

Sulforaphane synergizes with quercetin to inhibit self-renewal capacity of pancreatic cancer stem cells

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1. ABSTRACT

According to the cancer stem cell hypothesis, the aggressive growth and early metastasis of cancer may arise through dysregulation of self-renewal of stem cells. The objectives of this study were to examine the molecular mechanisms by which sulforaphane (SFN, an active compound in cruciferous vegetables) inhibits self-renewal capacity of pancreatic cancer stem cells (CSCs), and synergizes with quercetin, a major polyphenol and flavonoid commonly detected in many fruits and vegetables. Our data demonstrated that SFN inhibited self-renewal capacity of pancreatic CSCs. Inhibition of Nanog by lentiviral-mediated shRNA expression enhanced the inhibitory effects of sulforaphane on self-renewal capacity of CSCs. SFN induced apoptosis by inhibiting the expression of Bcl-2 and XIAP, phosphorylation of FKHR, and activating caspase-3. Moreover, SFN inhibited expression of proteins involved in the epithelial-mesenchymal transition (β -catenin, vimentin, twist-1, and ZEB1), suggesting the blockade of signaling involved in early metastasis. Furthermore, the combination of quercetin with SFN had synergistic effects on self-renewal capacity of pancreatic CSCs. These data suggest that SFN either alone or in combination with quercetin can eliminate cancer stem cell-characteristics.

2. INTRODUCTION

Cancer of the pancreas is the fourth leading cause of cancer death in the United States. This year approximately 32,000 Americans will die from cancer of the pancreas. With an overall 5-year survival rate of 3% (1), pancreatic cancer has one of the poorest prognoses among all cancers (2). Only 20% of pancreatic cancer patients are eligible for surgical resection, which currently remains the only potentially curative therapy (3). Unfortunately, many cancers of the pancreas are not resectable at the time of diagnosis. There are limited treatment options available for this disease because chemo- and radio-therapies are largely ineffective, and metastatic disease frequently redevelops even after surgery (1, 2). Therefore, developing effective strategies to prevent pancreatic neoplasms are of paramount importance.

Pancreatic cancer becomes clinically apparent at late stages and it resists all forms of conventional chemotherapy and radiotherapy (1, 2). Cancer stem cells (CSCs) have been proposed recently to be the cause of chemotherapy failure (4). Therefore, understanding the pathogenesis of the preinvasive stage, and developing effective strategies to prevent and/or treat pancreatic

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neoplasms are of paramount importance. CSCs and epithelial-mesenchymal transition (EMT)-type cells, which shares molecular characteristics with CSCs, have been believed to play critical roles in drug resistance and early cancer metastasis as demonstrated in several human malignancies including pancreatic cancer. Thus, the discovery of molecular knowledge of drug resistance and metastasis in relation to CSCs and EMT in pancreatic cancer is becoming an important area of research, and such knowledge is likely to be helpful in the discovery of newer drugs as well as designing novel therapeutic strategies for the treatment of pancreatic cancer with better outcome.

An increasing amount of scientific evidence indicates that tumors contain a small number of tumor-forming and self-renewing CSCs within a population of nontumor-forming cancer cells (5). CSCs have recently been identified in several types of human cancers including pancreatic cancer (6-12). It has been suggested that conventional chemotherapies kill differentiated or differentiating cells. These cells form the bulk of the tumor, but are unable to generate new cells. However, CSCs remain untouched, and therefore can cause a relapse of cancer (5). Removal of CSCs becomes more and more crucial to chemo- and radio-therapy. Unlike most cells within the tumor, CSCs, including pancreatic CSCs, are resistant to well-defined chemotherapy and radiotherapy, and may contribute to tumor metastasis and tumor recurrence after treatment (13-16). They can also regenerate all the cell types in the tumor through their stem cell-like behavior. For this reason, drugs that selectively target CSCs offer a greater promise for cancer therapy and prevention.

It is now clear that therapeutic failure/recurrence is due to ineffective targeting of CSC population. The clinical relevance of the cancer stem cell theory, however, has yet to be determined, along with the precise relationship between normal and cancer SCs. CD133 was reported as a marker of cancer stem cells in the brain (17-19), colon (20-22), liver (23, 24) and prostate (25-28). In pancreatic cancer, Li and colleagues have determined that pancreatic cancer is hierarchically organized and originates from a primitive stem/progenitor group of cells for which CD44⁺CD24⁺ESA⁺ precursors constitute one of the most immature stages (9). However, Hermann and colleagues have reported that a distinct subpopulation of CD133⁺ cancer stem cells determined the metastatic phenotype of individual tumors (29). Based on these studies it appears that, there are two possible sources for cancer stem cells in pancreatic cancer; the first source is CD44⁺CD24⁺ESA⁺ cells, and the second source is CD133⁺ cells. Furthermore, Hermann *et al.* reported that these 2 populations overlap but are not identical (29). Since CD44 expressed in almost 100% of pancreatic cancer cell lines, it seemed to be an inappropriate marker for isolating pancreatic cancer stem cells or cancer initiating cells. The CD44⁺CD24⁺ESA⁺ pancreatic cancer cells are highly tumorigenic and possess the stem cell-like properties of self-renewal and the ability to produce differentiated progeny (9). Pancreatic cancer stem cells also demonstrate upregulation of molecules important in developmental signaling pathways, including

sonic hedgehog (8, 10, 30, 31) and the polycomb gene family member Bmi-1 (8, 10). Of clinical importance, cancer stem cells in several tumor types have shown resistance to standard therapies and may play a role in treatment failure or disease recurrence. Identification of pancreatic cancer stem cells and further elucidation of the signaling pathways that regulate their growth and survival may provide novel therapeutic approaches to treat pancreatic cancer, which is notoriously resistant to standard chemotherapy.

