition, the effect of silymarin on cell cycle arrest and apoptosis has been reported in ovarian cancer (Fan *et al*., 2014) and lung cancer (Wu *et al*., 2016). These studies for anti-proliferative effect of silymarin have been focused on cyclin D1 associated with the cell cycle regulation. However, cancer cell growth has been controlled by a number of the cell cycle regulators.

Among the cell cycle regulators, c-Myc is overexpressed in various human cancers, including lung carcinoma (Little *et al*., 1983), breast carcinoma (Mariani-Costantini *et al*., 1988) and colon carcinoma (Augenlicht *et al*., 1997). c-Myc regulates the expression of various genes involved in controlling cell proliferation and apoptosis (Bretones *et al*., 2015). Thus, it has been accepted that c-Myc may be the potential target for cancer chemoprevention and therapy.

In this study, we elucidated the molecular mechanism of silymarin by which silymarin may inhibits cell proliferation in human colorectal cancer cells in order to search the new potential anti-cancer target associated with the cell growth arrest.

Materials and Methods

Reagents

Cell culture media, Dulbecco's Modified Eagle medium

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(DMEM)/F-12 1:1 Modified medium (DMEM/F-12) was purchased from Lonza (Walkersville, MD, USA). PD98059 (ERK1/2 inhibitor), SB203580 (p38 inhibitor), SP600125 (JNK inhibitor) and LiCl (GSK3β inhibitor) and MG132 were purchased from Sigma Aldrich (St. Louis, MO, USA). Antibodies against c-Myc, phospho-c-Myc (Thr58), V5-tag and β-actin were purchased from Cell Signaling (Bervely, MA, USA). All chemicals were purchased from Fisher Scientific (Hampton, NH, USA), unless otherwise specified.

Cell culture and treatment

Human colorectal cancer cell lines such as HCT116, SW480, LoVo and HT-29 were purchased from Korean Cell Line Bank (Seoul, Korea) and cultured in DMEM/F-12 supplemented with 10% fetal bovine serum (FBS), 100 U/㎖ penicillin and 100 ㎍/㎖ streptomycin. The cells were maintained at 37℃ under a humidified atmosphere of 5% CO_2. Silymarin was purchased from Sigma Aldrich (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO). DMSO was used as a vehicle and the final DMSO concentration did not exceed 0.1% (v/v).

Cell proliferation assay

Cell growth was measured using MTT assay system. Briefly, cells were plated onto 96-well plated and grown overnight. The cells were transfected with wild type c-Myc and T58A c-Myc expression factor for 24 h and then treated with silymarin for the additional 24 h. Then, the cells were incubated with 50 ㎕ of MTT solution (1 ㎎/㎖) for an additional 2 h. The resulting crystals were dissolved in DMSO. The formation of formazan was measured by reading absorbance at a wavelength of 570 ㎚.

SDS-PAGE and Western blot

After silymarin treatment, cells were washed with 1× phosphate-buffered saline (PBS), and lysed in radioimmunoprecipitation assay (RIPA) buffer (Boston Bio Products, Ashland, MA, USA) supplemented with protease inhibitor cocktail (Sigma-Aldrich) and phosphatase inhibitor cocktail (Sigma-Aldrich) for 30 min at 4℃. Then, cell lysates were centrifuged at 15,000 rpm for 10 min at 4℃. Protein concentration was determined by the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA). The equal proteins were separated on SDS-PAGE and transferred to PVDF membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membranes were blocked for non-specific binding with 5% non-fat dry milk in Tris-buffered saline containing 0.05% Tween 20 (TBS-T) for 1h at room temperature and then incubated with specific primary antibodies in 5% non-fat dry milk at 4℃ overnight. After three washes with TBS-T, the membranes were incubated with horse radish peroxidase (HRP)-conjugated immunoglobulin G (IgG) for 1 h at room temperature and chemiluminescence was detected with ECL Western blotting substrate (Amersham Biosciences, Piscataway, NJ, USA) and visualized in Polaroid film.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

