



RESEARCH ARTICLE

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Inhibition of PI3K/AKT and MAPK/ERK pathways causes activation of FOXO transcription factor, leading to cell cycle arrest and apoptosis in pancreatic cancer

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Abstract

Background: Mammalian forkhead members of the class O (FOXO) transcription factors, including FOXO1, FOXO3a, and FOXO4, are implicated in the regulation of several biological processes, including the stress resistance, metabolism, cell cycle, apoptosis and DNA repair. The objectives of this study were to examine the molecular mechanisms by which FOXO transcription factors induced cell cycle arrest and apoptosis and enhanced anti-proliferative effects of sulforaphane (SFN, an active compound in cruciferous vegetables) in pancreatic cancer cells.

Results: Our data demonstrated that SFN inhibited cell proliferation and colony formation, and induced apoptosis through caspase-3 activation in pancreatic cancer cells. The inhibition of PI3K/AKT and MEK/ERK pathways activated FOXO transcription factors. SFN inhibited phosphorylation of AKT and ERK, and activated FOXO transcription factors, leading to cell cycle arrest and apoptosis. Phosphorylation deficient mutants of FOXO proteins enhanced FOXO transcriptional activity, and further enhanced SFN-induced FOXO activity and apoptosis. SFN induced the expression of p21^{CIP1} and p27^{KIP1}, and inhibited the expression of cyclin D1.

Conclusion: These data suggest that inhibition of PI3K/AKT and ERK pathways acts together to activate FOXO transcription factor and enhances SFN-induced FOXO transcriptional activity, leading to cell cycle arrest and apoptosis.

Background

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.

Sulforaphane (SFN), a constituent of cruciferous vegetables, is a naturally occurring isothiocyanate with promising chemopreventive activity [4]. Epidemiological studies have shown that people who eat cruciferous vegetables have reduced incidence of breast and prostate cancer. SFN possesses anti-oxidant, anti-proliferative and anti-carcinogenic properties [5-7]. SFN is effective in preventing chemically induced breast [8,9], stomach [5] and colon [10] cancers in rats. We and others have shown that SFN inhibited the growth of prostate, breast, oral and squamous carcinoma xenografts [11-15]. SFN enhanced radiosensitivity of tumor cells *in vitro* and *in vivo* [16]. Furthermore, a pharmacokinetic study has demonstrated that it is rapidly absorbed and 82% bioavailable [17]. SFN induces a phase 2 enzyme, thereby neutralizing carcinogens before they

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can damage DNA [18,19]. SFN inhibits benzo[a]pyrene-DNA and 1,6-dinitropyrene-DNA adducts formation [20-23], and downregulates PI3K/AKT [24,25] and NF κ B [12,26,27] pathways. 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 [12]. *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 [12]. These studies strongly suggest that SFN can be developed as a cancer preventive agent.

PTEN (phosphatase and tensin homolog deleted on chromosome 10, also called MMAC1 or TEP1) is a tumor suppressor gene [28-30], which is frequently deleted or mutated in a wide range of human cancers, including glioblastoma [31], melanoma [32], and prostate [33], breast [34], and endometrial cancers [35]. While point mutations in PTEN rarely occur in pancreatic cancer [36,37], functional inactivation of PTEN through promoter methylation [38], loss of protein expression [39], reduction of mRNA levels [40], or loss of heterozygosity (LOH) of linked markers [37,41] occur with high frequency. Phosphatidylinositol 3,4,5-trisphosphate (PIP₃) is a substrate of PTEN [42-44]. AKT is a serine-threonine protein kinase regulated by PIP₃ that is implicated in survival signaling in a wide variety of cells, including fibroblastic, epithelial, and neuronal cells [45]. PTEN increases sensitivity to cell death in response to several apoptotic stimuli by negatively regulating the PI3K/AKT pathway [43]. In addition to its role in regulating the PI3K/AKT cell survival pathway, PTEN also inhibits growth factor-induced Shc phosphorylation and suppresses the MAP kinase signaling pathway [46], suggesting that PTEN has roles independent of PI3K/AKT signaling pathway. Hyperactivation of AKT is associated with resistance to apoptosis, increased cell growth, cell proliferation, metastasis, angiogenesis, and cellular energy metabolism [45,47-54]. Overexpression of AKT has been reported in a variety of human cancers, including pancreatic cancer, and cells expressing elevated levels of AKT are less sensitive to apoptosis stimuli [38,55-57]. Antagonizing PI3K activity negatively regulates AKT activity. Once activated, however, AKT exerts antiapoptotic effects through phosphorylation of substrates such as Bad [58,59] and caspase-9 [60] that directly regulate the apoptotic machinery, or human telomerase reverse transcriptase subunit [61], forkhead transcription family members [62,63] and IB kinases [64] that indirectly inhibit apoptosis [65]. Studies in pancreatic cancer cell lines have demonstrated that PI3K is required for growth and survival of tumor cells [66-68]. Furthermore, amplification or activation of

