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Silibinin Suppresses the Maintenance of Colorectal Cancer Stem-Like Cells by Inhibiting PP2A/AKT/mTOR Pathways

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ABSTRACT

Silibinin, an effective chemo-preventive agent in various cancer types, suppresses cancer cell growth, but its effects on cancer stem-like cells (CSLCs) remain unclear. This study aimed to examine whether silibinin inhibited the development of CSLCs and disclose the underlying signaling. The colorectal cancer spheroid culture system was used for enriching CSLCs. The effects of silibinin on CSLCs were evaluated by counting sphere numbers, and calculating the percentage of CD133+ cells by flow cytometry and immunofluorescence both in the absence and presence of different concentrations of silibinin. The results showed the sphere number of CCS was 36 ± 9.6 after 15 days of CSLC enrichment in spheroid culture, and the percentage of CD133+ cells increased to $18 \pm 6.4\%$ compared to $3 \pm 0.8\%$ before enrichment. Treatment with silibinin reduced the sphere formation to 5 ± 3.3 and decreased the CD133+ percentage to $8 \pm 2.3\%$. Interestingly, treatment of silibinin suppressed the activation of the AKT Ser473/mTOR pathway in spheroid culture through suppressing the activity of protein phosphatase 2Ac subunit (PP2Ac). In a xenograft tumor model, treatment with silibinin also inhibited tumor formation rate and tumor growth. Silibinin, which inhibits colon CSLCs self-renewal and sphere formation by suppressing the PP2Ac/AKT Ser473/mTOR pathway, may be a compound for developing new strategies in modulating CSLCs in cancer therapy. J. Cell. Biochem. 113: 1733–1743, 2012.

KEY WORDS: CANCER STEM-LIKE CELLS (CSLCS); PP2A/AKT/MTOR; SILIBININ; SELF-RENEWAL; SPHEROID CULTURE

Colorectal cancer is a major health concern around the world and is the third most common form of cancer and second leading cause of cancer-related deaths [Jemal et al., 2010]. Recently, a small subpopulation of cancer cells with the characteristics of stem cells such as self-renewal, differentiation potential and the ability of chemo- or radio-resistance, was identified in colorectal cancer samples and cultured cells lines and defined as cancer stem-like cells (CSLCs) or cancer initiation cells [Selgrad et al., 2009; Todaro et al., 2010]. The CSLC population in colorectal cancers, as identified by the presence of surface marker CD133 (also known as prominin-1), has been found to have undifferentiated and tumorigenic characteristics and provides a therapeutic target for colorectal cancer therapy [Ricci-Vitiani et al., 2007; Shmelkov et al., 2008]. Except for the isolation of cells positive for certain CSLC markers, CSLCs have also been enriched by culturing primary and developed cancer cell lines in medium with serum reduction in the presence of growth factors such as EGF, and FGF2 in solid cancer, such as colorectal, brain, and lung cancer [Singh et al., 2003; Eramo et al., 2008; Vermeulen et al., 2008].

Accumulating evidence has demonstrated activating AKT [Dubrovska et al., 2009; Krueger et al., 2009; Selgrad et al., 2009], extracellular signal-regulated kinase (ERK) [Krueger et al.,

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2009], or the mitogen-activated protein kinase (MAPK) pathway [Rountree et al., 2009] modulates the characteristics of CSLCs by controlling the signaling cascades of cell cycle entry, differentiation, or apoptosis. The upstream regulators of these signaling molecules include the serine/threonine protein kinase kinases PI3 kinase (PI3K) and RAF kinase, which activate AKT and ERK, respectively [McCubrey et al., 2007]. There are also several phosphatases including serine/threonine or tyrosine phosphatase that play a counter balance role in upstream kinases by dephosphorylating these molecules [Shi, 2009; Julien et al., 2011]. Using targeted agents to inhibit one or multiple kinase or phosphatase molecules has emerged as a new paradigm for anticancer treatment [Faivre et al., 2006].

Protein phosphatase 2A (PP2A), a serine/threonine protein phosphatase, modulates many signal transduction cascades by dephosphorylating its down-stream targets on the serine or threonine sites [Zhu et al., 2004; Andrabi et al., 2007]. The negative effects of PP2A on AKT [Switzer et al., 2009] or other signaling molecules related to cancer progression modulate the properties of cancer cells and thereby suppress tumor growth, impair tumor angiogenesis, or inhibit metastasis [Schonthal, 2001; Andrabi et al., 2007; Lu et al., 2009]. Low dose okadaic acid (OA) [Bialojan and Takai, 1988], derived from marine sponge, suppresses the activity of protein phosphatases with relatively high specificity to PP2A was studied as a tumor promoter. [Janssens et al., 2005]. However, the relationship between the expression and activity of PP2A and the modulation of cell growth or tumor initiation of CSLCs remains unknown.

Silibinin, a flavonoid compound, has been used clinically in treating several liver diseases as an antiviral agent or an inducer of cell apoptosis [Saller et al., 2001; Wellington and Jarvis, 2001]. Previous investigations have shown the strong anticancer and chemopreventive abilities of silibinin on many kinds of cancers by inhibiting cell proliferation and inducing apoptosis [Singh et al., 2008; Kaur et al., 2009]. The modulatory effects of silibinin on cell cycle entry were demonstrated to be related to the inhibition of the AKT [Switzer et al., 2009], ERK, or p38 MAPK (p38) [Li et al., 2008; Singh et al., 2009] pathway.

Despite the therapeutic effects on various cancers, the effects of silibinin on CSLCs are unclear. The current study first characterized the chemo-resistant properties of colorectal CSLCs and illustrated the chemotherapeutic effect of silibinin on targeting colorectal CSLCs. Further, we discovered the underlying pathway that silibinin mediated to modulate the development of CSLCs in colorectal cancer cells.

MATERIALS AND METHODS

REAGENTS

Silibinin ($C_{25}H_{22}O_{10}$, ≥98% HPLC) was purchased from Sigma-Aldrich (St Louis, MO) and dissolved in DMSO and stock as 100 mg/ml sock solution at −20°C.

