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Cancer Therapy: Preclinical

Clinical Cancer Research

Sulforaphane, a Dietary Component of Broccoli/Broccoli Sprouts, Inhibits Breast Cancer Stem Cells

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Abstract

Purpose: The existence of cancer stem cells (CSCs) in breast cancer has profound implications for cancer prevention. In this study, we evaluated sulforaphane, a natural compound derived from broccoli/broccoli sprouts, for its efficacy to inhibit breast CSCs and its potential mechanism.

Experimental Design: Aldefluor assay and mammosphere formation assay were used to evaluate the effect of sulforaphane on breast CSCs *in vitro*. A nonobese diabetic/severe combined immunodeficient xenograft model was used to determine whether sulforaphane could target breast CSCs *in vivo*, as assessed by Aldefluor assay, and tumor growth upon cell reimplantation in secondary mice. The potential mechanism was investigated using Western blotting analysis and β-catenin reporter assay.

Results: Sulforaphane (1-5 μmol/L) decreased aldehyde dehydrogenase–positive cell population by 65% to 80% in human breast cancer cells ($P < 0.01$) and reduced the size and number of primary mammospheres by 8- to 125-fold and 45% to 75% ($P < 0.01$), respectively. Daily injection with 50 mg/kg sulforaphane for 2 weeks reduced aldehyde dehydrogenase–positive cells by >50% in nonobese diabetic/severe combined immunodeficient xenograft tumors ($P = 0.003$). Sulforaphane eliminated breast CSCs *in vivo*, thereby abrogating tumor growth after the reimplantation of primary tumor cells into the secondary mice ($P < 0.01$). Western blotting analysis and β-catenin reporter assay showed that sulforaphane downregulated the Wnt/β-catenin self-renewal pathway.

Conclusions: Sulforaphane inhibits breast CSCs and downregulates the Wnt/β-catenin self-renewal pathway. These findings support the use of sulforaphane for the chemoprevention of breast cancer stem cells and warrant further clinical evaluation. *Clin Cancer Res; 16(9); 2580–90. ©2010 AACR.*

Broccoli and broccoli sprouts contain large amounts of glucosinolates (1). Numerous studies have substantiated the chemoprevention effect of increasing cruciferous vegetable intake against cancer, which has been attributed to the activity of various isothiocyanates that are enzymatically hydrolyzed from glucosinolates (2). Sulforaphane was found to be converted from glucoraphanin, a major glucosinolate in broccoli/broccoli sprouts (3). The chemoprevention properties of sulforaphane against cancer are through both "blocking" and "suppressing" effects (2). The blocking function of sulforaphane is achieved through inhibiting phase 1 metabolism enzymes that convert procarcinogens to carcinogens and inducing phase 2 metabolism enzymes that promote excretion of carcinogens (2). Subsequent studies revealed the suppressing effects of sulforaphane in modulating diverse cellular activities to inhibit the growth of transformed cells (2, 4). The ability of sulforaphane to induce apoptosis and cell cycle arrest is associated with regulation of many molecules including Bcl-2 family proteins, caspases, p21, cyclins, and cyclin-dependent kinases (4). Sulforaphane was also shown to suppress angiogenesis and metastasis by downregulating vascular endothelial growth factor, HIF-1α, matrix metalloproteinase-2 and matrix metalloproteinase-9 (4).

Accumulating evidence has shown that many types of cancer, including breast cancer, are initiated from and maintained by a small population of cancer stem cells (CSCs; refs. 5, 6). This minor population produces the tumor bulk through continuous self-renewal and differentiation, which may be regulated by similar signaling pathways occurring in normal stem cells (5–8). Several pathways including Wnt/β-catenin, Hedgehog, and Notch

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Translational Relevance

Sulforaphane, the natural compound derived from broccoli/broccoli sprouts, has been proved to possess anticancer activity. This study shows that sulforaphane inhibits breast cancer stem cells *in vitro* and *in vivo*, which provides a strong rationale for future clinical evaluation of sulforaphane or extract of broccoli/broccoli sprouts for breast cancer chemoprevention. Breast cancer is initiated from and maintained by a small population of breast cancer stem cells. Currently available chemotherapy and radiation therapy are incapable of suppressing cancer stem cell population. Aldefluor assay and mammosphere formation assay showed that sulforaphane inhibited breast cancer stem cells *in vitro*. Nonobese diabetic/severe combined immunodeficient mouse model exhibited that sulforaphane eliminated breast cancer stem cells *in vivo*.

have been identified to be critical to the self-renewal behavior of CSCs (7, 9, 10). Furthermore, CSCs have been suggested to contribute to tumor resistance/relapse because chemotherapy and radiation therapy are incapable of eradicating them (6, 11, 12). Thus, targeting these self-renewal pathways may provide an effective strategy to target CSCs and thereby overcome tumor resistance and reduce relapse (5). Several dietary compounds, such as curcumin (13, 14), quercetin, and epigallocatechin-gallate (15), were found to be potentially against CSC self-renewal.

Wnt/β-catenin signaling is one of the key pathways that promote self-renewal of breast CSCs (5). Activation of Wnt target genes are mediated by β-catenin, which translocates into the nucleus and binds to the transcription factors T-cell factor/lymphoid enhancer factor (TCF/LEF; refs. 5, 16). The level of intracellular β-catenin is modulated by a multiprotein complex consisting of glycogen synthase kinase3β (GSK3β), adenomatous polyposis coli, casein kinase1α, and axin (17). GSK3β promotes the ubiquitin-proteasome degradation of β-catenin by phosphorylating three specific amino acids, Ser33/Ser37/Thr41, on β-catenin (17).

Sulforaphane was shown to target pancreatic tumor–initiating cells in a very recent report (18). In the present study, we examined the efficacy of sulforaphane against breast CSCs in both breast cancer cell lines and breast cancer xenografts. We showed that sulforaphane eliminated breast CSCs *in vivo*, which was reflected by the inhibition of tumor growth in recipient mice that were inoculated with tumor cells derived from sulforaphane-treated primary xenografts. Furthermore, because sulforaphane was reported to induce the downregulation of β-catenin in human cervical carcinoma HeLa and hepatocarcinoma HepG2 cells (19), we investigated the suppressing activity of sulforaphane on the Wnt/β-catenin pathway.

Materials and Methods

Cell lines and reagents. Human breast cancer cell lines MCF7 and SUM159 were obtained from the American Type Culture Collection and from Dr. Stephen Ethier (Karmanos Cancer Center, Detroit, Michigan), respectively. The source of SUM159 cell line is primary breast anaplastic carcinoma. This cell line is estrogen receptor (ER) negative, progesterone receptor (PR) negative, and does not have Her2 overexpression. Both cell lines were tested and authenticated in their origin sources. Authentication of these cell lines included morphology analysis, growth curve analysis, isoenzyme analysis, short tandem repeat analysis, and *Mycoplasma* detection. Both cell lines were passaged in our laboratory for fewer than 6 mo after receipt. To maintain the integrity of collections, stocks of the earliest passage cells have been stored and cell lines have been carefully maintained in culture as described below. MCF7 was maintained in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine erum (Fisher Scientific), 1% antibiotic-antimycotic (Invitrogen), and 5 μg/mL insulin (Sigma-Aldrich). SUM159 was maintained in Ham's F12 medium (Invitrogen) supplemented with 5% fetal bovine serum, 1% antibiotic-antimycotic, 5 μg/mL insulin, 1 μg/mL hydrocortisone (Sigma-Aldrich), and 4 μg/mL gentamicin (Invitrogen).

Sulforaphane was obtained from LKT Laboratories. Propidium iodide was from Invitrogen. LiCl was purchased from Fisher Scientific; BIO (GSK3 inhibitor IX) was from Calbiochem (EMD Biosciences); and MG132 was obtained from Assay Designs (Stressgen).