AKT2 occurs in up to 60% of pancreatic cancer [39,69-71], supporting the participation of an activated PI3K-AKT axis in this disease.

FOXO subfamily of forkhead transcription factors include FOXO1a/FKHR, FOXO3a/FKHRL1, and FOXO4/AFX [72-75]. The PI3K pathway, via activation of its downstream kinase AKT, phosphorylates each of the FOXO proteins [62,76,77]. These phosphorylations result in impairment of DNA binding ability and increased binding affinity for the 14-3-3 protein [62,77]. Newly formed 14-3-3-FOXO complexes are then exported from the nucleus [78], thereby inhibiting FOXO-dependent transcription. Inhibition of the PI3K pathway leads to dephosphorylation and nuclear translocation of active FKHRL1, FKHR, and AFX; which induce cells cycle arrest and apoptosis [79]. 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 [80,81]. FOXO regulates cell cycle and apoptotic genes such as cyclin-dependent kinase inhibitor (CKI) p27^{KIP1} [78,80,82,83], Bim [84,85], Fas ligand [62], and Bcl-6 [86]. Consequently, activation of the PI3K pathway serves to repress FOXO-mediated growth arrest and apoptosis. However, regulation of FOXO target genes is multifactorial, and therefore other transcription factors and post-translation regulatory events will influence the final level of protein expression. Interestingly, overexpression of AKT, and inactivation and loss of PTEN are frequently observed in pancreatic cancer [39,66-71], indicating a potential role for FOXOs in modulating both cell cycle and apoptosis during tumorigenesis and treatment. Together, these results indicate that FOXO proteins are important downstream effectors of PTEN tumor suppressive activity; however, their molecular targets and mechanisms of action in pancreatic cancer are not well understood.

The Ras proteins are small (21 kDa) GTP-binding, membrane-associated proteins [87]. The Ras proteins transduce signals from ligand-activated tyrosine kinase receptors to downstream effectors [88]. Activating mutations can impair GTP hydrolysis and lead to constitutively activated Ras that impacts the cellular phenotype [89]. Oncogenic Ras can lead to cellular transformation [90], presumably by perturbing its signal transduction pathways. Ras regulates multiple signaling pathways [91]. Three major groups of MAP kinases are found in mammalian cells: extracellular signal-regulated protein kinase (ERK) [92], p38 MAP kinase [93], and c-Jun N-terminal kinase (JNK) [94-96]. MAP kinases regulate many cellular activities, which range from gene expression to mitosis, movement, metabolism, and apoptosis.

These MAP kinases are activated by the dual phosphorylations of neighboring threonine and tyrosine residues in response to various extracellular stimuli [97,98]. Specifically, p38 and JNK have been implicated in stress-responsive signaling leading to the initiation of adaptive events such as gene expression, differentiation, metabolism, and apoptosis [94,95,99]. ERKs are often activated by growth signals, such as epidermal growth factor (EGF) or platelet-derived growth factor [100]. We have recently demonstrated that inhibition of PI3K/AKT and MEK/ERK pathways act synergistically to regulate anti-angiogenic effects of EGCG and SFN through activation of FOXO transcription factors [24,101].