After silymarin treatment, total RNA was prepared using a RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and total RNA (1 ㎍) was reverse-transcribed using a Verso cDNA Kit (Thermo Scientific, Pittsburgh, PA, USA) according to the manufacturer's protocol for cDNA synthesis. PCR was carried out using PCR Master Mix Kit (Promega, Madison, WI, USA) with human primers for c-Myc and GAPDH as followed : c-Myc: forward 5'- cgcgctgagtataaaagccg -3' and reverse 5'- ctattcgctccggatctccc-3', GAPDH: forward 5'-acccagaagactgtggatgg-3' and reverse 5'-ttctagacggcaggtcaggt-3'.

Expression vectors

Wild type V5-tagged c-Myc and point mutation of T58A of V5-tagged c-Myc were provided from Addgene (Cambridge, MA, USA). Each vector was transfected to HCT116 cells for 24 h using the PolyJet DNA transfection reagent (SignaGen Laboratories, Ijamsville, MD, USA) according to the manufacturers' instruction.

Statistical analysis

All the data are shown as mean ± SEM (standard error of mean). Statistical analysis was performed with one-way ANOVA followed by Dunnett's test. Differences with *P <0.05 were considered statistically significant.

Results and Discussion

Effect of silymarin on c-Myc protein level in human colorectal cancer cells

As one of the transcription factors, c-Myc has been reported to regulate the expression of various genes involved in controlling cell proliferation and apoptosis (Bretones *et al*., 2015), which is associated with cancer progression and metastasis (Dang, 2012). Thus, c-Myc has been regarded as the potential molecular target for cancer therapy (Fletcher and Prochownik, 2015). Recently, c-Myc overexpression has been observed in human colorectal cancer (Wright *et al*., 2010). In our previous study of silymarin, we demonstrated that silymarin suppresses the growth of human colorectal cancer cells through cyclin D1 proteasomal degradation (Eo *et al*., 2015) and induces apoptosis through activating ATF3 expression (Eo *et al*., 2016). In this study, we investigated whether silymarin regulates c-Myc level, which affects the growth of human colorectal cancer cells. As shown in Fig. 1A, silymarin treatment dose-dependently decreased c-Myc protein level in HCT116 cells. In addition, the attenuation of c-Myc protein level was observed in SW480, LoVo and HT-29 cells treated with silymarin (Fig. 1B). In time-course experiment, c-Myc protein level started to be decreased at 10 h after silymarin treatment.

Contribution of proteasomal degradation to silymarin-mediated downregulation of c-Myc protein level

c-Myc expression has been reported to be regulated through its transcription via NF-κB signaling pathway (Liu *et al*., 2016). We have reported that silymarin induces NF-κB activation (Eo *et al*., 2015). Thus, we investigated that silymarin-mediated downregulation of c-Myc protein level is attributed to transcriptional regulation, mRNA level of c-Myc was determined in human colorectal cancer cells treated with silymarin. As shown in Fig. 2A and 2B, mRNA level of c-Myc was not changed by silymarin treatment. These data imply that silymarin-mediated downregulation of c-Myc may be independent on transcriptional regulation.