CANCER CELL AND SPHEROID CULTURE

The primary colorectal cancer cells, CCS cells, isolated from a primary tumor of a female patient with Duke C_3 colorectal adenocarcinoma were gifted by Dr. Wen K. Yang (Laboratory of Cell/Gene Therapy, China Medical University Hospital, Taichung, Taiwan). The human colorectal cancer cell line HT-29 was obtained from the American Type Culture Collection (ATCC). These cells were cultured in high-glucose DMEM (Gibco, Grand Island, NY) containing 10 units/ml penicillin, 10 μg/ml streptomycin, 2 mmol/l glutamine, and 10% fetal bovine serum (FBS; Gibco), in 37°C humidified atmosphere with 5% CO_2. Spheroid cultures were grown as described previously [Todaro et al., 2007]. In brief, CCS or HT-29 cells in high-glucose DMEM with 10% FBS were reseeded and medium was replaced with serum-free DMEM/F12 (Gibco) containing N2-supplement (Gibco), human EGF (20 ng/ml, Peprotech), and basic FGF (10 ng/ml, Peprotech). Sphere formation was noted since day 5 of culture and protein lysates were collected at day 5, 10, and 15 with or without silibinin treatment.

FLOW CYTOMETRY ANALYSIS OF CD133 EXPRESSION

Suspensions of cancer cells were trypsinized and washed with phosphate-buffered saline (PBS) and incubated for 30 min at 4°C with FITC-conjugated monoclonal antibodies to human CD133 (200× dilution) in 50μl of washing buffer (2% FBS in PBS). After incubation for 30 min, cells were washed twice with washing buffer and fixed in 1% paraformaldehyde. Cells stained with mouse isotype antibodies were served as respective controls. Cells were analyzed using a FACScan flow cytometer running CellQuest software (Becton Dickinson, San Jose, CA).

IMMUNOFLUORSCENCE FOR CD133, CDX2, CK20, AND β-CATENIN IN SPHERES

Spheres were blocked with 5% heat inactivated normal serum, then incubated with mouse or rabbit antibodies against human CD133 (10× dilution, AC133 mouse IgG, Miltenyi), CK 20 (50× dilution, Ks20.8 mouse IgG2a, GeneTex, San Antonio, TX), CDX2 (200× dilution, Chemicon, Temecula, CA), and β-catenin (50× dilution, H-102, Santa Cruz Biotechnology; Santa Cruz, CA) overnight at 4°C, washed extensively with PBS, reacted with corresponding fluorescein isothiocyanate (FITC)-conjugated secondary antibodies, and observed with fluorescence microscope.

APOPTOSIS BY TUNEL ASSAY

Apoptotic cells were determined by using a commercially available TUNEL kit (In Situ Cell Death Detection Kit, Roche), and visualized using a fluorescence microscope.

WESTERN BLOTTING

Cell extracts were prepared with M-PER (Pierce, Rockford, IL) plus protease inhibitor cocktail (Halt™; Pierce) and protein concentrations were determined using the BCA assay (Pierce). Aliquots of protein lysates were separated on SDS–10% polyacrylamide gels and transferred to PVDF membrane filters, followed by blocking with 5% blotting grade milk (Bio-Rad, Hercules, CA) in TBST (20 mM Tris-HCl [pH 7.6], 137 mM NaCl, 1% Tween 20). Membranes were then probed with indicated primary antibodies, reacted with corresponding secondary antibodies, and detected using a chemiluminescence assay (Millipore, Billerica, MA). Membranes were

exposed to X-ray film to visualize the bands (Amersham Pharmacia Biotech, Piscataway, NJ).

PP2A ACTIVITY ASSAY

PP2A activity was assayed using the phosphatase kit (Promega, V1260, Medison). Briefly, cells were lysed in phosphatase storage buffer, followed by removal of endogenous phosphate using spin columns provided by serine/threonine phosphatase assay system. One microgram protein samples in triplicates were incubated with phosphopeptide in PP2A reaction buffer for 2 h at room temperature. After incubation, the samples were colorized by dye mixture for 15 min followed by stopping the reaction, and the OD values at 600 nm were measured. The data were normalized with control cells as 100%.

SCREENING DETECTION OF PHOSPHORYLATION OF Rtks AND FAMILY OF MAPK

Membranes from the Human Phospho-MAPK Array Kit (Catalog Number ARY002; R&D Systems) were blocked with Array Buffer I for 1 h. Diluted protein lysates in Array Buffer I were incubate overnight at 2–8°C (or 2 h at room temperature). The membranes were washed with 1× Wash Buffer at room temperature, and incubated with freshly diluted Detection Antibodies for 2 h at room temperature. The membranes were washed and detected using a chemiluminescence assay. Membranes were exposed to X-ray film to visualize the dots.

CELL PROLIFERATION ASSAY

CCS or HT-29 cells cultured with basal medium with or without 10% FBS, or in spheroid culture were trypsinized and reseeded on 96-well plates with a density of 10^4 cells/well. Different concentrations of silibinin (5∼50 μg/ml) were treated overnight after seeding and the cell numbers were detected by WST-1 assay (Roche) at day 0, 1, 2, and 3.

IN VIVO TUMORIGENICITY EXPERIMENTS

Study protocols involving mice were approved by the Institutional Animal Committee of Taipei Veterans General Hospital. Non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and maintained as a clone at the National Taiwan University Animal Facility (Taipei, Taiwan) in specific pathogen-free conditions. The mice were used for experiments at 6–8 weeks of age. CCS primary tumor cells, CCS in spheroid culture at day 15, CCS in spheroid culture at day 15 with silibinin 5 μg/ml were injected subcutaneously at a dose of 5×10^3 cells per injection site. The same groups with colorectal cancer cell line, HT-29, were injected as further confirmation.

STATISTICAL ANALYSIS

All values are expressed as mean ± standard deviation. Independent *t*-test was performed for comparison of data of independent samples. More than two groups were compared by one-way ANOVA. A *P*-value <0.05 was considered significant.

RESULTS

ENRICHMENT OF COLORECTAL CANCER STEM-LIKE CELL POPULATIONS IN SPHEROID CULTURE

To enrich colorectal CSLCs, long-term spheroid culture system was applied in primary derived (CCS) and HT-29 colorectal cancer cells. After 15 days of spheroid culture in serum-free medium in the presence of EGF and FGF2 (TSM medium), cancer cells adopted sphere morphology and the sphere diameter increased as long as the increase of culture period (Fig. 1a). When spheres were defined as those with diameter more than 500 μm, we found the sphere numbers formed by these cancer cells increased in a time-dependent manner during the 15-day culture period (Fig. 1a). To confirm, the enrichment of CSLCs was associated with changes in surface markers related to differentiation status during the culture period, CD133, the undifferentiated marker, and CDX2, the differentiated marker, were examined by immunofluorescence. The expression level of CD133 increased and CDX2 decreased along with the increased culture period (Fig. 1b). In addition, the percentage of $CD133^+$ cells as analyzed by flow cytometry at day 15 (18.1 ± 6.38%) was significantly higher than that at day 0 (3.5 ± 0.83%) (Fig. 1c, $P < 0.05$, *t*-test). Moreover, the enriched CSLCs maintain the ability to form spheres under the same culture condition after subculture (Fig. 1d). These data suggest colorectal CSLCs can be enriched by serum reduction in the presence of EGF and FGF2.