A number of experimental studies have also support that certain dietary chemicals isolated from foodstuffs can protect against cancer. An important group of agents that have this property are the organosulfur compounds such as isothiocyanates (ITCs), abundant in cruciferous vegetables for which consumption has epidemiologically shown an inverse link with pancreatic cancer. ITC have been shown to exhibit several potential chemoprotective activities in cell and animal models (32-38). Epidemiological studies have suggested that increased risks of pancreatic cancer are associated with tobacco, obesity and high consumption of fat, fish, pork or beef, and that decreased risks are associated with consumption of cruciferous vegetables. In human pancreatic cancer cells, it has been reported that benzyl isothiocyanate (BITC) and sulforaphane (SFN) which are abundantly included in garden cress and broccoli, respectively, have anti-proliferative activity (32, 34, 35, 39-41). Oral administration of SFN inhibited or retarded experimental multistage carcinogenesis models including cancers of the breast, colon, stomach, prostate, and lung. Previously, these anticancer effects were attributed to modulation of carcinogen metabolism by the inhibition of metabolic activation of phase I enzymes and the induction of phase II detoxification enzymes and glutathione (GSH) levels (36, 42). Furthermore, we have recently demonstrated that SFN induces death receptors (DR4 and DR5) and proapoptotic members of Bcl-2 family, inhibits antiapoptotic Bcl-2 proteins, activates caspase(s), and enhances apoptosis-inducing potential of TRAIL *in vitro* (38). *In vivo*, SFN inhibits growth of PC-3 cells orthotopically implanted in nude mice by inducing apoptosis and inhibiting tumor cell proliferation, metastasis and angiogenesis (38). In a recent report, sulforaphane has been suggested to target pancreatic cancer stem cells (34). These studies strongly suggest that SFN can be developed as a pancreatic cancer preventive and/or therapeutic agent.

Flavonoids represent one of the most actively studied classes of molecules for their potential to prevent cancer. Quercetin, 3, 3', 4', 5, 7-pentahydroxyflavone, is a typical flavonol-type flavonoid ubiquitously present in fruits and vegetables, such as onion, tea, apples and berries. It exhibits anti-oxidative, anti-inflammatory and vasodilating effects, and has been proposed to be a potential anti-cancer agent (43). Epidemiological studies have estimated that the daily dietary intake of quercetin by an individual ranges from 4 to 68 mg (44-46). Quercetin exert antitumor activity, inhibit proliferation and induce apoptosis in human pancreatic cancer cells (47-49). Quercetin itself showed growth inhibitory activity on both

drug-sensitive and MDR cells (50-52). In addition, quercetin at a non-cytotoxic concentration has enhanced the effect of chemotherapeutic drug on MDR cells. Quercetin has also been shown to act as a chemosensitizer for the ABC pump-proteins in a number of MDR tumor cell lines. Furthermore, quercetin interacts directly with transporter proteins to inhibit drug efflux mediated by either MDR1 or MRP1 or BCRP (53-57).

FOXO subfamily of forkhead transcription factors include FOXO1a / FKHR, FOXO3a / FKHL1, and FOXO4 / AFX (58-61). The PI3K pathway, via activation of its downstream kinase AKT, phosphorylates each of the FOXO proteins (62-64). These phosphorylations result in impairment of DNA binding ability and increased binding affinity for the 14-3-3 protein (63, 64). Newly formed 14-3-3-FOXO complexes are then exported from the nucleus (65), thereby inhibiting FOXO-dependent transcription. Inhibition of the PI3K pathway leads to dephosphorylation and nuclear translocation of active FKHL1, FKHR, and AFX; which induce cells cycle arrest and apoptosis (66). Conversely, loss of PTEN activity results in increased AKT activity leading to inhibition of FOXO protein activity through phosphorylation and cytoplasmic sequestration. In addition, the data demonstrate that FOXO transcriptional activity controls cellular proliferation and apoptosis downstream of PTEN (67, 68). FOXO regulates cell cycle and apoptotic genes such as cyclin-dependent kinase inhibitor (CKI) p27^{KIP1} (65, 67, 69, 70), Bim (71, 72), Fas ligand (63), and Bcl-6 (73). Interestingly, overexpression of AKT, and inactivation and loss of PTEN are frequently observed in pancreatic cancer (74-80), indicating a potential role for FOXOs in modulating both cell cycle and apoptosis during tumorigenesis and treatment. We have recently demonstrated that SFN inhibited the activation of PI3K/AKT and MAPK/ERK pathways which resulted in activation of FOXO transcription factors, leading to cell cycle arrest and apoptosis in pancreatic cancer cells (32), and inhibition of angiogenesis by HUVECs (43). However, the molecular targets of FOXOs and their mechanisms of action in cancer stem cells have never been examined.

The objectives of our study were to examine the molecular mechanisms by which SFN inhibits growth and induces apoptosis in pancreatic cancer stem cells. In addition, the interactive effects of quercetin and SFN on self-renewal capacity of pancreatic CSCs were also examined. Our data indicate that: (i) SFN inhibits self-renewal capacity of pancreatic CSCs, and quercetin further enhances the biological effects of SFN; (ii) SFN induces apoptosis by inhibiting the expression of Bcl-2 and XIAP, and phosphorylation of FKHR, and (iii) SFN inhibits the expression of EMT markers (vimentin, β -catenin, twist-1 and Zeb-1) suggesting its effects of early metastasis. These data suggest that SFN alone or in combination with quercetin can be a beneficial agent for the treatment and/or prevention of pancreatic cancer.