Antibodies to β-catenin, phospho-β-catenin Ser33/Ser37/Thr41, phospho-GSK3β Ser9, and GSK3β were purchased from Cell Signaling Technology. Antibodies to cyclin D1 and β-actin were acquired from Santa Cruz Biotechnology.

MTS cell proliferation assay. MCF7 and SUM159 were seeded in 96-well microplates at a density of 3,000 to 5,000 cells per well. Cells were treated with increasing concentrations of sulforaphane as indicated. After 48 hours of incubation, cell viability was assessed by MTS assay (Promega) according to the manufacturer's instruction. The number of living cells is directly proportional to the absorbance at 490 nm of a formazan product reduced from MTS by living cells.

Caspase-3 activity assay. Cells were treated with different concentrations of sulforaphane and collected after 24 hours. The caspase-3 activity assay was based on the manufacturer's instruction of the Caspase-3/CPP32 Fluorometric Assay kit (Biovision Research Products). Cellular protein was extracted with the supplied lysis buffer, followed by the determination of protein concentration using BCA Protein Assay Reagents (Pierce). The cleavage of DEVD-AFC, a substrate of caspase-3, was quantified by using a fluorescence microtiter plate reader with a 400-nm excitation filter and a 505-nm emission filter.

Mammosphere formation assay. Stem/progenitor cells are enriched in mammospheres of breast cancer cells (20), based on the unique ability of stem/progenitor cells to grow and form spheres in serum-free medium (21). Mammosphere culture was done as previously described (22, 23) in a serum-free mammary epithelium basal medium (Lonza, Inc.) supplemented with B27 (Invitrogen), 1% antibiotic-antimycotic, 5 μg/mL insulin, 1 μg/mL hydrocortisone, 4 μg/mL gentamicin, 20 ng/mL EGF (Sigma-Aldrich), 20 ng/mL basic fibroblast growth factor (Sigma-Aldrich), and 1:25,000,000 β-mercaptoethanol (Sigma-Aldrich). Single cells prepared from mechanical and enzymatic dissociation were plated in six-well ultra-low attachment plates (Corning) at a density of 500 to 1,000 cells/mL in primary culture and 100 to 500 cells/mL in the following passages. Different concentrations of sulforaphane were added to primary culture, whereas the second and third passages were grown in the absence of drug. After 7 days of culture, the number of mammospheres was counted under a Nikon Eclipse TE2000-S microscope and the photos were acquired with MetaMorph 7.6.0.0.

Aldefluor assay. A cell population with a high aldehyde dehydrogenase (ALDH) enzyme activity was previously reported to enrich mammary stem/progenitor cells (23). Aldefluor assay was done according to the manufacturer's guidelines (StemCell Technologies). Single cells obtained from cell cultures or xenograft tumors were incubated in an Aldefluor assay buffer containing an ALDH substrate, bodipy-aminoacetaldehyde (1 μmol/L per 1,000,000 cells), for 40 to 50 minutes at 37°C. As a negative control, a fraction of cells from each sample was incubated under identical condition in the presence of the ALDH inhibitor diethylaminobenzaldehyde. Flow cytometry was used to measure ALDH-positive cell population.

Primary nonobese diabetic/severe combined immunodeficient mouse model. All experimentation involving mice were conducted in accordance with the standard protocol approved by the University Committee on the Use and Care of Animals at the University of Michigan. SUM159 cells (2,000,000) mixed with Matrigel (BD Biosciences) were injected to the mammary fat pads of 5-week-old female nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice (The Jackson Laboratory) as previously described (24). Tumors were measured with a caliper and the volume was calculated using $V = 1/2$ (width^2 × length). Two weeks after the cell injection, the mice were randomly separated into two groups: one group was i.p. injected with control (0.9% NaCl solution) and the other group was injected with 50 mg/kg sulforaphane (dissolved in 0.9% NaCl solution) daily for 2 weeks.

Dissociation of tumors. At the end of drug treatment, the mice were humanely euthanized and tumors were harvested. Tumor tissues were dissociated mechanically and enzymatically to obtain a single-cell suspension as previously described (25). Briefly, tumors were minced by scalpel and incubated in medium 199 (Invitrogen) mixed with collagenase/hyaluronidase (StemCell Technologies) at 37°C for 15 to 20 minutes. The tissues were further dissociated by pipette trituration and then passed through a 40-μm nylon mesh to produce a single-cell suspension, which was used for Aldeflour assay and flow cytometry.

Secondary NOD/SCID mouse model. Living cells from the dissociated tumors were sorted out by fluorescence-activated cell sorting. Two groups of mice (four in group 1 and three in group 2) were implanted with tumor cells separately. Each secondary NOD/SCID mouse was inoculated with 50,000 cells from control mouse tumors in one side of inguinal mammary fat pad and another 50,000 cells from sulforaphane-treated tumors in the contralateral mammary fat pad. The growth of tumors was monitored and tumor volumes were measured twice weekly. Mice were humanely euthanized when the larger one of the two tumors reached 300 to 500 mm^3.

Western blotting analysis. Cells were treated with sulforaphane at varying concentrations for indicated time periods in the figure legends. Cells were harvested, lysed in radioimmunoprecipitation assay buffer [20 mmol/L Tris-HCl, 150 mmol/L NaCl, 1% NP40, 5 mmol/L EDTA, 1 mmol/L Na_3VO_4 (pH 7.5)] supplemented with a protease inhibitor cocktail (Pierce) and a phosphatase inhibitor (Calbiochem, EMD Biosciences), and incubated on ice for 30 minutes. Cell lysate was centrifuged at 14,000 rpm for 15 minutes and the supernatant

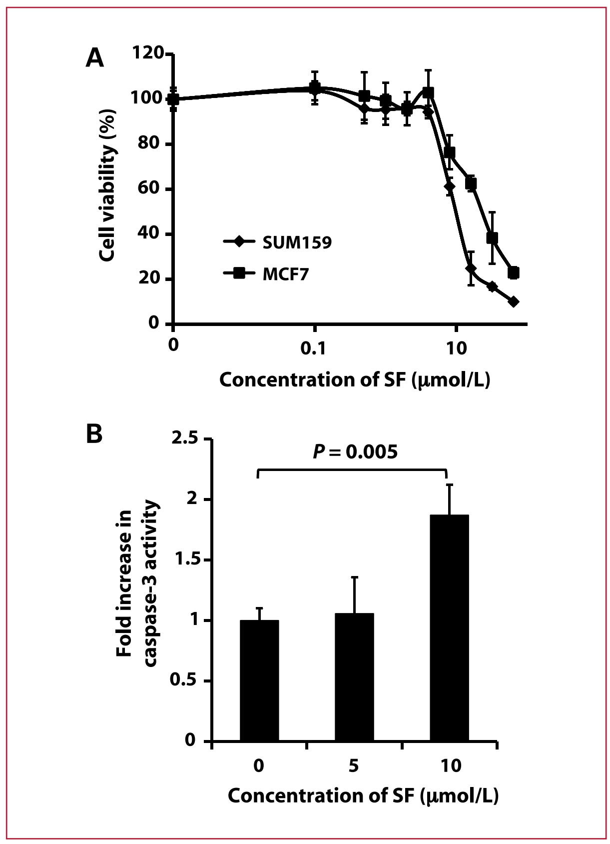

Fig. 1. Sulforaphane inhibited proliferation and induced apoptosis in breast cancer cells. A, SUM159 and MCF7 cells growing in log phase were treated with increasing concentrations of sulforaphane for 48 hours. The antiproliferation effect of sulforaphane was measured by MTS assay. B, sulforaphane enhanced caspase-3 activity in SUM159 cells. Columns, mean ($n \geq 3$); bars, SD; SF, sulforaphane.