SILIBININ INHIBITED THE ENRICHMENT OF CSLCs BUT DID NOT INDUCE APOPTOSIS IN CSLCs

Since silibinin affects cell proliferation and survival in numerous types of cancer cells, the effects of silibinin on the enrichment of CSLCs or sphere formation in spheroid culture should be investigated at concentrations that do not induce any cell cycle arrest and cell apoptosis. Silibinin at a concentration of 5 μg/ml or below did not inhibit cell proliferation or induce apoptosis in colorectal cancer cells used here, whereas more than 25 μg/ml of silibinin did inhibit cell growth in a dose-dependent manner in colorectal cancer cells grown in growth medium (Fig. 2a). In contrast, silibinin up to 100 μg/ml induced no effects on cell growth in 15-day spheroid culture-enriched CSLCs (Fig. 2b). Moreover silibinin up to 25 μg/ml also induced significant apoptosis in cells grown in growth medium, while did not cause cell apoptosis in spheroid culture-enriched CSLCs (Fig. 2c). Interestingly, the sphere numbers formed in TSM medium were obviously decreased upon treatment with silibinin as low as 5 μg/ml (Fig. 3a). Except for the inhibition of sphere formation in spheroid culture, silibinin also inhibited the expression of CD133 undifferentiated marker (Fig. 3b,c) and enhanced the expression of differentiation markers such as CDX2 (Fig. 3b). Similar results were also demonstrated in the HT-29 cell line (data not shown). These data all together suggest CSLCs resist silibinin-induced cell death at concentrations that killed ordinary colorectal cancer cells. Although silibinin did not induce cell death, silibinin inhibited sphere formation and the expression of CD133 marker, and increased the expression of differentiated marker in spheroid culture.

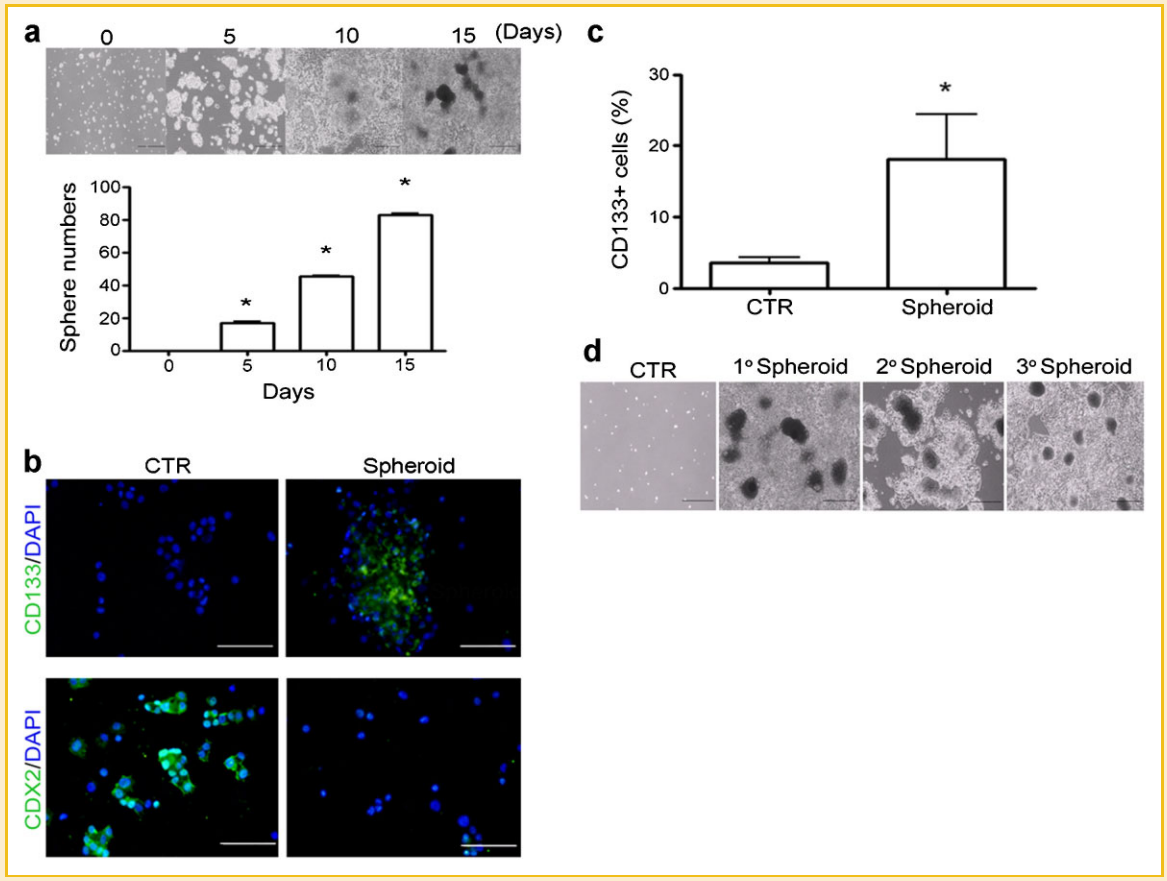

Fig. 1. Enrichment of colon cancer stem-like cell populations in spheroid culture. a: Pictures show primary colorectal cancer cells-formed spheres at indicated time of spheroid culture (scale bar 1 mm). Spheres were defined as cell clusters with diameter more than 500 μm. Graph shows the sphere numbers averaged from five high-power fields ($^*P < 0.05$ compared with day 0). b, c Primary colorectal cancer cells were grown in spheroid culture for indicated time. b: Immuno-fluorescence for CD133 and CDX2 were performed. c: The percentages of CD133+ cells were assayed by flow cytometry. ($^*P < 0.05$ as compared with day 0). d: The enriched CSLCs maintained the ability to form spheres after subculture for three passages (scale bar 1 mm).