3. MATERIAL AND METHODS

3.1. Reagents

Antibodies against β -catenine, vimentin, phospho-FKHR, twist-1, ZEB-1, GAPDH, XIAP, caspase-

3, Bcl-2, and Nanog were purchased from Cell Signaling Technology, Inc. (Danvers, MA). Sulforaphane and quercetin were purchased from LKT Laboratories, Inc. (St. Paul, MN). Enhanced chemiluminescence (ECL) Western blot detection reagents were from Amersham Life Sciences Inc. (Arlington Heights, IL). Terminal Deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling (TUNEL) assay kit was purchased from EMD Biosciences / Calbiochem (San Diego, CA). All other chemicals were purchased from Sigma-Aldrich (St Louis, MO).

3.2. Cell culture

PANC-1, MIA PaCa-2, AsPC-1 and Bx PC-3 cells were obtained from the American Type Culture Collection (Manassas, VA). Human pancreatic cancer stem cells (CD44⁺/CD24⁺/ESA⁺) were from Celprogen Inc. (San Pedro, CA). CSCs were cultured in DMEM supplemented with 1% N2 Supplement (Invitrogen), 2% B27 Supplement (Invitrogen), 20 ng/ml human platelet growth factor (Sigma-Aldrich), 100 ng/ml epidermal growth factor (Invitrogen) and 1% antibiotic-antimycotic (Invitrogen) at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

3.3. Tumor spheroid assay

Spheroid forming assays were performed as described elsewhere (9, 10). In brief, single cells were plated in six-well ultralow attachment plates (Corning Inc., Corning, NY) at a density of 1,000 cells/ml in DMEM supplemented with 1% N2 Supplement (Invitrogen), 2% B27 Supplement (Invitrogen), 20 ng/ml human platelet growth factor- (Sigma-Aldrich), 100 ng/ml epidermal growth factor (Invitrogen) and 1% antibiotic-antimycotic (Invitrogen) at 37°C in a humidified atmosphere of 95% air and 5% CO₂. Spheroid were collected after 7 days and dissociated with Accutase (Innovative Cell Technologies, Inc.). The cells obtained from dissociation were sieved through a 40- μ m filter, and counted by coulter counter using trypan blue dye.

3.4. Soft agar colony assay for assessment of tumorigenic potential *in vitro*

To examine the anchorage independent growth, the CSCs from both pancreatic cancer cell lines and primary tumors were suspended (10³ cells/ml) in 2 ml of 0.3% agar with 1% N2 Supplement (Invitrogen), 2% B27 Supplement (Invitrogen), 20 ng/ml human platelet growth factor- (Sigma-Aldrich), 100 ng/ml epidermal growth factor (Invitrogen) and 1% antibiotic-antimycotic (Invitrogen) overlaid into six-well plates containing a 0.5% agar base. All samples were plated in triplicate. Colonies with >0.2 mm in diameter were counted on day 21. Colonies were stained with 0.001% crystal violet blue and counted.

3.5. Lentiviral vector-mediated transduction of pancreatic cancer stem cells

Lentiviral human Nanog construct (LL-hNANOGi) is described elsewhere (81). Target sequence for the Nanog was GGGTAAAGCTGTAACATACTT (NM_024865: bp 1857-1877). The shRNA was cloned under the control of the U6 promoter in the vector Lentilox 37. Lentiviral vectors, pseudotyped with the vesicular