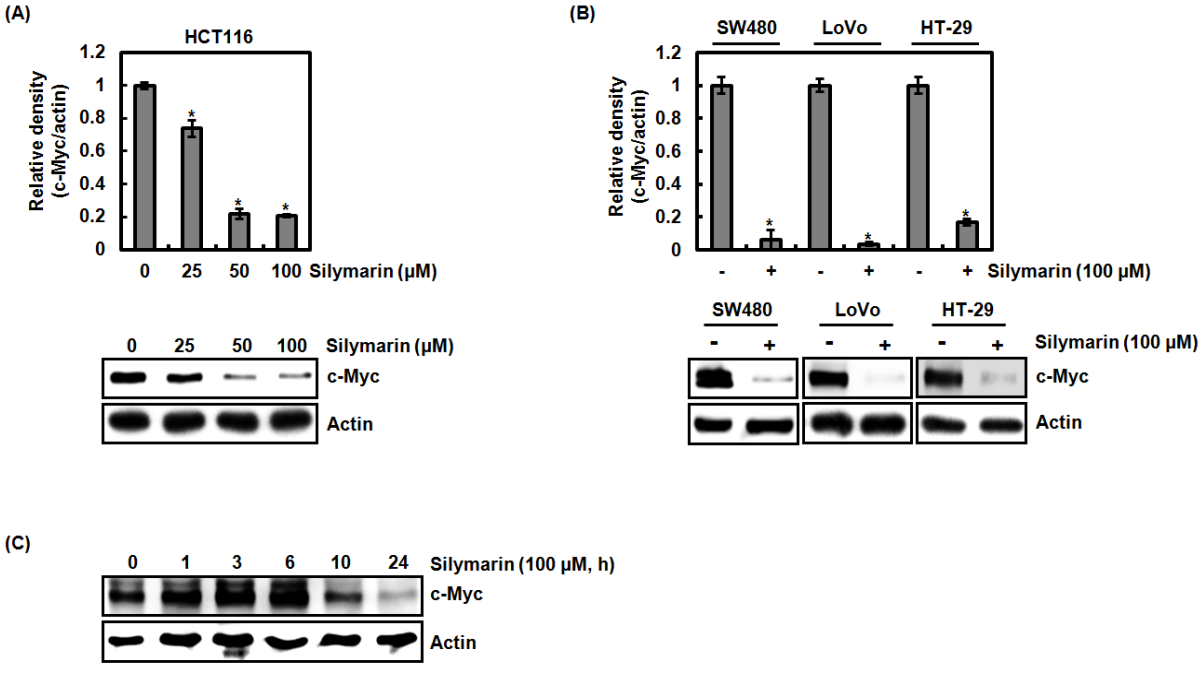

Fig. 1. Effect of silymarin on the protein level of c-Myc in human colorectal cancer cells. (A, B) The cells were plated onto 6-well plate for 24 h. Then the cells were treated with silymarin at the indicated concentrations for the additional 24 h. (C) HCT116 cells were plated onto 6-well plate for 24 h. Then the cells were treated with 100 μM of silymarin for the indicated times. Each cell lysate was subjected to SDS-PAGE and Western blot was performed using antibodies c-Myc and actin. Actin was used as internal control for Western blot analysis. *P<0.05 compared to cell without silymarin treatment.