PP2A/AKT/mTOR PATHWAY IS INVOLVED IN COLORECTAL CSLC ENRICHMENT IN SPHEROID CULTURE

To clarify the signaling pathways involved in enrichment of colorectal CSLCs in spheroid culture, the phosphorylation levels of several CSLC-related signaling molecules such as AKT, and its downstream molecules such as mTOR and FOXO [Selgrad et al., 2009], were evaluated. Enriched CSLCs increased in the phosphorylation levels of AKT Ser473 and its downstream signals such as mTOR (Fig. 4a), while the phosphorylation level at Thr308 of AKT, downstream pathways such as GSK3β or FOXO, or other signal transduction pathways including ERK and p38 were only slightly increased or not changed (Fig. 4a and data not shown).

Since the signals with the phosphorylation sites on serine or threonine of AKT Ser473 were dephosphorylated by PP2A, we also checked the involvement of PP2A in the enrichment of CSLCs. Along with the start of the spheroid culture, a time-dependent decrease in PP2A activity was found in spheroid culture compared to the control growth culture (Fig. 4b). Because the phosphorylation of PP2A at Tyr307 in C subunit induces a decrease of PP2A activity, we compared the phosphorylation of protein phosphatase 2Ac subunit (PP2Ac) (Tyr307) between colorectal cancer cells and enriched CSLCs. The results demonstrated that the phosphorylation level of PP2Ac was increased along with the spheroid culture, while the total level of PP2Ac remained unchanged (Fig. 4c). To demonstrate decreased PP2Ac activity is important in activating the AKT Ser473/mTOR pathway, we found similar activation of the pathway was induced by treatment with PP2A inhibitors, caliculin A, and OA (Fig. 4d). These data suggest the involvement of PP2Ac in activating AKT Ser473 and downstream signaling molecules during the enrichment of colorectal CSLCs in spheroid culture. To demonstrate the causational relationship of the AKT/mTOR pathway in enriching CSLCs, we evaluated the effects of several specific inhibitors of the pathway on CSLC sphere formation. The administration of PP2Ac activator, C6-ceramide 1 μM, PI3K inhibitor, LY294002 10 μM, and mTOR inhibitor, rapamycin 10 μM, but not the inhibitors of ERK (PD98059) and p38 (SB203580), inhibited the sphere formation, suggesting the involvement of PP2Ac/AKT Ser473/mTOR in enriching the CSLC population (Fig. 4e and data not shown).

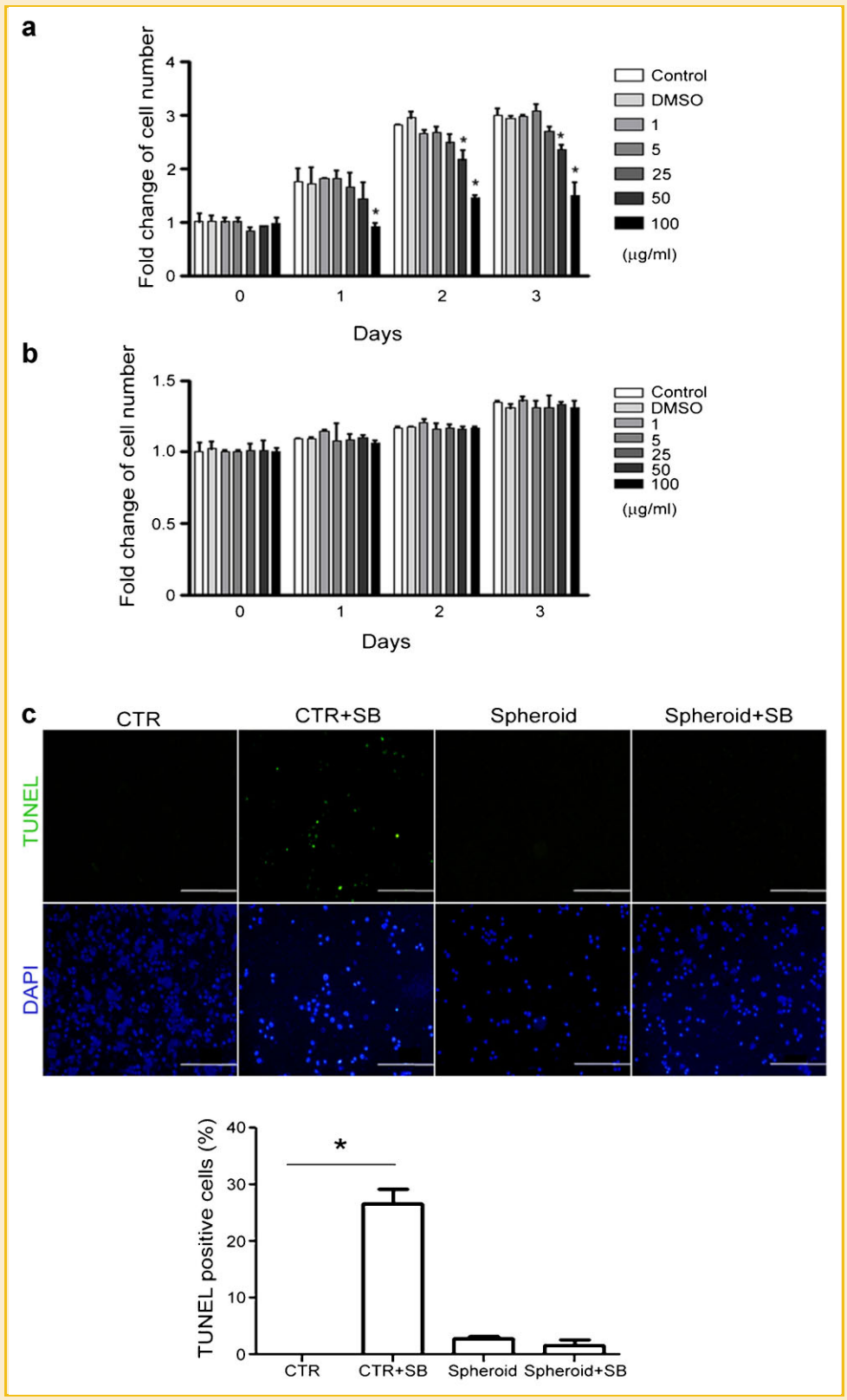

Fig. 2. Silibinin suppresses survival in colorectal cancer cells but not in CSLCs. a: Primary colorectal cancer cells or (b) enriched CSLCs were treated without or with silibinin for indicated days at various concentrations, and cell numbers were calculated by WST-1 kit. Treatment of silibinin on colorectal CSCs within 5 μg/ml did not cause cell apoptosis while more than 25 μg/ml induced cytotoxicity under growth condition (a). Compared with cancer cells cultured under growth condition, the concentration of silibinin higher than 25 μg/ml did not cause cell death on colorectal CSLCs. b: The apoptotic cells were detected on silibinin 25 μg/ml treated groups under growth condition by TUNEL assay while no significant detections on CSLCs groups (c, scale bar, 500 μm). Followed the safety concentration without leading cell death, 5 μg/ml silibinin was treated for further studies. $^{*}P < 0.05$ compare with DMSO