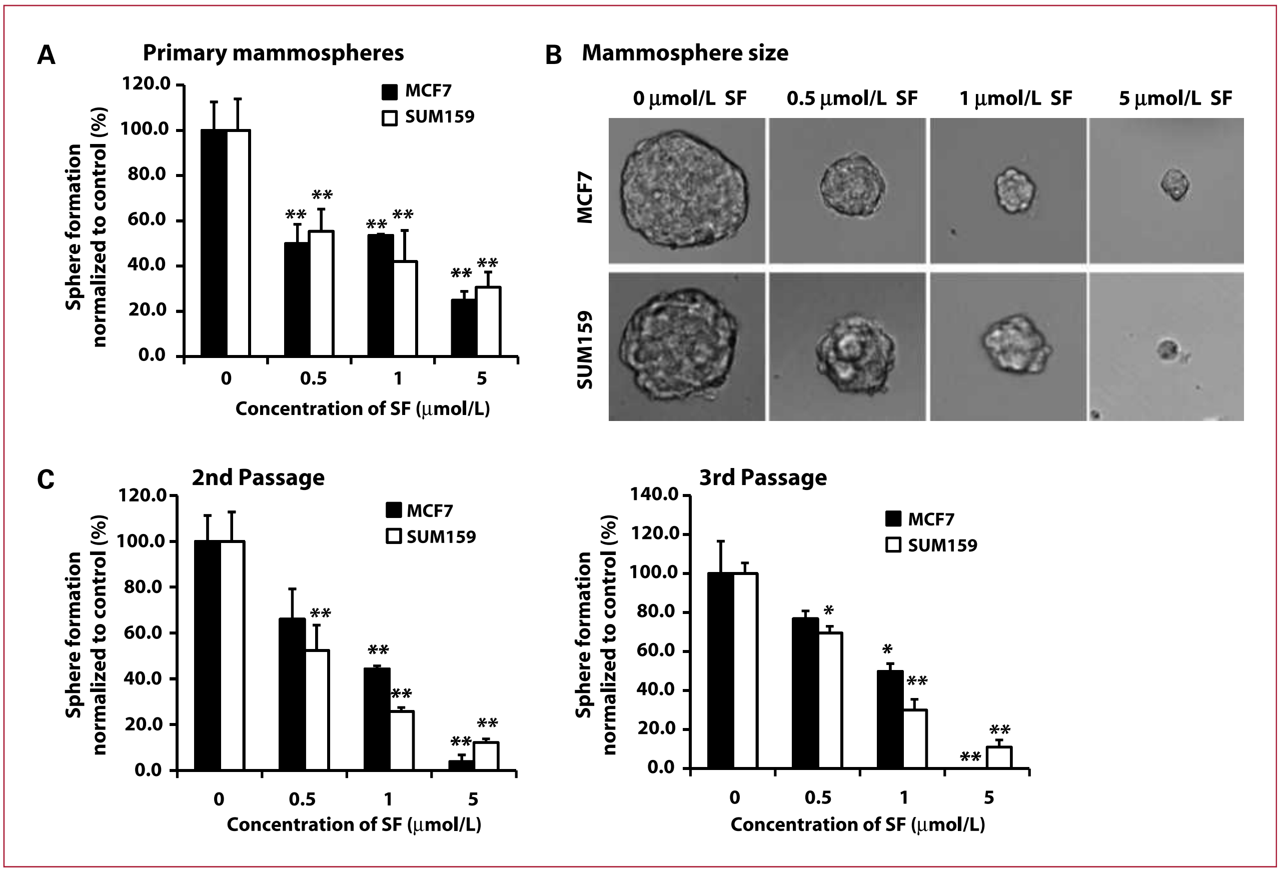

Fig. 2. Inhibitory effect of sulforaphane on mammosphere formation. MCF7 and SUM159 cells were cultured in mammosphere-forming conditions. A, primary mammospheres were incubated with sulforaphane (0.5, 1, and 5 μmol/L) or DMSO for 7 days. Sulforaphane treatment reduced the number of primary mammospheres. B, sulforaphane reduced the size of primary mammospheres (magnification, ×100). The size of mammospheres was estimated using $V = (4/3)\,\pi\,R^3$. C, in the absence of drug, the second and third passages that were derived from sulforaphane-treated primary mammospheres yielded smaller numbers of spheres in comparison with control. Columns, mean (n = 3); bars, SD. *, $P < 0.05$; **, $P < 0.01$; SF, sulforaphane.

was recovered. Protein concentration was determined with BCA Protein Assay Reagents (Pierce). Equal amounts of protein were subject to SDS-PAGE and transferred to polyvinylidene difluoride membrane (Bio-Rad, Richmond, CA). The membrane was then incubated with appropriate antibodies.

TOP-dGFP lentiviral β-catenin reporter assay. TCF/LEF-1 (TOP-dGFP, FOP-dGFP) lentiviral reporter system was kindly gifted by Dr. Wiessman at Ludwig Center, Stanford University School of Medicine (Stanford, CA)(26). Cells were infected with TOP-dGFP or control reporter FOP-dGFP with mutated TCF/LEF-1 binding sites. TOP-dGFP MCF7 and FOP-dGFP MCF7 cells were maintained in the same RPMI 1640 as MCF7 cells. MCF7, TOP-dGFP MCF7, and FOP-dGFP MCF7 cells were cultured in the same serum-free mammary epithelium basal medium as mammospheres in six-well ultralow attachment plates at a density of 1,000 to 1,500 cells/mL for 5 days. Single cells prepared from the primary spheres were incubated in a medium containing 5 μmol/L sulforaphane or/and 0.5 μmol/L BIO for 48 hours. After dissociation, single-cell suspension was subject to flow cytometry analysis for dGFP-positive cell population. Parental MCF7 cells served as a control for autofluorescence. The photos of mammospheres were taken with a Nikon Eclipse TE2000-S microscope and acquired with MetaMorph 7.6.0.0.

Statistical analysis. Statistical differences were determined using two-tailed Student's t test. Data are presented as mean ± SD ($n \geq 3$).

Results

Sulforaphane inhibits proliferation and induces apoptosis of breast cancer cells. Sulforaphane was previously shown to inhibit proliferation (27) and induce apoptosis (28) in breast cancer cells. We first evaluated the antiproliferative effects of sulforaphane in two human breast cancer cell lines, SUM159 and MCF7, by MTS assay. Cells were treated with increasing concentrations of sulforaphane for 48 hours and the ratio of viable cells of treatment relative to control is plotted in Fig. 1A. Cell survival decreased as the concentration of sulforaphane increased, with an IC_{50} of ~10 μmol/L for SUM159 and 16 μmol/L for MCF7. Caspase-3 fluorometric assay showed that sulforaphane

(10 μmol/L) significantly ($P = 0.005$) induced the activation of caspase-3 (Fig. 1B).

***Sulforaphane inhibits breast cancer stem/progenitor cells* in vitro.** It has been shown that mammary stem/progenitor cells are enriched in nonadherent spherical clusters of cells, termed mammospheres (22). These cells are capable of yielding secondary spheres and differentiating along multiple lineages (22). To evaluate whether sulforaphane could suppress the formation of mammospheres *in vitro*, we exposed primary MCF7 and SUM159 spheres to varying concentrations of sulforaphane and then cultured them two additional passages in the absence of drug. As shown in Fig. 2A and B, sulforaphane inhibited the formation of primary spheres. Not only the number of spheres declined by 45% to 75% ($P < 0.01$; Fig. 2A) but the size of the spheres was also reduced by 8- to 125-fold (Fig. 2B). Furthermore, a significant decrease in the number of sphere-forming cells in subsequent passages indicated a reduced self-renewal capacity of these stem/progenitor cells (Fig. 2C; ref. 22). MCF7 cells initially propagated in the presence of 5 μmol/L sulforaphane barely produced secondary spheres, with no cells passaged to third generation (Fig. 2C). It is worth noting that the concentrations of sulforaphane that were capable of suppressing mammosphere formation (IC_{50}, approximately 0.5-1 μmol/L for both SUM159 and MCF7 spheres) were ~10-fold lower than those exhibiting antiproliferative effects in the MTS assay (IC_{50}, ~10 μmol/L for SUM159 and 16 μmol/L for MCF7).