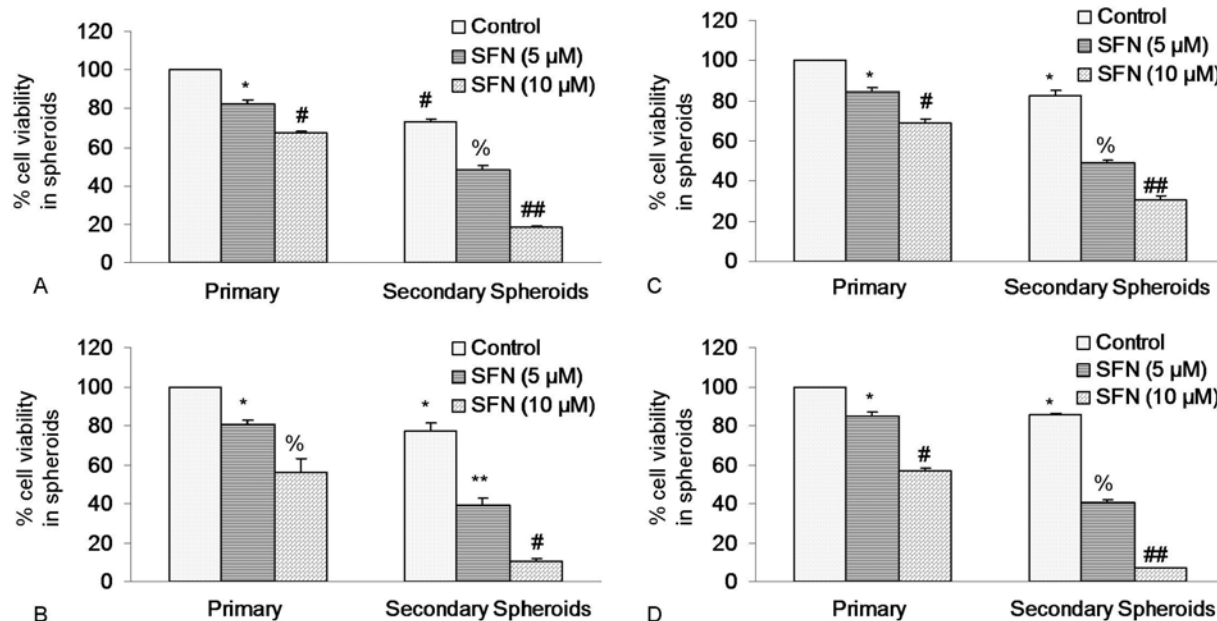


Figure 1. Effects of SFN on spheroid cell viability in cancer stem cells (CSCs) derived from human pancreatic cancer cell lines. (A), Pancreatic CSCs were isolated from MIA PaCa-2 cells, seeded in suspension and treated with SFN (0-10 μ M) for 7 days. At the end of incubation period, all the spheroids were collected and resuspended. Cell viability was measured by trypan blue assay. Data represent mean \pm SD. *, #, % or ## = significantly different from control, $P < 0.05$. (B), Pancreatic cancer stem cells were isolated from PANC-1 cells, seeded in suspension and treated with SFN (0-10 μ M) for 7 days. At the end of incubation period, all the spheroids were collected and resuspended. Cell viability was measured by trypan blue assay. Data represent mean \pm SD. *, #, % or ** = significantly different from control, $P < 0.05$. (C), Pancreatic cancer stem cells were isolated from AsPC-1 cells. CSCs were seeded in suspension and treated with SFN (0-10 μ M) for 7 days. At the end of incubation period, all the spheroids were collected and resuspended. Cell viability was measured by trypan blue assay. Data represent mean \pm SD. *, %, # or ## = significantly different from control, $P < 0.05$. (D), Pancreatic cancer stem cells were isolated from Bx PC-3 cells. CSCs were seeded in suspension and treated with SFN (0-10 μ M) for 7 days. At the end of incubation period, all the spheroids were collected and resuspended. Cell viability was measured by trypan blue assay. Data represent mean \pm SD. *, #, % or ## = significantly different from control, $P < 0.05$.

stomatitis virus (VSV) G protein, were produced in 293T cells as described (82-84). Viral supernatants were concentrated by ultracentrifugation to produce virus stocks with titers of 1×10^8 to 1×10^9 infectious units per milliliter. Titers were determined on 293 T cells. Human pancreatic cancer cells were transduced with viral particles with two rounds of infections.

3.6. Western blot analysis

Western blots were performed as we described earlier (38, 85). In brief, cells were lysed in RIPA buffer containing 1 X protease inhibitor cocktail, and protein concentrations were determined using the Bradford assay (Bio-Rad, Philadelphia, PA). Proteins were separated by 12.5% SDS/PAGE and transferred to membranes (Millipore, Bedford, MA) at 55 V for 4 h at 4°C. After blocking in 5% nonfat dry milk in TBS, the membranes were incubated with primary antibodies at 1:1,000 dilution in TBS overnight at 4°C, washed three times with TBS-Tween 20, and then incubated with secondary antibodies conjugated with horseradish peroxidase at 1:5,000 dilution in TBS for 1 hour at room temperature. Membranes were washed again in TBS-Tween 20 for three times at room

temperature. Protein bands were visualized on X-ray film using an enhanced chemiluminescence detection system.

3.7. Statistical analysis

The mean and SD were calculated for each experimental group. Differences between groups were analyzed by one or two way ANOVA, followed by Bonferroni's multiple comparison tests using PRISM statistical analysis software (GraphPad Software, Inc., San Diego, CA). Significant differences among groups were calculated at $P < 0.05$.

4. RESULTS

4.1. Sulforaphane inhibits the growth of pancreatic cancer stem cells isolated from human pancreatic cancer cell lines

Since CSCs has been successfully isolated from established human cancer cells lines, we examined the effects of SFN on cancer stem cells (CD44⁺CD24⁺ESA⁺) isolated from human pancreatic cancer cell lines (Figure 1). Isolated CSCs were grown in pancreatic cancer stem cell medium in suspension (as described in Materials and Methods) and treated with various doses SFN (0-10 μ M)