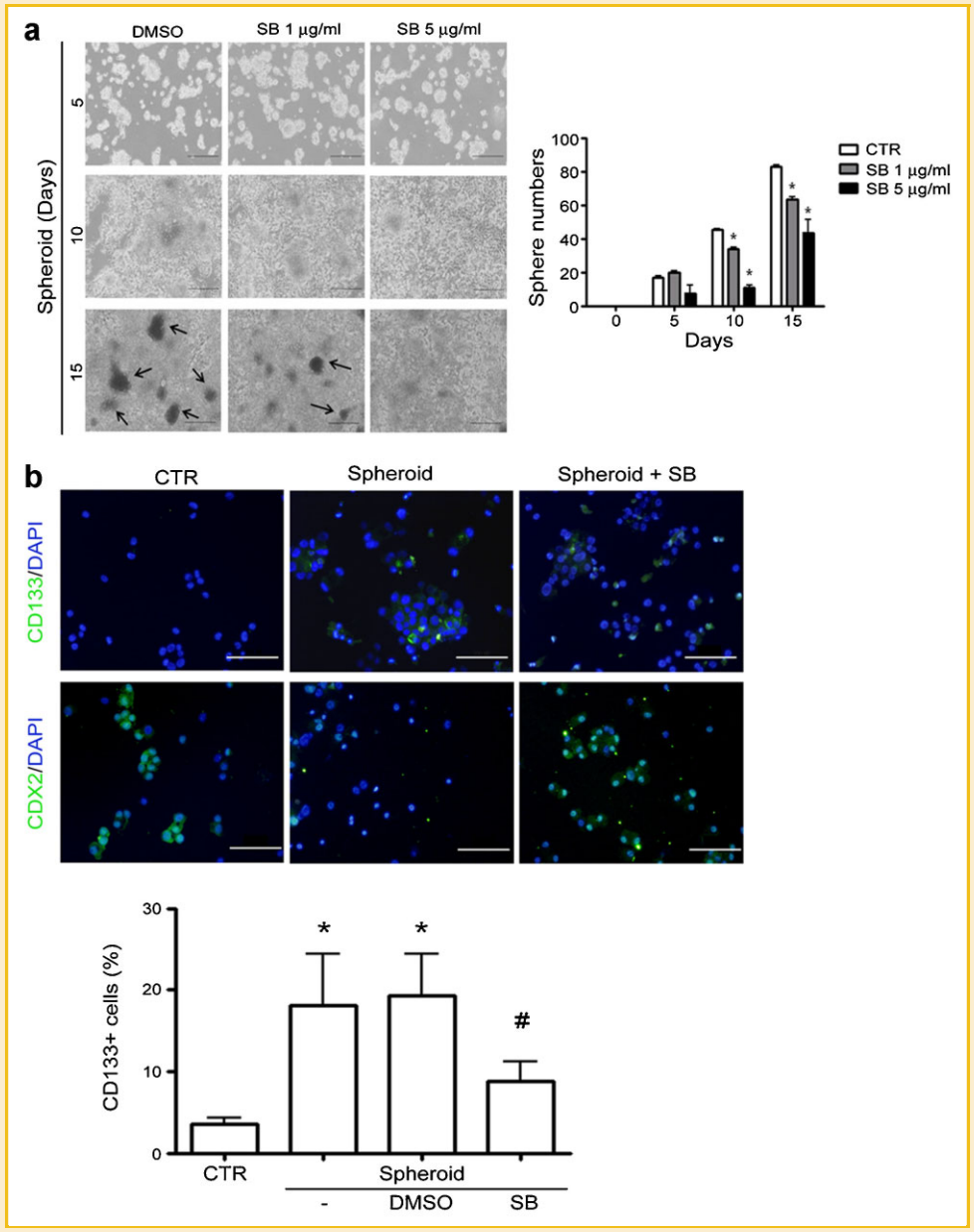

Fig. 3. Silibinin suppresses sphere formation, decreases CD133, and increased CDX2 expression in spheroid culture. a: Pictures show primary colorectal cancer cells-formed spheres at indicated time of spheroid culture in the absence (DMSO, vehicle control) or presence of silibinin at indicated concentrations. (scale bar, 1 mm). Graph shows the sphere numbers averaged from five high-power fields, spheres were defined as cell clusters with diameter more than 500 μm (arrow head). ($^{*}P < 0.05$ as compared with DMSO). b, c: Primary colorectal cancer cells were grown under growth condition (CTR) or in spheroid culture in the absence or presence of 5 μg/ml silibinin for 15 days (scale bar, 1 mm). b: Immuno-fluorescence for CD133 and CDX2 were performed (scale bar, 200 μm). c: The percentages of CD133+ cells were assayed by flow cytometry ($^{*}P < 0.05$ as compared with CTR; $^{\#}P < 0.05$ as compared with DMSO).

SILIBININ SUPPRESSED COLORECTAL CSLC DEVELOPMENT THROUGH INACTIVATION OF THE PP2Ac/AKT Ser473/mTOR PATHWAY

To examine the underlying mechanisms that silibinin mediated to modulate sphere formation and expression of CSLC markers in spheroid culture, MAPK Ser/Thr protein array was performed and the results showed obvious changes in JNK, p38, GSK3β, RSK, and AKT Ser473 phosphorylation. Western blotting also confirmed the phosphorylation level of AKT Ser473 and the level of downstream signal mTOR, but not rictor protein (one component of mTORC2 complex), were increased in spheroid culture and further suppressed by silibinin treatment (Fig. 5a). Spheroid culture increased the phosphorylation level of PP2Ac and thereby decreased the PP2Ac activity, while silibinin treatment reversed the effects of spheroid culture on phosphorylation and activation of PP2Ac (Fig. 5b). However, the expression levels of B55alpha or B56gamma subunits, which have been reported to enhance PP2A activity, were upregulated in spheroid culture and silibinin slightly suppress