In breast carcinomas, a cell population with high ALDH activity as assessed by the Aldefluor assay has been shown to enrich tumorigenic stem/progenitor cells (23). This cell population is capable of self-renewal and generating tumors resembling the parental tumor (23). Because SUM159 has a relatively high percentage of ALDH-positive cells, we selected SUM159 to examine whether sulforaphane inhibits the tumor-initiating ALDH-positive cells *in vitro*. As shown in Fig. 3A, 1 μmol/L sulforaphane significantly decreased the ALDH-positive population of SUM159 cells by over 65% ($P = 0.008$), whereas 5 μmol/L produced greater than an 80% reduction of ALDH-positive population ($P < 0.008$). Representative flow cytometry dot plots are presented in Fig. 3B. These data showed that sulforaphane inhibited the ALDH-positive cells at similar concentrations to those inhibited mammosphere formation and at 10-fold lower concentrations than those inhibited cancer cells as determined by MTS assay.

Therefore, these findings demonstrate sulforaphane in reducing the breast cancer stem/progenitor cell population *in vitro*. An interesting observation is that sulforaphane was able to inhibit stem/progenitor cells at the concentrations (0.5-5 μmol/L) that hardly affected the bulk population of cancer cells, implying that sulforaphane is likely to

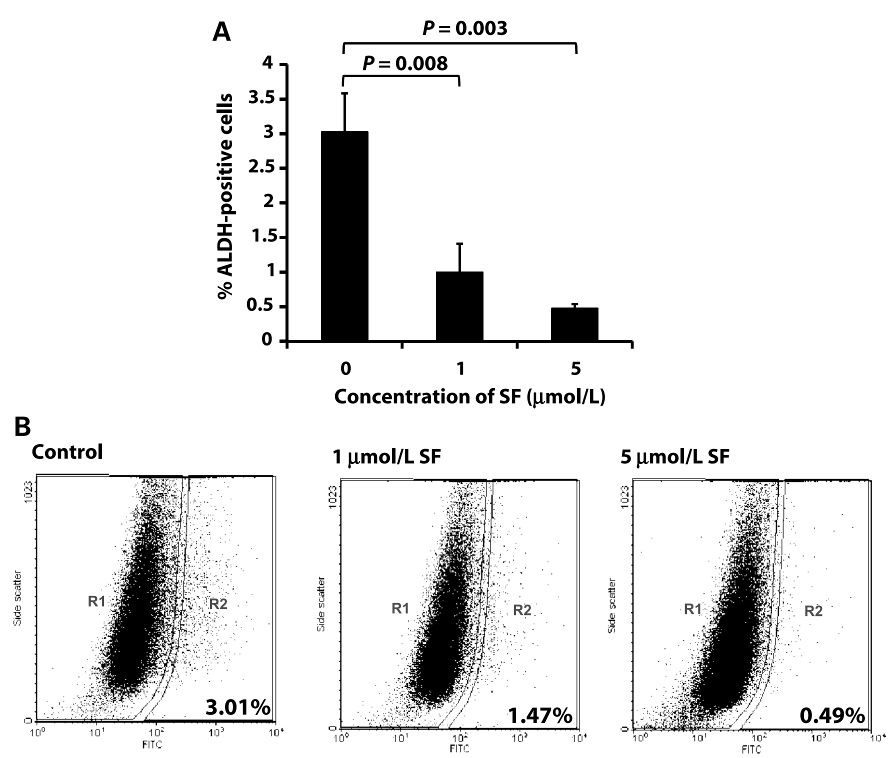

Fig. 3. Inhibitory effect of sulforaphane on ALDH-positive cell population. SUM159 cells were treated with sulforaphane (1 and 5 μmol/L) or DMSO for 4 days and subject to Aldefluor assay and flow cytometry analysis. A, sulforaphane decreased the percentage of ALDH-positive cells. Columns, mean ($n = 3$); bars, SD. B, a set of representative flow cytometry dot plots. R2 covers the region of ALDH-positive cells. SF, sulforaphane.

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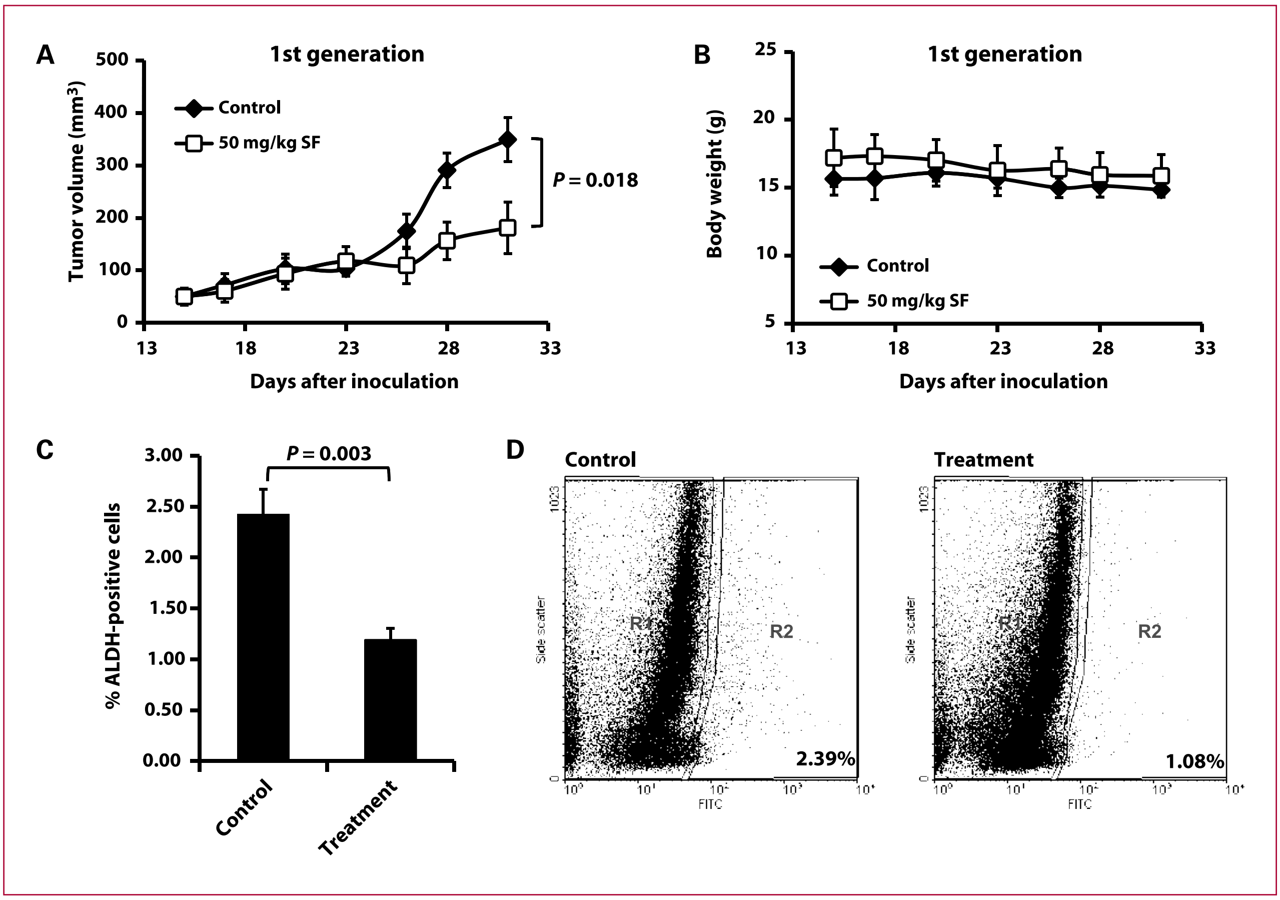

Fig. 4. Sulforaphane (SF) decreased tumor size and ALDH-positive cell population in primary breast cancer xenografts. NOD/SCID mice bearing SUM159 cells in fat pads as xenografts were treated with daily i.p. injection of control or 50 mg/kg sulforaphane for 2 weeks. Tumor volumes (A) and mouse body weights (B) were determined as described in Materials and Methods. Tumors in sulforaphane-treated mice were 50% the size of control animals at the end of drug treatment. C, sulforaphane decreased the percentage of ALDH-positive cells in xenograft breast tumors. D, a set of representative flow cytometry plots. Data are presented as mean ± SD (n = 6).

preferentially target stem/progenitor cells compared with the differentiated cancer cells.