Furthermore, FOXO transcription factors play a crucial role in the regulation of tissue homeostasis in organs such as the pancreas, and complex diseases such as diabetes and cancer. Unfortunately, the intracellular mechanisms by which SFN inhibits growth and induces apoptosis in pancreatic cancer cells through regulation of FOXO transcription factors have never been examined. The objectives of our study were to examine the molecular mechanisms by which FOXO transcription factors induce cell cycle arrest and apoptosis and enhances the anti-proliferative effects of SFN in pancreatic cancer cells. Our results demonstrate that inhibition of PI3K/AKT and ERK pathways activates FOXO transcription factors. SFN inhibited phosphorylation of AKT and ERK, and dephosphorylated FOXO transcription factors, leading to cell cycle arrest and apoptosis. Phosphorylation deficient mutants of FOXO proteins enhanced FOXO transcriptional activity, and further enhanced SFN-induced FOXO activity.

Results

Sulforaphane (SFN) inhibits cell growth in human pancreatic cancer cells

We first examined the effects of SFN on cell proliferation in four pancreatic cancer cell lines by XTT assay. We have selected four pancreatic cancer cell lines (MIA PaCa-2, AsPC-1, PANC-1 and Hs766T) because they have been derived from different pathological stages and may thus respond differently to SFN [102,103]. MIA PaCa-2 harbors a point mutation on Kras gene resulting in amino acid substitution from the wild-type glycine to a valine at codon 12. AsPC-1 and PANC-1 harbor a point mutation on Kras gene resulting in amino acid substitution from glycine to aspartate. Hs766T cell line does not possess a point mutation in codon 12 of the Kras gene. SFN inhibited cell viability in a dose dependent manner (Fig. 1). PANC-1 and MIA PaCa-2 cell lines were most sensitive, AsPC-1 cell line was moderately sensitive, and Hs 766T cell line was least sensitive. These data suggest that SFN can be a viable agent for inhibiting pancreatic cancer cell proliferation.

Sulforaphane inhibits colony formation in human pancreatic cancer cells

We next examined the effects of SFN on colony formation (a characteristic of cancer) on four pancreatic cancer cell lines by soft agar assay. SFN inhibited colony formation in a dose dependent manner (Fig. 2). Colonies formed by PANC-1 and MIA PaCa-2 cells were most sensitive, AsPC-1 cell line was moderately sensitive, and Hs 766T cell line was least sensitive. These data suggest that SFN can be used as a potent chemopreventive agent for pancreatic cancer.