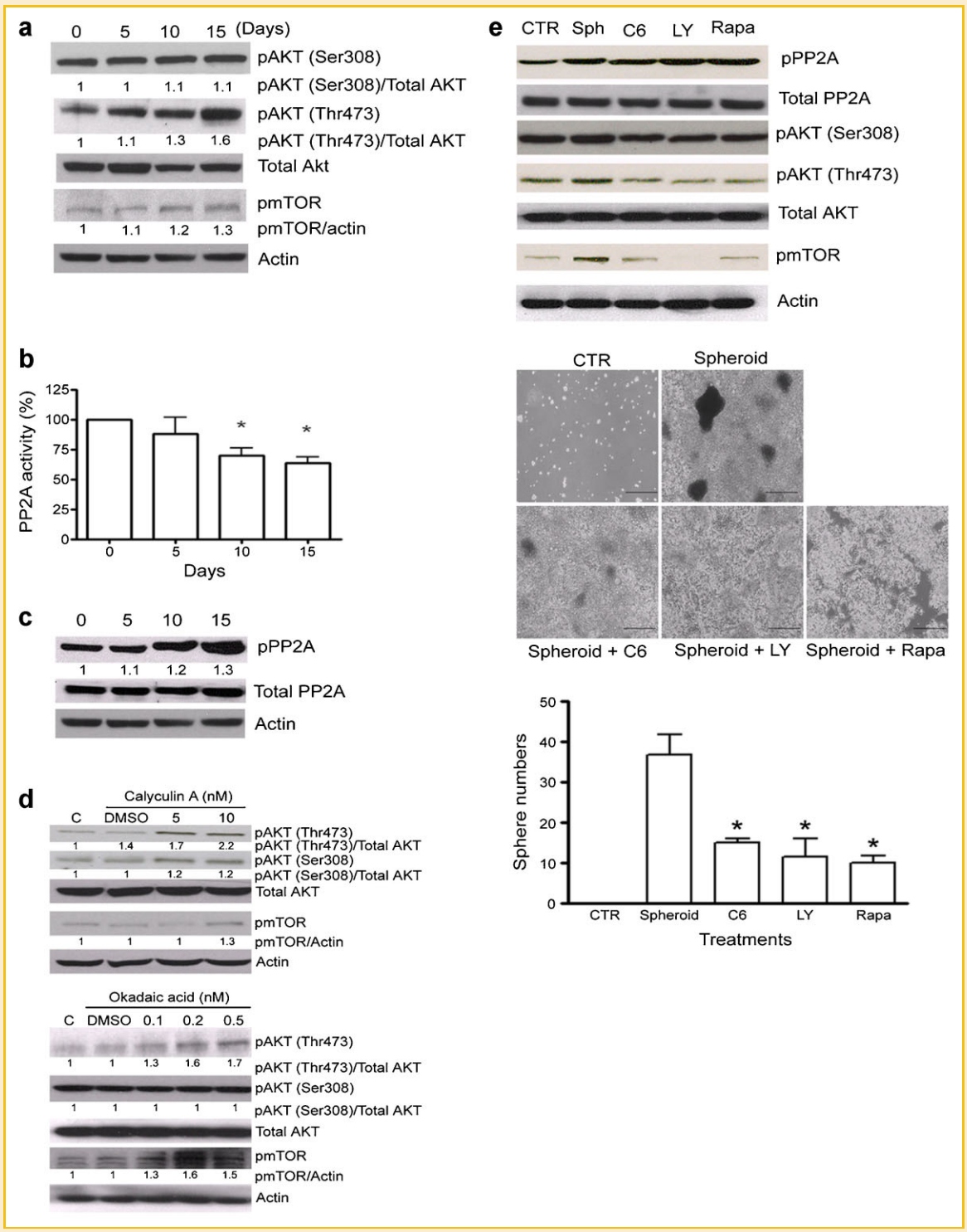

Fig. 4. The PP2A/AKT/mTOR pathway is involved in enrichment of CSLCs in spheroid culture. Primary colorectal cancer cells were grown in spheroid culture for up to 15 days. a, c: Western blotting for the protein levels. b: PP2A activity as assayed by phosphatase kit ($^{*}P < 0.05$ as compared with day 0). d: Primary colorectal cancer cells were grown in spheroid culture for 0 (Control) and 15 days in the absence (DMSO) or presence of calyculin A at indicated concentrations. Cell lysates were used for western blotting. e (upper panel): Pictures show western blotting for protein levels of primary colorectal cancer cells-formed spheres in spheroid culture for 0 (CTR) and 15 days in the absence or presence of PP2Ac activator C6-ceramide (C6, 1 μM), AKT inhibitor LY294002 (LY, 10 μM), or mTOR inhibitor rapamycin (Rapa, 10 μM). e (middle panel): Graph shows the morphology of sphere formation with or without inhibitors (scale bar, 1 mm). e (lower panel): Graph shows numbers averaged from five high-power fields. ($^{*}P < 0.05$ as compared with CTR; $^{\#}P < 0.05$ as compared with DMSO)

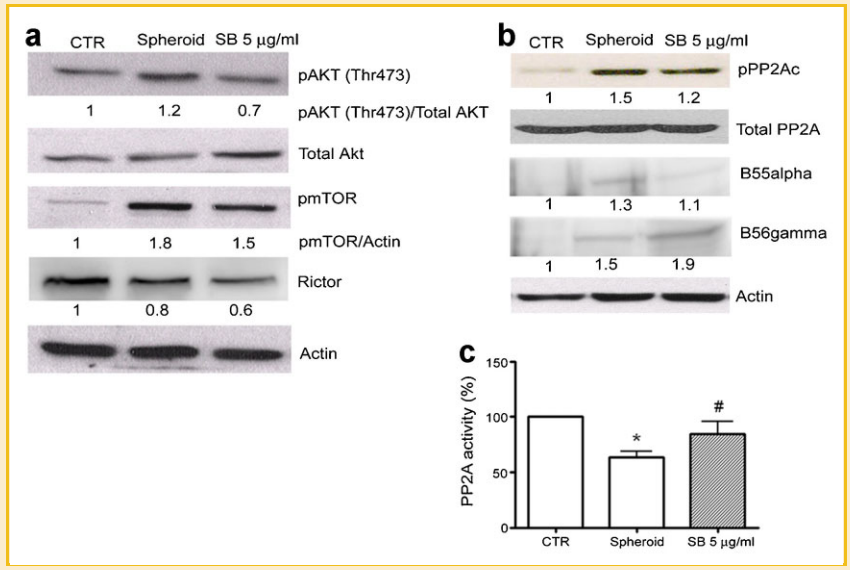

Fig. 5. Silibinin suppresses colorectal CSLC self-renewal through upregulation of PP2Ac activity. Primary colorectal cancer cells were grown in spheroid culture for 0 and 15 days in the absence (CTR) or presence of silibinin at 5 μg/ml. a, b: Western blotting for protein levels. c: PP2Ac activity as assayed by phosphatase kit ($^{*}P < 0.05$ as compared with CTR, $^{\#}P < 0.05$ as compared with spheroid).