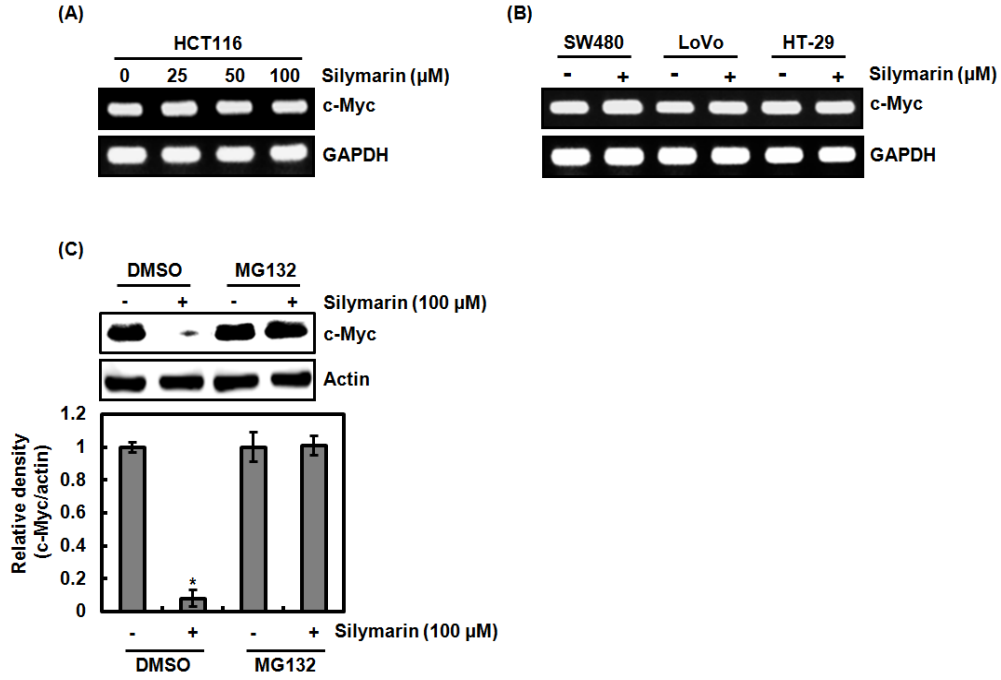

Fig. 2. Proteasomal degradation contributes to silymarin-mediated downregulation of c-Myc protein level. (A, B) The cells were plated onto 6-well plate for 24 h. Then the cells were treated with silymarin at the indicated concentrations for the additional 24 h. For RT-PCR analysis of c-Myc gene expression, total RNA was prepared after silymarin treatment. GAPDH was used as internal control for RT-PCR. (C) HCT116 cells were pretreated with 20 μM of MG132 for 2 h and then co-treated with 100 μM of silymarin for the additional 10 h. Each cell lysate was subjected to SDS-PAGE and Western blot was performed using antibodies c-Myc and actin. Actin was used as internal control for Western blot analysis. *P<0.05 compared to cell without silymarin treatment.

There is growing evidence that c-Myc protein level can be regulated through its degradation (Guo *et al*., 2012; Jing *et al*., 2016). Thus, we investigated whether silymarin induces c-Myc degradation using MG132 as a proteasome inhibitor. As shown in Fig. 2C, the inhibition of proteasome activity by MG132 blocked silymarin-mediated attenuation of c-Myc protein level. This finding suggests that silymarin-mediated downregulation of c-Myc may result from the proteasomal degradation.

Involvement of the phosphorylation on c-Myc threonine-58 (Thr58) in silymarin-mediated degradation of c-Myc

The phosphorylation site, Thr58 is associated with c-Myc degradation through the ubiquitin-proteasome pathway (Yeh *et al*., 2004). De-phosphorylation of Thr58 results in the increase of c-Myc stabilization and subsequently induces accumulation of c-Myc, which contributes to human cell oncogenesis (Yeh *et al*., 2004). In this study, we observed that silymarin treatment increased the phosphorylation status of c-Myc Thr58 at 3 and 6 h after silymarin treatment (Fig. 3A). To elucidate the contribution of silymarin-mediated phosphorylation of Thr58 to c-Myc degradation, we applied to Wild type c-Myc expression vector and T58A c-Myc expression vector. As shown in Fig. 3B, silymarin treatment reduced V5-c-Myc in the cell transfected with Wild type c-Myc expression vector. However, T58A transfection abolished silymarin-mediated decrease of V5-c-Myc. These findings indicate that silymarin may phosphorylate Thr58 of c-Myc protein, which may contribute to c-Myc degradation.

Elucidation of upstream kinases involved in silymarin-mediated degradation of c-Myc

The posttranslational modification by various kinases such as MAPK (ERK1/2, p38, JNK) and GSK3β has been reported to be associated with protein degradation (Diehl *et al*., 1997). However, the upstream kinases attributed to c-Myc degradation have been not elucidated. Thus, we investigated whether MAPK (ERK1/2, p38, JNK) and GSK3β affects silymarin-