Sulforaphane induces caspase-3 activation in human pancreatic cancer cell

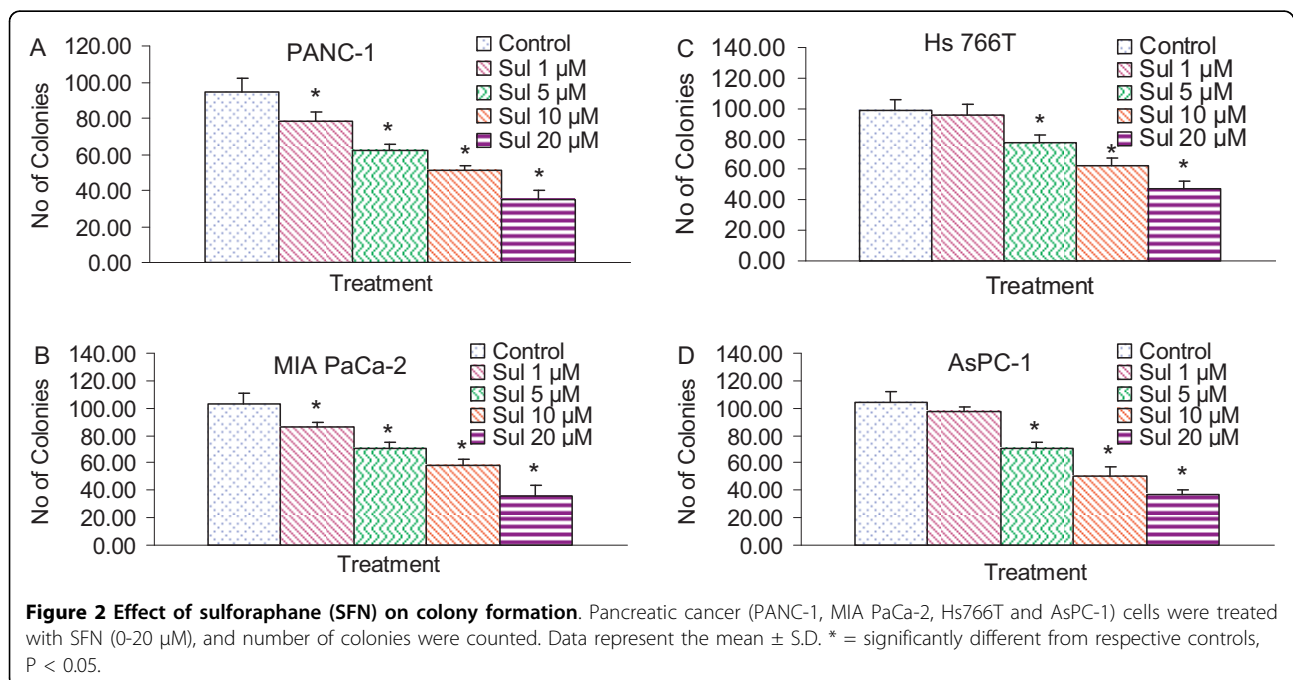
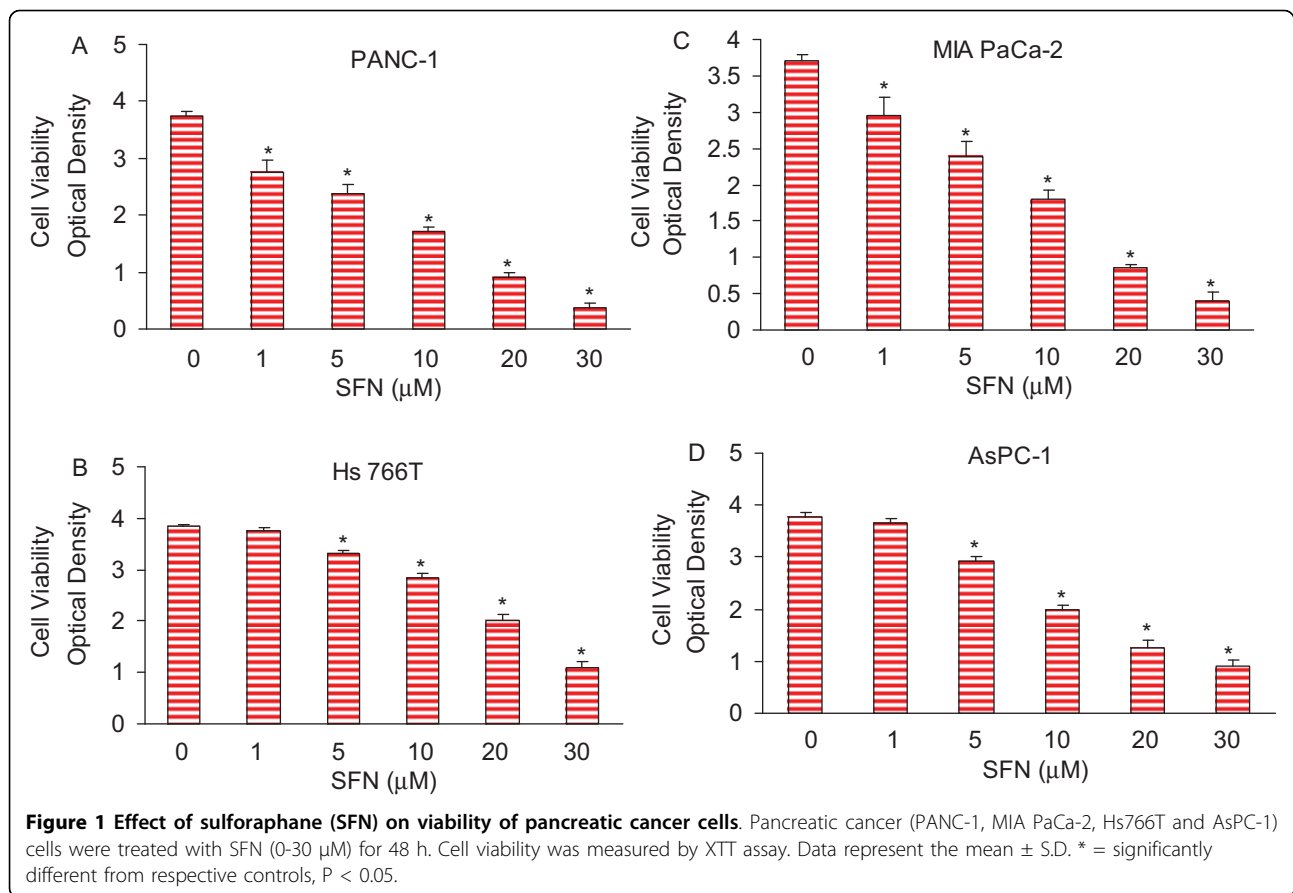
Most chemopreventive agents induce apoptosis through mitochondrial pathway, which activates caspase-3 [104]. We therefore examined whether SFN-induced apoptosis through caspase-3 activation in pancreatic cancer cell lines (Fig. 3). SFN induced caspase-3 activity in PANC-1, MIA PaCa-2, Hs 766T and AsPC-1 cells. However, a relatively high dose of SFN was required to activate caspase-3 in Hs 766T cells compared to other pancreatic cancer cell lines. These data suggest that SFN induced apoptosis through caspase-3 activation and may engage the mitochondria.