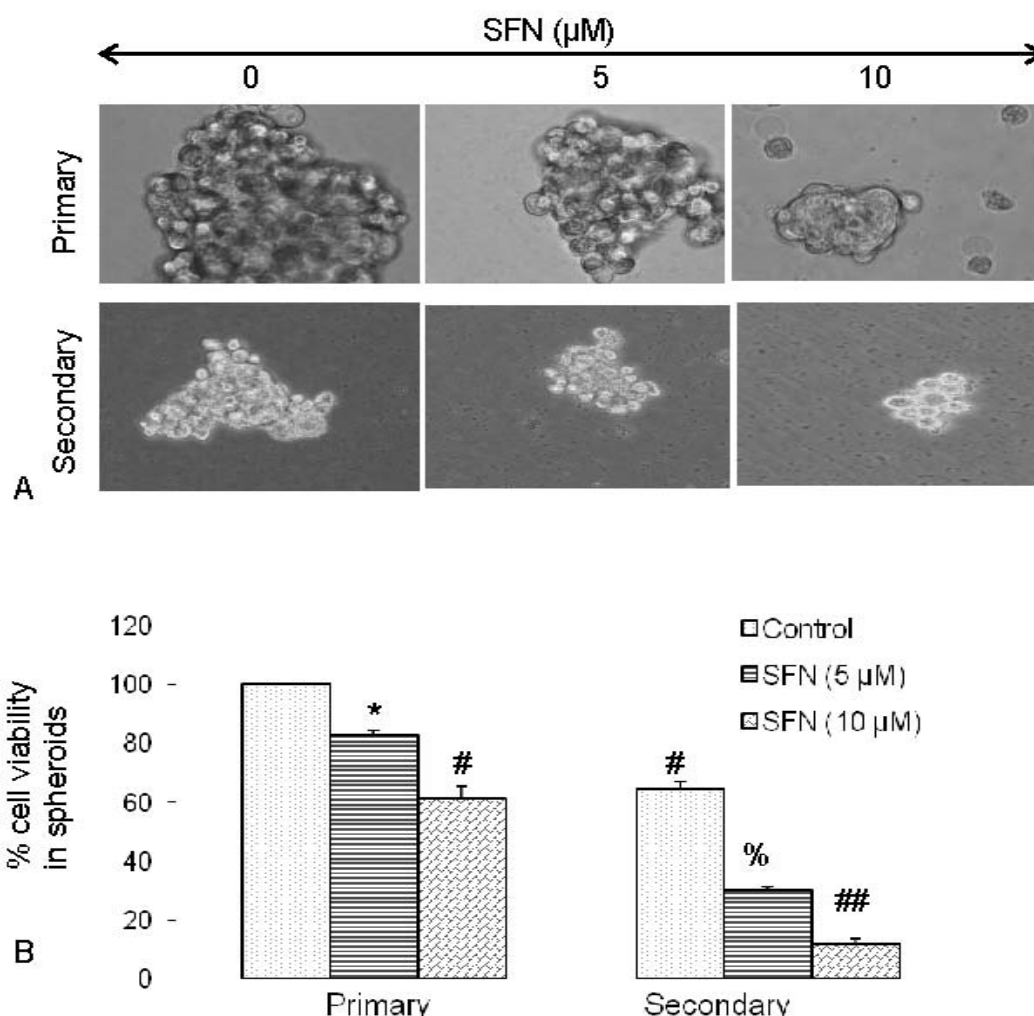


Figure 2. Effects of SFN on tumor spheroids and cell viability of pancreatic cancer stem cells (CSCs). (A), Pancreatic CSCs were seeded in suspension and treated with SFN (0-10 μ M) for 7 days. Pictures of spheroids formed in suspension were taken by a microscope. (B), Pancreatic CSCs were seeded in suspension and treated with SFN (0-10 μ M) for 7 days. At the end of incubation period, all the spheroids were collected and resuspended. Cell viability was measured by trypan blue assay. Data represent mean \pm SD. *, #, % or ## = significantly different from control, $P < 0.05$.

for 7 days. At the end of incubation period, spheroids were harvested, resuspended, and cell viability was measured. SFN inhibited cell viability of pancreatic CSCs isolated from MIA-PACA-2, PANC-1, AsPC-1 and Bx-PC-3 cell lines in a dose-dependent manner. These data suggest that human pancreatic cancer cell lines possess a small population of CSCs which are responsive to SFN treatment.

4.2. Sulforaphane inhibits the formation of primary and secondary tumor spheroids and cell viability of pancreatic cancer stem cells

Since SFN inhibited the growth of CSCs isolated from established pancreatic cancer cell lines, we sought to examine whether SFN could also inhibit the growth of CSCs isolated from human primary pancreatic tumors. We first examined the effects of SFN on the CSC growth by measuring spheroids formation and cell viability. CSCs were grown in pancreatic cancer stem cell defined medium

in suspension, and treated with SFN for 7 days. At the end of incubation period, spheroids in each well were photographed. SFN inhibited the growth of spheroids in suspension in a dose dependent manner (Figure 2A). The spheroids from each treatment group were collected and resuspended for counting cell viability. SFN inhibited stem cell viability of CSCs in a dose-dependent manner (Figure 2B). These data suggest that SFN can be effective in inhibiting the growth of pancreatic cancer stem cells.

4.3. Sulforaphane inhibits the growth of colonies formed by pancreatic cancer stem cells

Since SFN inhibited the growth of tumor spheroid and cell viability of CSCs, we sought to examine the effects of SFN on colony formation. Pancreatic CSCs were grown in agar, and treated with various doses of SFN for 3 weeks. At the end of incubation period, number of colonies were counted. SFN inhibited the growth of colonies in a

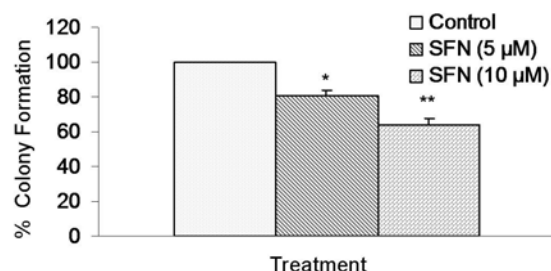


Figure 3. SFN inhibits colony formation by pancreatic CSCs. Pancreatic CSCs were seeded in soft agar and treated with various doses of SFN and incubated at 4°C for 21 days. At the end of incubation period, colonies were counted. Data represent mean \pm SD. * or ** = significantly different from respective controls, $P < 0.05$.