B55alpha while enhance B56gamma suggesting these changes were not compatible with PP2A activity in our system (Fig. 5b,c). These data suggest phosphorylation of PP2Ac subunit, rather than changes in the levels of B55alpha, B56gamma, or rictor protein, is involved in the effects of spheroid culture on AKT Ser473 phosphoryaltion or CSLCs development. The other factors such as JNK, p38, and RSK that upregulated during spheroid culture showed no responses under silibinin treatment (data not shown). These data all together suggest spheroid culture reduced the PP2A activity by inhibiting PP2Ac phosphorylation, thereby activating the AKT Ser473/mTOR pathway, while treatment with silibinin reversed the effects of spheroid culture on the PP2Ac/AKT Ser473/mTOR pathway.

SILIBININ-TREATED COLORECTAL CSLCs SHOWED LOWER TUMORIGENESITY FOR THE IN VIVO XENOGRAFT MODEL

To clarify the influence of silibinin and the involvement of PP2Ac/AKT Ser473/mTOR pathway in the ability of CSLCs-induced xenograft tumor formation, aliquot (10^4) of enriched colorectal CSLCs treated without or with silibinin, the PP2Ac activator C6-ceramide, the PI3K inhibitor LY294002, or the mTOR inhibitor rapamycin were subcutaneously injected into the flank of NOD-SCID mice, and the tumor formation rate was calculated for more than half a year (Table I). Tumors were formed by five of the six injections of enriched CSLCs without treatment with silibinin or any of the pathway modulators. In contrast, only four of the 12 injections of enriched CSLCs treated with silibinin formed tumors. Treatment with C6-ceramide, LY294002, or rapamycin completely blocked tumor formation for up to 7 months. Similarly, treatment with silibinin, C6-ceramide, LY294002, or rapamycin also inhibited tumor growth (Table I). Taken together, these data suggest silibinin or blockage of the signaling pathway involved in spheroid culture inhibited the tumorigenecity and tumor growth of CSLCs.

DISCUSSION

CSLCs are pluripotent cells characterized by self-renewal potential, an enhanced ability to initiate tumor formation, and resistance to current cancer therapies [Todaro et al., 2007]. Although controversy exists, cancer cell spheroid culture remains a reliable method to increase self-renewal of CSLCs and a suitable model to study signaling pathways involved in properties of CSLCs including self-renewal, chemo- and radio-resistance [Kaur et al., 2010; Polyak et al., 2010]. In colorectal cancer, we demonstrate here silibinin inhibits the development of CSLCs in vitro and thereby suppresses tumor initiation in vivo, through inactivation of the signaling pathway involved in self-renewal of CSLCs. To our knowledge,

TABLE I. The Xenograft Tumor Formation on Nud-Mice Model.

	Tumor formation	Tumor size (mm^3)
2×10^4 bulk	0/4	0 ± 0
1×10^6 bulk	4/4	4.2 ± 0.6
2×10^4 CSLCs-CTR	5/6	4.5 ± 1.5
2×10^4 CSLCs-SB	4/12	0.9 ± 0.1
2×10^4 CSLCs-C6	0/8	0 ± 0
2×10^4 CSLCs-LY	0/8	0 ± 0
2×10^4 CSLCs-Rapa	0/8	0 ± 0

Tumor formation and tumor size were measured every week and mice were sacrificed and the final tumor volume were analyzed at 6th week.
CTR, CSLCs without treating SB; SB, silibinin 5 μg/ml; C6, C6-ceramide 1 μM; LY, LY294002 10 μM; Rapa, rapamycin 10 μM
Silibinin decreases tumorigenecity of CSLCs and inhibits tumor growth in vivo. For in vivo xenograft tumorigenecity assay, 2×10^4 spheroid culture-enriched CSLCs (CSLCs) treated without (CTR) or with silibinin (SB, 5 μg/ml), C6-ceramide (C6, 1 μM), LY294002 (LY, 10 μM), or rapamycin (Rapa, 10 μM) for 15 days, followed by subcutaneous injection into the flank of nude mice, while 2×10^4 and 10^6 of primary colon cancer cell (bulk) were also injected to serve as controls. Tumor formation was monitored for up to 6 months, and tumor volume was calculated with the formulae: Tumor volume = height × width × depth × 0.523. ($^{*}P < 0.05$ as compared with CTR).

silibinin is one of the few botanic compounds demonstrated to have inhibitory effects on CSLCs. These results further support the concept of using the spheroid culture of primary cancer cells or cell lines to recapitulate the signaling pathways involved in the self-renewal of CSLCs, and develop new drugs or molecules that target the signaling pathways, thereby reducing tumor initiation or inhibiting tumor resistance in vivo. Similar results were demonstrated in a previous study where spheroid culture helped develop strategies to block chemo-resistance in lung cancer by targeting the signaling pathways involved in anti-apoptosis of CSLCs [Hsu et al., 2010].

The AKT cascades were demonstrated to control cell survival and proliferation in various kinds of cancer cells. Here, we demonstrated that treatment with the PI3K inhibitor LY294002, or mTOR inhibitor rapamycin, also successfully suppressed sphere formation in vitro and tumor initiation in vivo, suggesting the phosphorylation of AKT and mTOR was important for colorectal CSLCs enrichment. Similar to the current study, brain and hepatocellular carcinoma (HCC) CSLCs also display preferential sensitivity to AKT inhibition [Eyler et al., 2008; Ma et al., 2008]. Treatment with an AKT inhibitor more potently induced cell apoptosis, reduced the numbers of viable brain CSLCs, and suppressed neurosphere formation compared to matched non-CSLCs. Similarly, targeting the AKT survival pathway in CD133+ HCC CSLCs reduced the expression of the survival proteins and induced cell apoptosis [Ma et al., 2008].