***Sulforaphane eliminates breast CSCs* in vivo.** To determine whether sulforaphane could target breast CSCs *in vivo*, we used a xenograft model of SUM159 cells in NOD/SCID mice. Two weeks after cell inoculation, animals were daily injected with 50 mg/kg sulforaphane. After 2 weeks of treatment, tumors in sulforaphane-treated mice were 50% of the size of 0.9% NaCl solution control animals (P = 0.018; Fig. 4A), whereas sulforaphane had no apparent toxicity as determined by body weight (Fig. 4B). Tumors were isolated from the animals and the tumor cells were analyzed by Aldefluor assay. As shown in Fig. 4C and D, sulforaphane reduced the ALDH-positive population by >50% compared with that from control mice (P = 0.003).

Although the decreased ALDH-positive cell population in sulforaphane-treated tumors suggests that sulforaphane may target breast cancer stem/progenitor cells, the ability of residual cancer cells to initiate tumors upon reimplantation in secondary mice is a more definitive assay (6). Therefore, we examined the growth of secondary tumors in NOD/SCID mice inoculated with primary tumor cells obtained from primary xenografts. To avoid potential variations due to mouse heterogeneity, each recipient mouse was injected with 50,000 cells obtained from sulforaphane-treated tumors in one side of inguinal mammary fat pad and another 50,000 cells obtained from control tumors in the contralateral fat pad. The results showed that cancer cells from control animals exhibited rapid tumor regrowth, reaching a final tumor size ranging from 300 to 500 mm^3 in secondary NOD/SCID mice. However, the cancer cells obtained from sulforaphane-treated mice largely failed to produce any tumors in the recipient mice up to 33 days after implantation (Fig. 5A). Figure 5A and B showed that tumor cells derived from sulforaphane-treated mice only caused one small tumor (6 mm^3) of seven inoculations at day 19, whereas control tumor cells yielded tumors as early as day 7 ($P < 0.01$). All control inoculations produced tumors by day 15 (Fig. 5B). These results suggest that sulforaphane was able to eliminate breast CSCs in primary xenografts, thereby abrogating the

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regrowth of tumors in secondary mice. Taken together with the *in vivo* Aldefluor assay results, these findings suggest that sulforaphane targets breast CSCs with high potency.

Sulforaphane downregulates Wnt/β-catenin pathway in breast cancer cells. Next, we investigated the mechanisms that may contribute to the effects of sulforaphane on breast CSCs. The Wnt/β-catenin pathway is an important regulator of stem cell self-renewal (8). Because sulforaphane was reported to downregulate β-catenin in human cervical carcinoma and hepatocarcinoma cell lines (19), we examined whether β-catenin and Wnt/β-catenin downstream targets are downregulated by sulforaphane in human breast cancer cells. As shown in Fig. 6A, sulforaphane decreased the protein level of β-catenin by up to 85% in MCF7 and SUM159 cells, and the expression of *cyclin D1*, one of the Wnt/β-catenin target genes, declined by up to 77% as well. To further confirm that the downregulation of β-catenin protein level decreased its transcriptional activity, we used a TCF/LEF TOP-dGFP lentiviral reporter system. The β-catenin activates TCF/LEF in the nucleus, driving the transcription of the destabilized green fluorescent protein (*dGFP*) gene. In addition, the

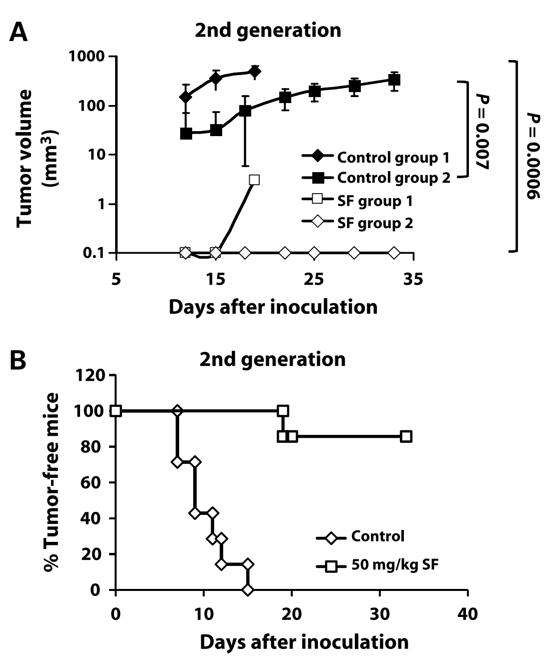

Fig. 5. Sulforaphane (SF) eradicated breast CSCs *in vivo* as assessed by reimplantation in secondary mice. Each secondary NOD/SCID mouse received 50,000 cells from control tumors in one side of mammary fat pad and another 50,000 cells from sulforaphane-treated tumors in the contralateral fat pad. A, tumor growth curves of the recipient NOD/SCID mice. Points, mean (group 1, $n = 4$; group 2, $n = 3$); bars, SD. Sulforaphane abrogated the tumorigenicity of breast CSCs. B, percentage of tumor-free mice by the day of euthanization for each group. Four mice were euthanized at day 20 and three mice were euthanized at day 33 due to the mass tumor burden on the control side.

dGFP expression was analyzed by fluorescence microscopy and quantified by flow cytometry. As determined by flow cytometry, ~3% of transfected cells are dGFP positive and 5 μmol/L sulforaphane reduced this population by 30% to 40% ($P = 0.002$; Fig. 6B).

The intracellular level of β-catenin is regulated by its phosphoryaltion status and subsequent proteasomal degradation. When β-catenin is phosphorylated at Ser33/Ser37/Thr41 by GSK3β, it is immediately subject to ubiquitin-proteasome degradation (17). Phospharylation of GSK3β at Ser9 may decrease the activity of GSK3β, thereby stabiliz ing β-catenin (29, 30). Thus, we used a proteasome inhibitor, MG132, to block proteasome function and observed an accumulation of phospho-β-catenin (Ser33/Ser37/Thr41) in response to sulforaphane (Fig. 6C, top). The sulforaphane-induced β-catenin phosphorylation was reversed when LiCl, a GSK3β inhibitor, was present (Fig. 6C, top; ref. 31). As shown in Fig. 6B, 0.5 μmol/L BIO, another specific GSK3β inhibitor (31, 32), enhanced the dGFP-positive cell population by >5-fold ($P < 0.0001$) and sulforaphane (5 μmol/L) decreased this population by over 60% in the presence of BIO ($P < 0.0001$). Furthermore, our result showed a decreased level of phospho-GSK3β (Ser9) by up to 74% in cells with increasing concentrations of sulforaphane (Fig. 6C, middle). LiCl was shown to inactivate GSK3β through Ser9 phosphorylation, which in turn reduce the phosphorylation of β-catenin at Ser33/Ser37/Thr41 and its degradation (31, 32). As shown in the bottom panel of Fig. 6C, sulforaphane was able to attenuate LiCl-induced GSK3β phosphorylation and β-catenin accumulation.