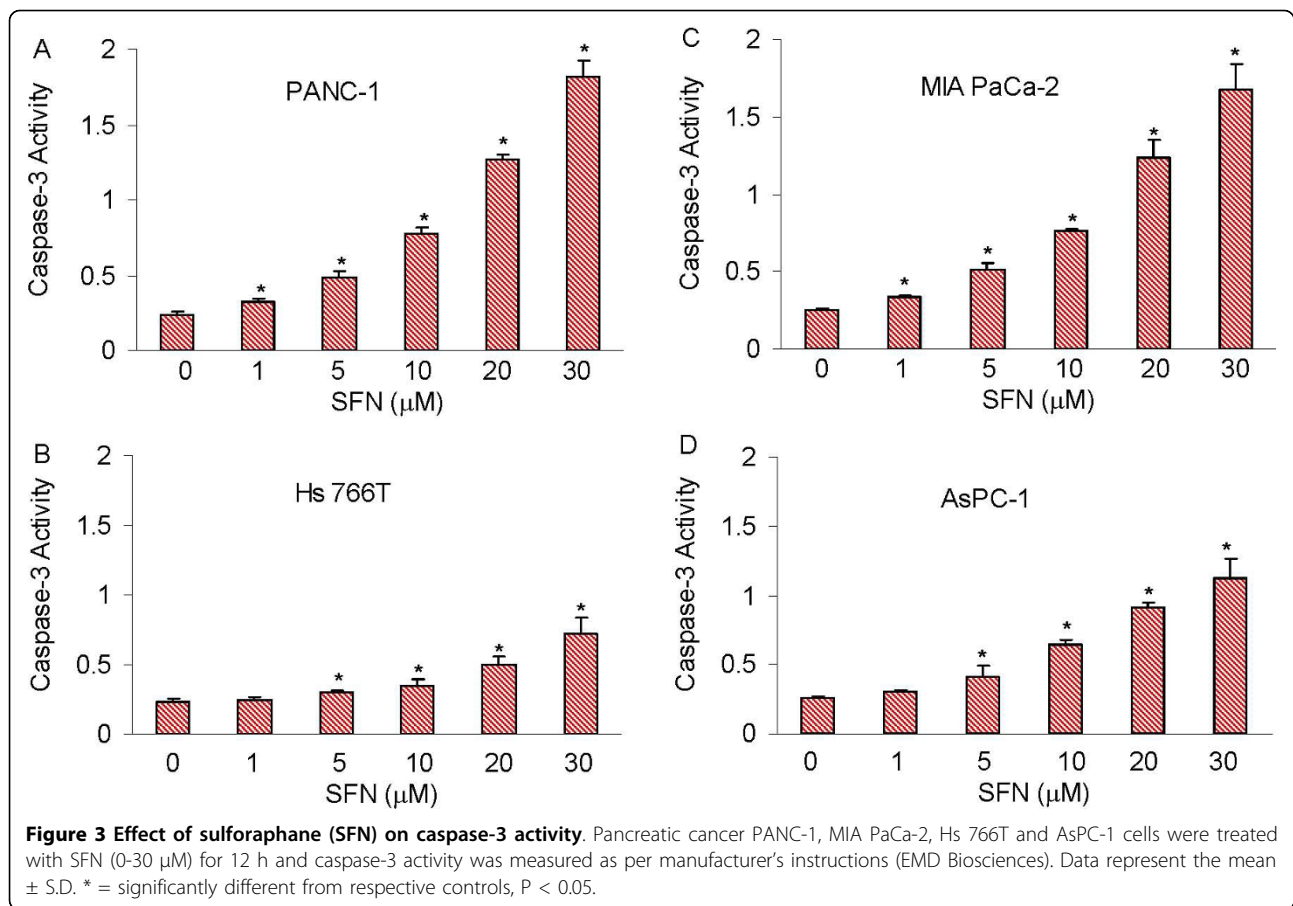
Regulation and function of PI3K/AKT and MAP kinase pathways by sulforaphane