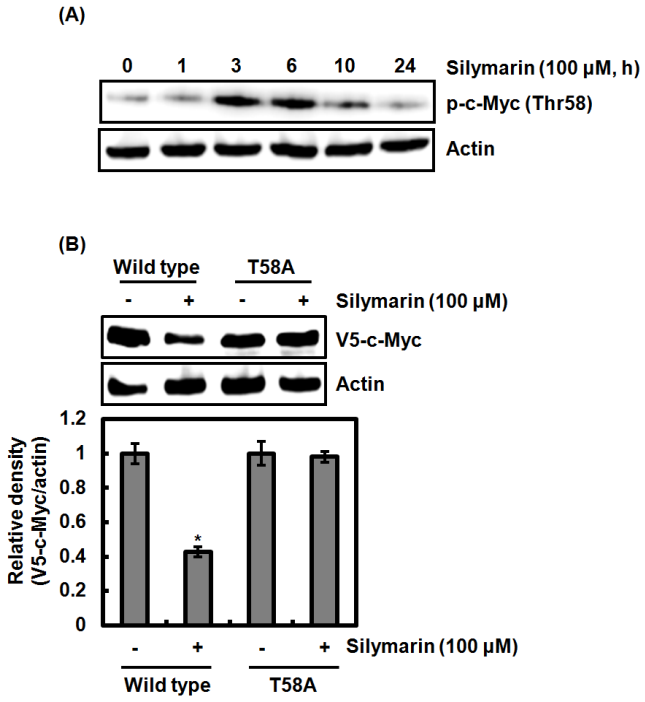

Fig. 3. Effect of silymarin on Thr58 phosphorylation of c-Myc and its contribution to c-Myc degradation. (A) HCT116 cells were plated onto 6-well plate for 24 h. Then, the cells were treated with 100 μM of silymarin for the indicated times. (B) HCT116 cells were plated onto 6-well plate for 24 h. Then, the cells were transfected with V5-tagged Wild type c-Myc or V5-tagged T58A c-Myc expression vector for 24h. After transfection, the cells were treated with 100 μM of silymarin for 24 h. Each cell lysate was subjected to SDS-PAGE and Western blot was performed using antibodies p-c-Myc (Thr58), V5-tag and actin. Actin was used as internal control for Western blot analysis. *P<0.05 compared to cell without silymarin treatment.

mediated c-Myc degradation in order to search the upstream kinases related to c-Myc degradation. As shown in Fig. 4A, the inhibition of ERK1/2 by PD98059, p38 by SB203580 and GSK3β by LiCl attenuated c-Myc degradation by silymarin, but not JNK inhibition. Next, we investigated the effect of the kinases such as ERK1/2, p38 and GSK3β on silymarin-mediated Thr58 phosphorylation in order to determine whether these kinases affect the protein modification of c-Myc. As shown in Fig. 4B, inhibitions of these kinases abolished Thr58 phosphorylation of c-Myc. These findings imply that ERK1/2, p38 and GSK3β may be the potential upstream kinases for silymarin-mediated Thr58 phosphorylation and subsequent degradation of c-Myc. Lastly, we investigated whether silymarin-mediated degradation of c-Myc contributes to the cell proliferation, MTT assay was performed. As shown in Fig. 4C, silymarin inhibited the proliferation of HCT116 cells transfected with Wild type c-Myc expression vector by 42%. Although silymarin suppressed the cell proliferation in T58A c-Myc expression vector by 15%, T58A c-Myc expression vector attenuated silymarin-mediated inhibition of the cell proliferation compared to the cells transfected with Wild type c-Myc expression vector. This finding suggests that silymarin-mediated c-Myc degradation may contribute partially to the inhibition of cell growth.

Taken together, our findings indicate that silymarin induces c-myc degradation through Thr58 phosphorylation dependent on ERK1/2, p38 and GSK3β, which results in the cell growth arrest. Therefore, these findings can provide information on the anti-proliferative effect and the potential molecular mechanism of silymarin.