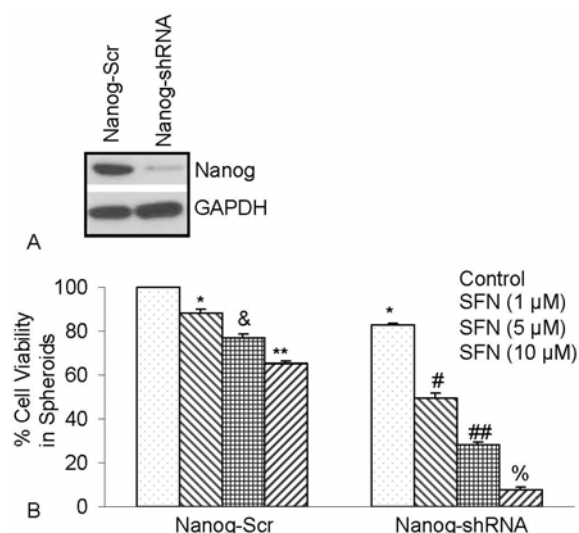


Figure 4. Inhibition of Nanog by shRNA enhances the antiproliferative effects of SFN. Isolated pancreatic CSCs were transduced with either Nanog scrambled or Nanog shRNA. Transduced cells were treated with various doses of SFN and maintained in pancreatic cancer stem cell medium for 7 days. At the end of incubation period, all the spheroids were collected and resuspended. Cell viability was measured by trypan blue assay. Data represent mean \pm SD. *, &, #, %, ## or ** = significantly different from control, $P < 0.05$.

dose-dependent manner (Figure 3). These data suggest that SFN can be an useful agent in targeting pancreatic cancer stem cells.

4.4. Inhibition of Nanog enhances the effects of sulforaphane on spheroid formation by human pancreatic cancer stem cells

Since pluripotent transcription factor Nanog is highly expressed in cancer stem cells compared to normal cells, we examined the effects of inhibiting Nanog on antiproliferative effects of SFN in human pancreatic CSCs expressing CD44⁺/CD24⁺/ESA⁺. Lentiviral mediated transduction of Nanog ShRNA inhibited Nanog protein

expression (Figure 4). SFN inhibited stem cell viability in CSC spheroids transduced with Nanog-scrambled shRNA in a dose-dependent manner. The inhibition of Nanog by shRNA further enhanced the antiproliferative effects of SFN on CSCs. These data suggest that inhibition of Nanog may be an attractive target to enhance the anticancer activities of SFN in CSCs.

4.5. Sulforaphane inhibits the expression of XIAP and Bcl-2 and phosphorylation of FKHR, and cleaves caspase-3 in human pancreatic cancer stem cells

We next examined the effects of SFN on the expression of XIAP and Bcl-2, phosphorylation of FKHR, cleavage of caspase-3, and apoptosis (Figure 5). SFN inhibited the expression of XIAP, Bcl-2 and cleaved pro-caspase-3 in pancreatic CSCs (Figure 5A). SFN also inhibited the phosphorylation of FKHR suggesting the inhibition of PI3K/AKT pathway leading to activation of FOXO transcription factor. SFN induced apoptosis in pancreatic CSCs in a dose-dependent manner as measured by TUNEL assay (Figure 5B). These data suggest that SFN can induce apoptosis in CSCs by engaging mitochondria because Bcl-2 mainly exerts its effects at the level of mitochondria. The inhibition of XIAP by SFN will further release caspases to induce apoptosis in CSCs.

4.6. Sulforaphane inhibits the expression of epithelial-mesenchymal transition markers (EMT) in human pancreatic cancer stem cells

Cancer stem cells have been shown to express EMT markers. FOXO proteins are mainly regulated through phosphorylation by upstream kinase AKT and ERK (32, 43). We therefore examined the regulation of EMT markers by SFN. As expected, SFN inhibited the expression of β -catenin, vimentin, Twist-1 and Zeb-1 (Figure 6). These data suggest that inhibition of EMT markers by SFN could inhibit early metastasis of cancer stem cells.

4.7. Quercetin enhances the effects of sulforaphane on spheroid and colony formation by pancreatic cancer stem cells

Quercetin has been shown to enhance the effects of anticancer drugs and sensitize cancer cells to chemotherapy and radiotherapy. We therefore examined whether quercetin enhances the effects of SFN on spheroid and colony formation by pancreatic CSCs (Figure 7). SFN inhibited the cell viability and colony formation of pancreatic CSCs in a dose-dependent manner. Quercetin, although effective alone, further enhanced the biological effects of SFN on cell viability (in spheroids) and colony formation. These data suggest that quercetin can be used with SFN to selectively target pancreatic CSCs.

5. DISCUSSION

Our study demonstrates, for the first time, that cancer preventive effects of SFN are regulated through activation of FOXO transcription factor FKHR and inhibition of stem-cell pluripotent transcription factor Nanog. Specifically, we have demonstrated that (i) SFN inhibits the expression of EMT markers (vimentin, β

Regulation of pancreatic cancer stem cells

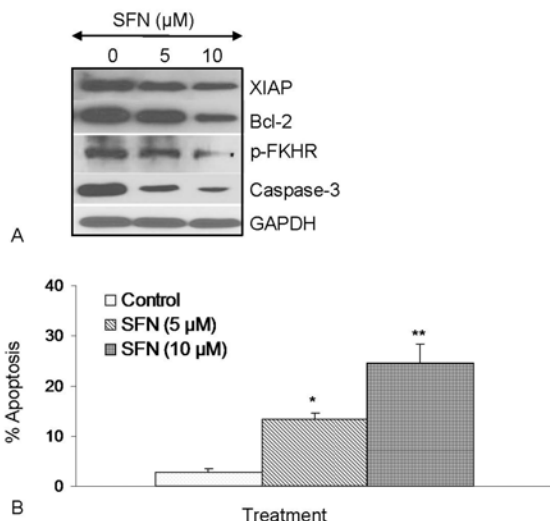


Figure 5. Regulation of apoptosis-related proteins and apoptosis by SFN in pancreatic cancer stem cells. (A), Regulation of apoptosis-related proteins by SFN. Pancreatic CSCs were treated with SFN (0-10 μ M) for 48 h. The Western blot analyses were performed to examine the expression of XIAP, Bcl-2, total caspase-3, phospho-FKHR and GAPDH. (B), Regulation of apoptosis by SFN. Pancreatic CSCs were treated with SFN (0-10 μ M) for 48 h, and apoptosis was measured by TUNEL assay.