PP2A is a trimeric holoenzyme, consisting of A, B, and C subunits, which are responsible for conformation, regulation, and catalyze, respectively. In this study, we found that during sphere formation, the level of Tyr307 phosphorylation of PP2Ac subunit was increased with the reduction in PP2A activity, and thereby induced AKT activation. Moreover, sphere formation-induced PP2A inactivation and AKT activation were inhibited in the presence of silibinin. The recent article [Ruvolo et al., 2011] pointed out that the expression level of PP2A B55alpha subunit targeted on the phosphorylation on AKT Thr308, but showed no effects on Ser473. In our system, we first found both of B55alpha and B56gamma levels were increased, while PP2A activity was decreased in spheroid culture. Moreover, silibinin mainly suppressed AKT phosphorylation at Ser473 rather than at Thr308. Since increases in B subunits levels often increase PP2A holoenzyme activity, these results therefore suggest the decreased PP2A activity in current study was not due to the change in B55alpha or B56gamma subunit levels. The other B subunit of PP2A, B56gamma, is also reported to be responsible for AKT phosphorylation [Rocher et al., 2007]. In this study, the mRNA and protein levels of B56gamma were upregulated during spheroid culture and enhanced by silibinin treatment (Fig. 5b). These data together suggest that the decrease in PP2A activity during sphere formation was due to changes in the phosphorylation levels of PP2Ac subunit rather than the levels of B subunits such as B55alpha or B56gamma. The tumor suppressor PP2A, long known to control several signaling molecules including AKT, ERK, or p38 on serine and threonine sites [Janssens et al., 2005], has recently been demonstrated in CSLCs of glioblastoma to control the survival of CSLCs through modulating the AKT/mTOR/GSK3β pathway [Lu et al., 2010]. In our study, reduced PP2A activity in spheroid culture modulated the AKT Ser473/mTOR pathway to regulate the self-renewal of CSLCs, increase sphere formation in vitro, and induce tumor initiation in vivo, where silibinin activated PP2A activity, thereby suppressing the self-renewal of CSLCs and inhibiting sphere formation and tumor initiation. Ceramide, containing many kinds of isoforms, is known as a PP2A activator. The affinity of each ceramide isoform to PP2A is dependent on the numbers of carbon chains and each isoform targets on specific subunits of PP2A [Dobrowsky et al., 1993; Chalfant et al., 1999, 2004; Lin et al., 2007]. C6-ceramide, we used in the current study, targets specifically to PP2Ac subunit with the affinity threefold higher than to B subunits [Chalfant et al., 2004]. Thus, we used this isoform to confirm the involvement of PP2Ac subunit in sphere formation and the effect of silibinin on CSLCs development. Treatment with the PP2Ac activator C6-ceramide also successfully suppressed the sphere formation in vitro and tumor initiation in vivo. All of these results indicate that the activity of PP2Ac was important for CSLC enrichment.

Silibinin is commonly used for hepato-protection against clinical hepatitis [Ferenci et al., 2008; Polyak et al., 2010] and possesses tumor suppressor activity by inhibiting cell proliferation and inducing apoptosis through arresting the cell cycle progression and activating caspase cascades, respectively [Singh et al., 2008, 2009]. Accumulating evidence suggests silibinin suppresses the in vitro growth of many kinds of cancer cells in vitro such as prostate cancer cell PC-3 [Singh et al., 2009], colorectal cancer cell HT-29 [Singh et al., 2008] and SW480 [Kaur et al., 2010], and glioblastoma cell U87MG [Momeny et al., 2010]. Recent studies also demonstrated silibinin has anti-angiogenesis abilities through suppressing the VEGF/Flt-1 related pathways [Yang et al., 2005]. When combining with cisplatin and doxorubisin, silibinin shows synergistic effects in anti-proliferative effects on gynaecological malignancies [Scambia et al., 1996]. Under a concentration of 100 μM (~25 μg/ml), silibinin caused prominent cytotoxicity in primary cultures and cell lines of colorectal cancer [Kaur et al., 2009], but not in colorectal CSLCs. Here, silibinin was used as low as 5 μg/ml to decrease the confounding of overdose cytotoxicity, and showed a significant effects in inhibiting colorectal CSLCs enrichment, suppressing sphere formation, and reducing tumorigenecity. Since self-renewal is the characteristic that CSLCs mediate to form sphere in culture [Bisson and Prowse, 2009], our results also indicate the self-renewal of CSLCs was also suppressed by this compound.

The effects of silibinin on arresting the cell cycle or inducing apoptosis has been reported through activating AKT, ERK, or p38-related signaling pathways [Hsieh et al., 2007]. In this study, silibinin decreased sphere formation and impaired the enrichment of CSLCs by suppressing the phosphorylation of AKT Ser473 and the downstream mTOR. In contrast, no significant change in the activation of ERK and p38 was noticed in silibinin-treated CSLCs. The phosphorylation sites of AKT, Ser473 and Thr308, both control AKT activity but have different effects. Phosphorylation of Ser473 and Thr308 involves cell growth, while phosphorylation of AKT at Thr308 alone leads to growth arrest [Andrabi et al., 2007]. Here, we showed treatment of silibinin suppressed the phosphorylation of AKT at Ser473, but not significant at Thr308, in spheroid culture of colorectal cancer cells. To figure out the mechanism that silibinin mediated to modulate AKT Ser473, we found PP2Ac activity, which was suppressed in spheroid culture, was upregulated upon treatment

with silibinin. Although PP2Ac has been reported to influence the phosphorylation of ERK and p38, no changes in the activation of these pathways were noted in our study. Because the concentration of silibinin we used in the current study was much lower than that commonly used in cancer cells; it may help to explain why the phosphorylation of ERK and p38 was not changed. Since the differentiation of stem cell through AKT Ser473/mTOR pathway was reported related to free radicals [Chen et al., 2008; Ivanovic, 2009], whether the mechanisms of silibinin on CSLCs involved on this pathway need to be further investigated.

There are still no useful compounds reported for suppressing CSLCs because of the chemoresistant characteristics [Ahmed et al., 2010; Misawa et al., 2010]. Recently, some chemical compounds such as paclitaxel/8-quinolinol [Zhou et al., 2009] or sorafenib/sulforaphane [Rausch et al., 2010] were combined with chemotoxic drugs to control CSLCs. Because there are still some side effects of chemical compounds, we investigated natural compounds for modulating CSLCs sphere formation in this study. We were the first to find the suppressive effects of silibinin on enrichment of colorectal CSLCs. Without cytotoxicity, silibinin should be a supporting agent combined with chemotherapy drugs to target CSLCs enrichment, the key component of chemo-resistance, recurrence, and metastasis.

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