Taken together, these data suggest that the downregulation of Wnt/β-catenin self-renewal pathway might contribute to the inhibitory effects of sulforaphane on breast CSCs. This warrants further studies to establish the conclusive role of this downregulation in the inhibition of breast CSCs by sulforaphane.

Discussion

The anticancer efficacy of sulforaphane, a natural compound derived from broccoli/broccoli sprouts, has been evaluated in various cancers. For instance, oral or i.p. administration of sulforaphane inhibited the tumor growth in prostate PC-3 and pancreatic Panc-1 xenografts (33, 34). The risk of premenopausal breast cancer was shown to be inversely associated with broccoli consumption (35). The orally administered sulforaphane reached mammary gland and increased the detoxification enzyme activity (36). Additionally, it has been suggested that sulforaphane may have the potential to act against tumor resistance and relapse/recurrence (37). A very recent study showed the effectiveness of sulforaphane in abrogating pancreatic tumor resistance to tumor necrosis factor-α-related apoptosis-inducing ligand by interfering with NF-κB–induced antiapoptotic signaling (18). Another study indicated that sulforaphane could overcome doxorubicin resistance and restore apoptosis induction in cells (38). These findings

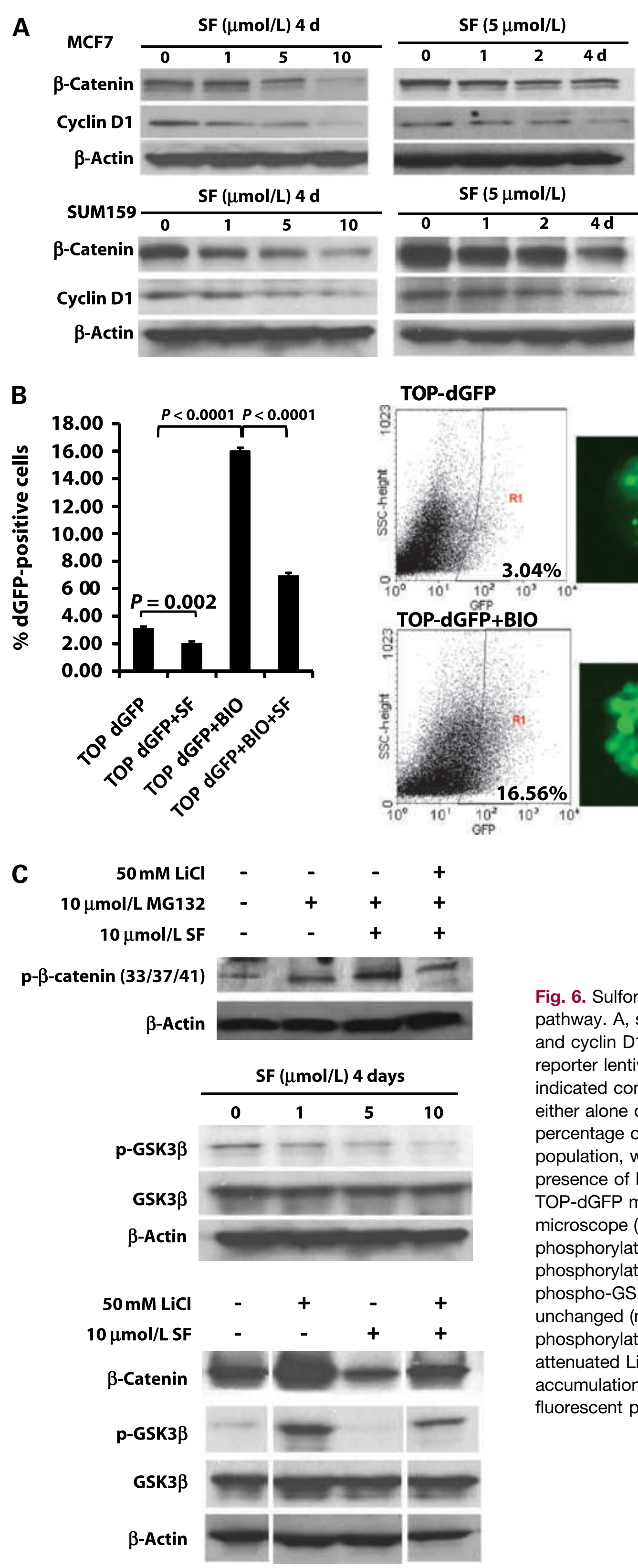

Fig. 6. Sulforaphane downregulated the Wnt/β-catenin self-renewal pathway. A, sulforaphane decreased the protein levels of β-catenin and cyclin D1 in both SUM159 and MCF7 cell lines. B, TOP-dGFP reporter lentivirus-infected MCF7 mammospheres were treated with indicated compounds (0.5 μmol/L BIO and 5 μmol/L sulforaphane) either alone or in combination for 2 days. Sulforaphane decreased the percentage of dGFP-positive cells by 30% to 40%. BIO increased this population, whereas sulforaphane decreased it by over 60% in the presence of BIO. Right, representative flow cytometry results of TOP-dGFP mammospheres and their pictures under fluorescence microscope (magnification, ×100). C, sulforaphane promoted β-catenin phosphorylation at Ser33/37/Thr41, whereas LiCl suppressed the phosphorylation by inactivating GSK3β (top). Sulforaphane decreased phospho-GSK3β (Ser9) level, whereas total GSK3β remained unchanged (middle). LiCl increased the protein level of β-catenin by phosphorylating/inactivating GSK3β at Ser9, whereas sulforaphane attenuated LiCl-induced GSK3β phosphorylation and β-catenin accumulation (bottom). SF, sulforaphane; dGFP, destabilized green fluorescent protein.

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provide a strong rationale for investigating the chemoprevention property of sulforaphane or broccoli/broccoli sprouts in clinical trials.

Increasing evidence supports the CSC theory, which states that a variety of cancers are driven and sustained by a small proportion of CSCs (8). The concept of CSCs has profound clinical implications for cancer therapeutics and prevention (8, 39). Recent studies indicate that CSCs have the capacity to drive tumor resistance and relapse/recurrence (40, 41). Lack of efficacy of current chemotherapies in advance and metastatic disease requires novel approaches to specifically target CSC population (8, 42, 43). Thus, therapies that are directed against both differentiated cancer cells and CSCs may provide advantages to treat these diseases. Researchers have found that several dietary compounds are promising chemoprevention agents against CSCs, such as curcumin (13, 14). Therefore, based on the chemopreventive activity of sulforaphane and the implications of CSC theory, we have used both *in vitro* and *in vivo* systems to determine whether sulforaphane acts against breast CSCs.

Several techniques have been developed to isolate and characterize breast CSCs *in vitro*. Mammosphere culture was first used to isolate and expand mammary stem/progenitor cells by Dontu et al. (22), based on the ability of stem/progenitor cells to grow in serum-free suspension, whereas differentiated cells fail to survive under the same condition (21). By using this technique, we have shown that sulforaphane (0.5-5 μmol/L) significantly suppressed the mammosphere formation of both SUM159 and MCF7 cells (Fig. 2). Another technique is to use cell makers, e.g., $CD44^{+}CD24^{-/low}lin^{-}$ and ALDH positive (21, 23, 25), to distinguish mammary stem/progenitor cells from differentiated cancer cells. It has been reported that as few as 500 ALDH-positive cells were able to generate a breast tumor within 40 days, whereas 50,000 ALDH-negative cells failed to form tumor (23). ALDH-positive cells and $CD44^{+}CD24^{-/low}lin^{-}$ were identified as small overlaps that have the highest tumorigenic capacity, generating tumors from as few as 20 cells (23). In contrast, ALDH-positive cells without the $CD44^{+}CD24^{-/low}lin^{-}$ marker were able to produce tumors from 1,500 cells, whereas 50,000 $CD44^{+}CD24^{-/low}lin^{-}$ ALDH-negative cells did not (23). Thus, we used Aldefluor assay to evaluate the ability of sulforaphane to target breast cancer stem/progenitor cells. We have shown that sulforaphane (1-5 μmol/L) could inhibit the tumor-initiating ALDH-positive cells *in vitro* by 65% to 80% (Fig. 3). Of special note, concentrations of sulforaphane that inhibit stem/progenitor cells in both the mammosphere formation assay and Aldefluor assay had only minimal effects on the bulk population of breast cancer cell lines, which implies the preferential targeting of stem/progenitor cells by sulforaphane.