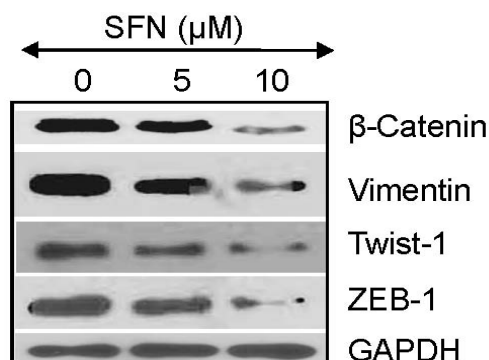


Figure 6. Regulation of epithelial mesenchymal transition factors by SFN in pancreatic cancer stem cells. Pancreatic CSCs were treated with SFN (0-10 μ M) for 48 h. At the end of incubation period, the expression of β -catenin, vimentin, Twist-1 and Zeb-1 was measured by the Western blot analysis.

catenin, twist-1, and Zeb-1), (ii) SFN induces the activation of FOXO transcription factor by inhibiting the phosphorylation of FKHR, (iii) SFN induces apoptosis by inhibiting Bcl-2 and XIAP expression, and activating caspase-3; and (iv) SFN inhibits self-renewal capacity of pancreatic CSC and synergizes with quercetin. Furthermore, we have convincingly demonstrated that inhibition of Nanog may be an attractive target to enhance the anticancer activities of SFN. Our data are in agreement

with others who demonstrated the anticancer activity of SFN in pancreatic cancer stem cells (33, 34, 36, 86).

SFN inhibits the factors required for maintaining the pluripotency in CSCs. Nanog, Oct-4 and Sox-2 co-occupy and regulate their own promoters together with other developmental genes with diverse functions and collaborate to form an extensive regulatory circuitry including autoregulatory and feed-forward loops (87-89). A high level of Nanog is a key regulator of embryonic stem cell (ESC) self-renewal and pluripotency. Nanog-deficient ES cells and embryos lose their pluripotency (90). Nanog overexpression leads to the clonal expansion of ES cells through circumvention of the LIF-dependent Stat-3 pathway and sustained Oct-4 expression levels (90, 91). Genome-wide gene expression profiling shows that Nanog is expressed at high levels in testicular carcinoma *in situ* and germ cell tumors (92). In the present study, the inhibition of Nanog attenuated the self-renewal capacity of pancreatic cancer stem cells, and enhanced the antiproliferative effects of SFN. These data suggest that inhibition of Nanog expression could be a novel strategy to kill CSCs.

Epithelial-to-mesenchymal transition (EMT) is an embryonic program in which epithelial cells lose their characteristics and gain mesenchymal features. Therefore, EMT might play a very important role during malignant tumor progression. Accumulating evidence suggest that transformed epithelial cells can activate embryonic programs of epithelial plasticity and switch from a sessile, epithelial phenotype to a motile, mesenchymal phenotype. Induction of EMT can, therefore, lead to invasion of surrounding stroma, intravasation, dissemination and colonization of distant sites. Under the cancer stem cell hypothesis, sustained metastatic growth requires the dissemination of a CSC from the primary tumor followed by its re-establishment in a secondary site. The EMT, a differentiation process crucial to normal development, has been implicated in conferring metastatic ability on carcinomas. In the present study, sulforaphane inhibited the expression of EMT markers and also inhibited the transcription factors which are required for induction of EMT.

The combinations of chemopreventive agents have been shown to exert synergistic effects on cancer cell growth. In our study, quercetin acted with SFN in a synergistic manner to inhibit the self-renewal capacity of pancreatic cancer stem cells. In a recent report, quercetin inhibited growth of cancer stem cell-enriched xenografts associated with reduced proliferation, angiogenesis, cancer stem cell-marker expression and induction of apoptosis (93). Furthermore, co-incubation with SFN increased these effects and no pronounced toxicity on normal cells or mice was observed. Since carcinogenesis is a complex process, combination of bioactive dietary agents with complementary activities will be beneficial for pancreatic cancer treatment and prevention.

Flavonols are a class of flavonoids, polyphenols, which are ubiquitous in plant foods and are known