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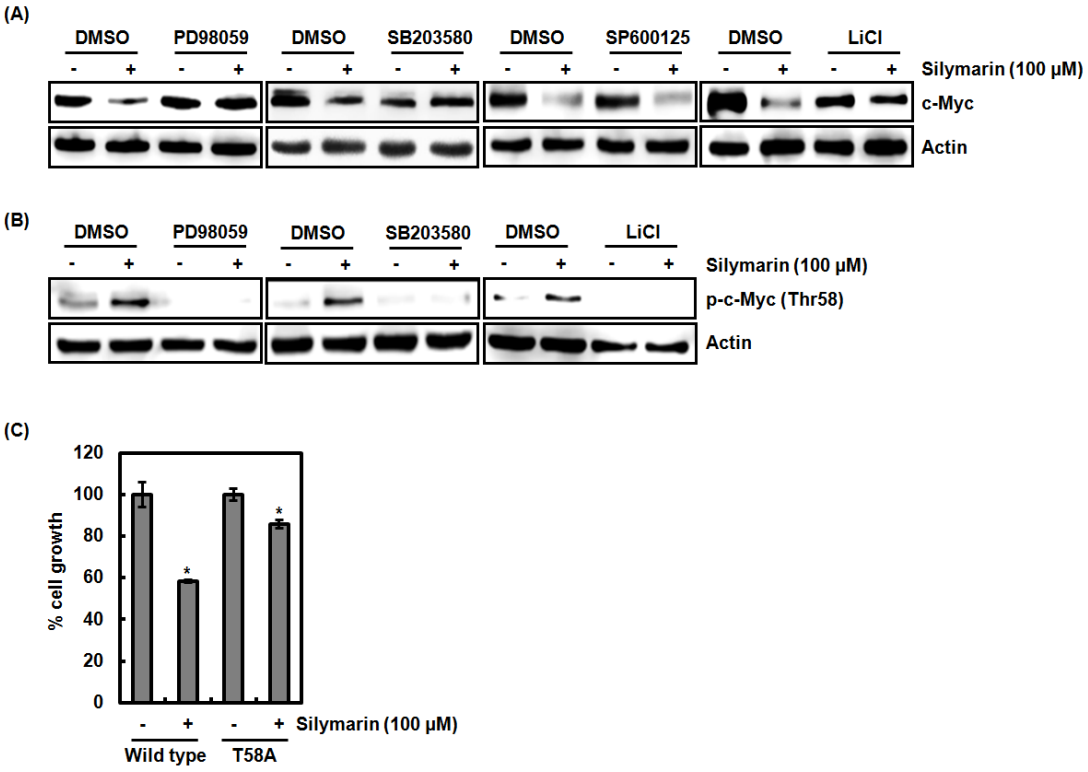

Fig. 4. Effect of ERK1/2, p38, JNK and GSK3β on silymarin-mediated Thr58 phosphorylation and subsequent degradation of c-Myc, and contribution of silymarin-mediated c-Myc degradation to the cell proliferation. (A) HCT116 cells were pretreated with PD98059 (40 μM, ERK1/2 inhibitor), SB203580 (40 μM, p38 inhibitor), SP900125 (40 μM, JNK inhibitor) or LiCl (20 mM, GSK3β inhibitor) for 2 h and then co-treated with 100 μM of silymarin for the additional 10 h. (B) HCT116 cells were pretreated with PD98059 (40 μM, ERK1/2 inhibitor), SB203580 (40 μM, p38 inhibitor) or LiCl (20 mM, GSK3β inhibitor) for 2 h and then co-treated with 100 μM of silymarin for the additional 6 h. Each cell lysate was subjected to SDS-PAGE and Western blot was performed using antibodies c-Myc, p-c-Myc (Thr58) and actin. Actin was used as internal control for Western blot analysis. (C) HCT116 cells were plated onto 6-well plate for 24 h. Then, the cells were transfected with V5-tagged Wild type c-Myc or V5-tagged T58A c-Myc expression vector for 24h. After transfection, MTT assay was performed. *P<0.05 compared to cell without silymarin treatment.

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