The injection of human breast cancer cells into the mammary fat pad of immunodeficient NOD/SCID mice provides a reliable and sensitive *in vivo* system for studying human breast cancer (25, 44). We showed that sulforaphane was able to target breast CSCs *in vivo* by using this xenograft model. Daily injection of sulforaphane for 2 weeks suppressed tumor growth in primary NOD/SCID mice and reduced ALDH-positive cell population of the tumors by ~50% (Fig. 4). More importantly, we found that the tumor cells derived from sulforaphane-treated mice were not able to form secondary tumors in recipient mice up to 33 days (Fig. 5). There are two possible reasons that may explain the difference between the 50% reduction of ALDH-positive population and the failure of tumor growth in secondary mice. One is that although ALDH-positive cells are enriched with stem/progenitor cells, not all ALDH-positive cells have tumor-initiating capacity. Another possible reason is the experimental setting we used for the primary NOD/SCID mice. We inoculated 2,000,000 SUM159 cells into the primary NOD/SCID mice and treated them with the drug after 2 weeks of cell inoculation, both of which could lead to an underestimation of the effect of sulforaphane on ALDH-positive cell population. However, the ability of CSCs to self-renew and differentiate as determined by the reimplantation of primary tumor cells in secondary animals is a more definitive functional assay (6). These are consistent with the *in vitro* observation that sulforaphane preferentially targeted cancer stem/progenitor cells instead of bulk cell population. The preference of sulforaphane in killing CSCs may be significant for chemoprevention.

The well-known curcumin was shown to interfere with self-renewal pathways, Wnt and Notch, in colon and pancreatic cancer cells, respectively (13, 14). Apple-derived quercetin and green tea epigallocatechin-gallate were reported to regulate key elements of Wnt and Notch pathways in human colon cancer cells (15). Park et al. (19) previously reported that β-catenin was downregulated in HeLa and HepG2 cells. In consistent with this study, we showed that sulforaphane was able to downregulate the Wnt/β-catenin self-renewal pathway in breast cancer cells, and sulforaphane-induced β-catenin phosphorylation (Ser33/Ser37/Thr41) and proteasome degradation was possibly through the activation of GSK3β (Fig. 6). Myzak et al. (45) reported that sulforaphane increased β-catenin activity without altering its protein level in HDAC1-transfected HEK293 cells. The differences among the studies could arise from distinct cell lines and treatment conditions.

As a chemoprevention agent, sulforaphane possesses many advantages, such as high bioavailability and low toxicity (4). Sulforaphane from broccoli extracts is efficiently and rapidly absorbed in the human small intestine and distributed throughout the body (2, 46). Plasma concentrations of sulforaphane equivalents peaked 0.94 to 2.27 μmol/L in humans 1 hour after a single dose of 200 μmol broccoli sprout isothiocyanates (mainly sulforaphane; ref. 47). A recent pilot study detected an accumulation of sulforaphane in human breast tissue, with 1.45 ± 1.12 pmol/mg for the right breast and 2.00 ± 1.95 pmol/mg for the left, in eight women who consumed broccoli sprout preparation containing 200 μmol sulforaphane ~1 hour before the surgery (36). These concentrations of sulforaphane are expected to be effective against breast CSCs, based on our *in vitro* results. Although sulforaphane itself has not been evaluated in humans, broccoli sprouts were tested for toxicity in clinical trials (4). A phase I trial showed that broccoli sprouts caused no significant toxicity when administered orally at 8-hour intervals for 7 days as 25 μmol isothiocyanates (mainly sulforaphane; ref. 48). In another study, it was well tolerated in 200 adults who consumed broccoli sprout solution containing 400 μmol glucoraphanin (precursor of sulforaphane) nightly for 2 weeks (49). Additionally, sulforaphane at concentrations below 10 μmol/L did not show significant effect on cell cycle arrest and apoptosis induction of human nontransformed T lymphocytes (50).

In conclusion, we have shown that sulforaphane was able to target breast CSCs as determined by the mammosphere formation assay, Aldefluor assay, and tumor growth upon reimplantation in secondary mice. Furthermore, our study identified the downregulation of Wnt/β-catenin self-renewal pathway by sulforaphane as one of the possible mechanisms for its efficacy. These studies support the use of sulforaphane for breast cancer chemoprevention. These findings provide a strong rationale for preclinical and clinical evaluation of sulforaphane or broccoli/broccoli sprouts for breast cancer therapies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Zhang Y, Talalay P, Cho CG, Posner GH. A major inducer of anticarcinogenic protective enzymes from broccoli: isolation and elucidation of structure. Proc Natl Acad Sci U S A 1992;89:2399–403.
2. Clarke JD, Dashwood RH, Ho E. Multi-targeted prevention of cancer by sulforaphane. Cancer Lett 2008;269:291–304.
3. Fahey JW, Zhang Y, Talalay P. Broccoli sprouts: an exceptionally rich source of inducers of enzymes that protect against chemical carcinogens. Proc Natl Acad Sci U S A 1997;94:10367–72.
4. Zhang Y, Tang L. Discovery and development of sulforaphane as a cancer chemopreventive phytochemical. Acta Pharmacol Sin 2007; 28:1343–54.
5. Liu S, Dontu G, Wicha MS. Mammary stem cells, self-renewal pathways, and carcinogenesis. Breast Cancer Res 2005;7:86–95.
6. Korkaya H, Paulson A, Charafe-Jauffret E, et al. Regulation of mammary stem/progenitor cells by PTEN/Akt/β-catenin signaling. PLoS Biol 2009;7:e1000121.
7. Liu S, Dontu G, Mantle ID, et al. Hedgehog signaling and Bmi-1 regulate self-renewal of normal and malignant human mammary stem cells. Cancer Res 2006;66:6063–71.
8. Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. Nature 2001;414:105–11.
9. Dontu G, Jackson KW, McNicholas E, Kawamura MJ, Abdallah WM, Wicha MS. Role of Notch signaling in cell-fate determination of human mammary stem/progenitor cells. Breast Cancer Res 2004;6: R605–15.
10. Smalley MJ, Dale TC. Wnt signalling in mammalian development and cancer. Cancer Metastasis Rev 1999;18:215–30.
11. Shafee N, Smith CR, Wei S, et al. Cancer stem cells contribute to cisplatin resistance in Brca1/p53-mediated mouse mammary tumors. Cancer Res 2008;68:3243–50.
12. Hambardzumyan D, Squatrito M, Holland EC. Radiation resistance and stem-like cells in brain tumors. Cancer Cell 2006;10:454–6.
13. Wang Z, Zhang Y, Banerjee S, Li Y, Sarkar FH. Notch-1 down-regulation by curcumin is associated with the inhibition of cell growth and the induction of apoptosis in pancreatic cancer cells. Cancer 2006;106:2503–13.
14. Jaiswal AS, Marlow BP, Gupta N, Narayan S. β-Catenin-mediated transactivation and cell-cell adhesion pathways are important in curcumin (diferuylmethane)-induced growth arrest and apoptosis in colon cancer cells. Oncogene 2002;21:8414–27.
15. Pahlke G, Ngiewih Y, Kern M, Jakobs S, Marko D, Eisenbrand G. Impact of quercetin and EGCG on key elements of the Wnt pathway in human colon carcinoma cells. J Agric Food Chem 2006;54: 7075–82.
16. Clevers H. Wnt/β-catenin signaling in development and disease. Cell 2006;127:469–80.
17. Liu C, Li Y, Semenov M, et al. Control of β-catenin phosphorylation/ degradation by a dual-kinase mechanism. Cell 2002;108:837–47.
18. Kallifatidis G, Rausch V, Baumann B, et al. Sulforaphane targets pancreatic tumour-initiating cells by NF-κB-induced antiapoptotic signalling. Gut 2009;58:949–63.
19. Park SY, Kim GY, Bae SJ, Yoo YH, Choi YH. Induction of apoptosis by isothiocyanate sulforaphane in human cervical carcinoma HeLa and hepatocarcinoma HepG2 cells through activation of caspase-3. Oncol Rep 2007;18:181–7.
20. Ponti D, Costa A, Zaffaroni N, et al. Isolation and *in vitro* propagation of tumorigenic breast cancer cells with stem/progenitor cell properties. Cancer Res 2005;65:5506–11.
21. Charafe-Jauffret E, Monville F, Ginestier C, Dontu G, Birnbaum D, Wicha MS. Cancer stem cells in breast: current opinion and future challenges. Pathobiology 2008;75:75–84.
22. Dontu G, Abdallah WM, Foley JM, et al. *In vitro* propagation and transcriptional profiling of human mammary stem/progenitor cells. Genes Dev 2003;17:1253–70.
23. Ginestier C, Hur MH, Charafe-Jauffret E, et al. ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. Cell Stem Cell 2007;1:555–67.
24. Luo M, Fan H, Nagy T, et al. Mammary epithelial-specific ablation of the focal adhesion kinase suppresses mammary tumorigenesis by affecting mammary cancer stem/progenitor cells. Cancer Res 2009;69:466–74.
25. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci U S A 2003;100:3983–8.