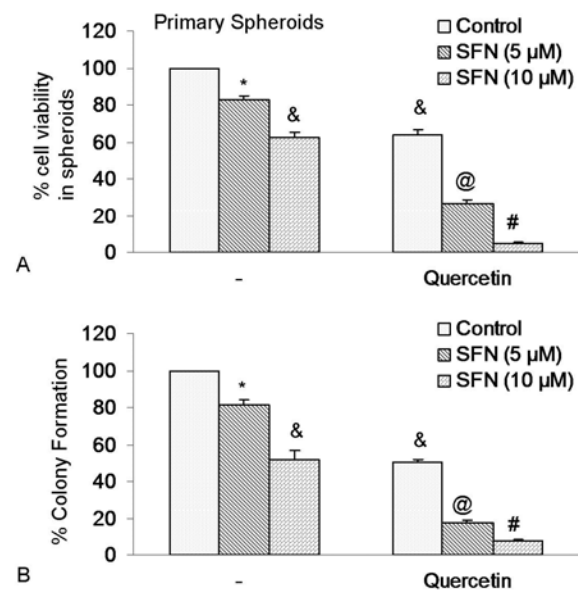


Figure 7. Quercetin synergizes with SFN to inhibit self-renewal capacity of pancreatic cancer CSCs. (A), Quercetin synergizes with SFN to inhibit spheroid cell viability. Pancreatic CSCs were seeded in suspension and treated with SFN (0-10 μ M) with or without quercetin (20 μ M) for 7 days. At the end of incubation period, all the spheroids were collected and resuspended. Cell viability was measured by trypan blue assay. Data represent mean \pm SD. *, &, @ or # = significantly different from control, $P < 0.05$. (B), Quercetin synergizes with SFN to inhibit colony formation. SFN inhibits colony formation by pancreatic CSCs. Pancreatic CSCs were seeded in soft agar and treated with various doses of SFN and incubated at 4°C for 21 days. At the end of incubation period, colonies were counted. Data represent mean \pm SD. *, &, @ or # = significantly different from respective controls, $P < 0.05$.

compounds of beer. Flavonol intake reduces the risk for developing pancreatic cancer (94-96). The pharmacokinetics of quercetin has been carried out both in animals and humans (97-99). Flavonoid glycosides are believed to pass through the small intestine, be hydrolyzed to aglycone by enterobacteria in the cecum and colon and absorbed into epithelial cells via lipophilicity-dependent simple diffusion (100). Quercetin glucosides can also be directly absorbed via the sodium-dependent glucose transporter-1 (SGLT-1) or excreted into the lumen via multidrug resistance protein 2 (MRP-2) (101). After their facilitated uptake by means of carrier-mediated transport, quercetin glycosides are often hydrolyzed by intracellular β -glucosidases (99). The intestinal lactase phlorizin hydrolase (LPH) displays a specific activity towards flavonoid glycosides (102). Hydrolysis to a glycone by enterocytes or enterobacteria is crucial for the efficient absorption of quercetin glucosides in the intestinal tract. Quercetin absorbed from the intestinal lumen is mostly converted to conjugated metabolites before entering circulation, and the major metabolites present in human plasma are quercetin 3'-O- β -d-glucuronide (Q3'GA) and

quercetin 4'-O- β -d-glucuronide (Q4'GA). Interestingly, some metabolites still possess considerable activity, including Q3GA, Q3'GA and Q4'GA (103). Furthermore, quercetin is concentrated in lungs, testes, kidneys, thymus, heart and liver, with the highest concentrations of quercetin and its methylated derivatives detected in the pulmonary tissue (104). Urinary elimination of quercetin is not the main excretion routes in human subjects or in rats. A substantial portion of the metabolites may be excreted in the bile (101). The low bioavailability of quercetin and high metabolite concentrations indicate an extensive first-pass metabolism in the gut and/or the liver (105, 106). Additionally, the high concentration of conjugated quercetin observed in the bile indicates potential enterohepatic recirculation (107). Following ingestion of quercetin (100 mg), a half-life range of 31–50 h was observed in humans, with peak plasma levels observed at 30 min and again at 8 h post-treatment (108). The half lives of the quercetin metabolites are 11 to 28 h, indicating that the metabolites could attain a considerable plasma level upon repeated quercetin supplementation (98, 109).

FOXO transcription factors play a crucial role in the regulation of tissue homeostasis in organs such as the pancreas and the ovaries and complex diseases such as diabetes and cancer (110-113). FOXO transcription factors are emerging as critical transcriptional integrators among pathways regulating differentiation, proliferation, survival, and angiogenesis (114-117). Gene expression profiling showed that FOXO1 and FOXO3a specifically regulate a nonredundant but overlapping set of angiogenesis- and vascular remodeling-related genes (117). We have recently demonstrated that inhibition of the MEK/ERK and PI3K/AKT pathways synergistically induced FOXO transcriptional activity and inhibited cancer cell growth and angiogenesis; these events were further enhanced in the presence of sulforaphane, resveratrol and EGCG (43, 118, 119). Phosphorylation deficient mutants of FOXO enhanced anti-angiogenic effects of sulforaphane, resveratrol and EGCG by activating the FOXO transcription factors. These studies suggest that activation of FOXO transcription factors by these dietary agents could be an important physiological process to inhibit tumor growth and angiogenesis. The ability of sulforaphane to inhibit the phosphorylation of FKHR suggests the activation of PI3K/AKT pathway in pancreatic cancer stem cells. Thus, FKHR may be a crucial molecular target for regulation of self-renewal capacity of cancer stem cells.

In conclusion, we have demonstrated that sulforaphane inhibited self-renewal capacity of pancreatic cancer stem cells, and these properties of SFN were enhanced with quercetin. SFN inhibited the expression of transcription factors which are required for maintaining stem-cell pluripotency. Inhibition of Nanog could be considered as a novel strategy to enhance the biological effects of anticancer and chemopreventive agents or sensitize those cells which are resistant to chemotherapy or irradiation. Moreover, sulforaphane inhibited expression of proteins involved in the epithelial-mesenchymal transition, suggesting the blockade of signaling involved in early metastasis. Furthermore, combination of quercetin with sulforaphane had synergistic effects on self-renewal

capacity of pancreatic cancer stem cells. These data suggest that sulforaphane either alone or in combination with quercetin can be used for the prevention and/or treatment of pancreatic cancer. However, further studies are needed to validate the combination of SFN and quercetin in an appropriate *in vivo* model.

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