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26. Reya T, Duncan AW, Ailles L, et al. A role for Wnt signalling in self-renewal of haematopoietic stem cells. Nature 2003;423:409–14.
27. Azarenko O, Okouneva T, Singletary KW, Jordan MA, Wilson L. Suppression of microtubule dynamic instability and turnover in MCF7 breast cancer cells by sulforaphane. Carcinogenesis 2008;29:2360–8.
28. Pledgie-Tracy A, Sobolewski MD, Davidson NE. Sulforaphane induces cell type-specific apoptosis in human breast cancer cell lines. Mol Cancer Ther 2007;6:1013–21.
29. Pap M, Cooper GM. Role of glycogen synthase kinase-3 in the phosphatidylinositol 3-Kinase/Akt cell survival pathway. J Biol Chem 1998;273:19929–32.
30. Cohen P, Frame S. The renaissance of GSK3. Nat Rev Mol Cell Biol 2001;2:769–76.
31. Hedgepeth CM, Conrad LJ, Zhang J, Huang HC, Lee VM, Klein PS. Activation of the Wnt signaling pathway: a molecular mechanism for lithium action. Dev Biol 1997;185:82–91.
32. Klein PS, Melton DA. A molecular mechanism for the effect of lithium on development. Proc Natl Acad Sci U S A 1996;93:8455–9.
33. Pham NA, Jacobberger JW, Schimmer AD, Cao P, Gronda M, Hedley DW. The dietary isothiocyanate sulforaphane targets pathways of apoptosis, cell cycle arrest, and oxidative stress in human pancreatic cancer cells and inhibits tumor growth in severe combined immunodeficient mice. Mol Cancer Ther 2004;3:1239–48.
34. Singh AV, Xiao D, Lew KL, Dhir R, Singh SV. Sulforaphane induces caspase-mediated apoptosis in cultured PC-3 human prostate cancer cells and retards growth of PC-3 xenografts *in vivo*. Carcinogenesis 2004;25:83–90.
35. Ambrosone CB, McCann SE, Freudenheim JL, Marshall JR, Zhang Y, Shields PG. Breast cancer risk in premenopausal women is inversely associated with consumption of broccoli, a source of isothiocyanates, but is not modified by GST genotype. J Nutr 2004;134:1134–8.
36. Cornblatt BS, Ye L, Dinkova-Kostova AT, et al. Preclinical and clinical evaluation of sulforaphane for chemoprevention in the breast. Carcinogenesis 2007;28:1485–90.
37. Myzak MC, Dashwood RH. Chemoprotection by sulforaphane: keep one eye beyond Keap1. Cancer Lett 2006;233:208–18.
38. Fimognari C, Nusse M, Lenzi M, Sciuscio D, Cantelli-Forti G, Hrelia P. Sulforaphane increases the efficacy of doxorubicin in mouse fibroblasts characterized by p53 mutations. Mutat Res 2006;601:92–101.
39. Kakarala M, Wicha MS. Implications of the cancer stem-cell hypothesis for breast cancer prevention and therapy. J Clin Oncol 2008;26:2813–20.
40. Sakariassen PO, Immervoll H, Chekenya M. Cancer stem cells as mediators of treatment resistance in brain tumors: status and controversies. Neoplasia 2007;9:882–92.
41. Tang C, Chua CL, Ang BT. Insights into the cancer stem cell model of glioma tumorigenesis. Ann Acad Med Singapore 2007;36:352–7.
42. Lippman ME. High-dose chemotherapy plus autologous bone marrow transplantation for metastatic breast cancer. N Engl J Med 2000;342:1119–20.
43. Williams SD, Birch R, Einhorn LH, Irwin L, Greco FA, Loehrer PJ. Treatment of disseminated germ-cell tumors with cisplatin, bleomycin, and either vinblastine or etoposide. N Engl J Med 1987;316:1435–40.
44. Dick JE. Breast cancer stem cells revealed. Proc Natl Acad Sci U S A 2003;100:3547–9.
45. Myzak MC, Karplus PA, Chung FL, Dashwood RH. A novel mechanism of chemoprotection by sulforaphane: inhibition of histone deacetylase. Cancer Res 2004;64:5767–74.
46. Petri N, Tannergren C, Holst B, et al. Absorption/metabolism of sulforaphane and quercetin, and regulation of phase II enzymes, in human jejunum *in vivo*. Drug Metab Dispos 2003;31:805–13.
47. Ye L, Dinkova-Kostova AT, Wade KL, Zhang Y, Shapiro TA, Talalay P. Quantitative determination of dithiocarbamates in human plasma, serum, erythrocytes and urine: pharmacokinetics of broccoli sprout isothiocyanates in humans. Clin Chim Acta 2002;316:43–53.
48. Shapiro TA, Fahey JW, Dinkova-Kostova AT, et al. Safety, tolerance, and metabolism of broccoli sprout glucosinolates and isothiocyanates: a clinical phase I study. Nutr Cancer 2006;55:53–62.
49. Kensler TW, Chen JG, Egner PA, et al. Effects of glucosinolate-rich broccoli sprouts on urinary levels of aflatoxin-DNA adducts and phenanthrene tetraols in a randomized clinical trial in He Zuo township, Qidong, People's Republic of China. Cancer Epidemiol Biomarkers Prev 2005;14:2605–13.
50. Fimognari C, Nusse M, Berti F, Iori R, Cantelli-Forti G, Hrelia P. Isothiocyanates as novel cytotoxic and cytostatic agents: molecular pathway on human transformed and non-transformed cells. Biochem Pharmacol 2004;68:1133–8.

Sulforaphane, a Dietary Component of Broccoli/Broccoli Sprouts, Inhibits Breast Cancer Stem Cells

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