In most cancer cells, AKT is constitutively active and enhances cell proliferation [105]. In order to understand a relationship between PTEN and AKT in SFN-induced apoptosis, we measured the expression of PTEN and phosphorylation status of AKT in cells treated with SFN (Fig. 4A). SFN induces PTEN expression and inhibits AKT phosphorylation in pancreatic cancer PANC-1 cells. By comparison, SFN has no effect on total AKT expression. These data suggest that SFN inhibits cell proliferation by regulating PI3K/AKT pathway.

Ras/Raf/MAP kinase pathway regulates many cellular activities, which range from gene expression to mitosis, movement, metabolism, and apoptosis [94,106-109]. We therefore examined the effects of SFN on the expression of Ras, and activation of ERK, JNK and p38 MAP kinases. SFN inhibited Ras expression in PANC-1 cells (Fig. 4A). Treatment of PANC-1 cells with SFN caused a decrease in ERK phosphorylation, and an increase in JNK phosphorylation. SFN has no significant effect on p38 MAP kinase activity in PANC-1 cells. These data suggest that SFN inhibits growth and induces apoptosis through regulation of Ras/Raf/MAP kinase pathway.

We next examined whether SFN induces apoptosis through PI3K/AKT pathway (Fig. 4B). Pancreatic cancer cells were transfected with empty vector, wild type PTEN, dominant negative AKT (DN-AKT), and apoptosis was measured. Overexpression of wild type PTEN or





DN-AKT induced apoptosis in AsPC-1 and PANC-1 cells. Treatment of transfected cells with SFN further enhanced apoptosis. These data suggest that inhibition of PI3K/AKT pathway enhances SFN-induced apoptosis in pancreatic cancer cells.

We next examined whether inhibition of MEK/ERK pathway enhances SFN-induced apoptosis in pancreatic cancer cells. MEK1/2 inhibitor (PD98059) induced apoptosis in PANC-1 and AsPC-1 cells (Fig. 4C). PD98059 enhanced SFN-induced apoptosis. Overall, these data suggest that inhibition of PI3K/AKT and MEK/ERK pathways enhanced SFN-induced apoptosis.

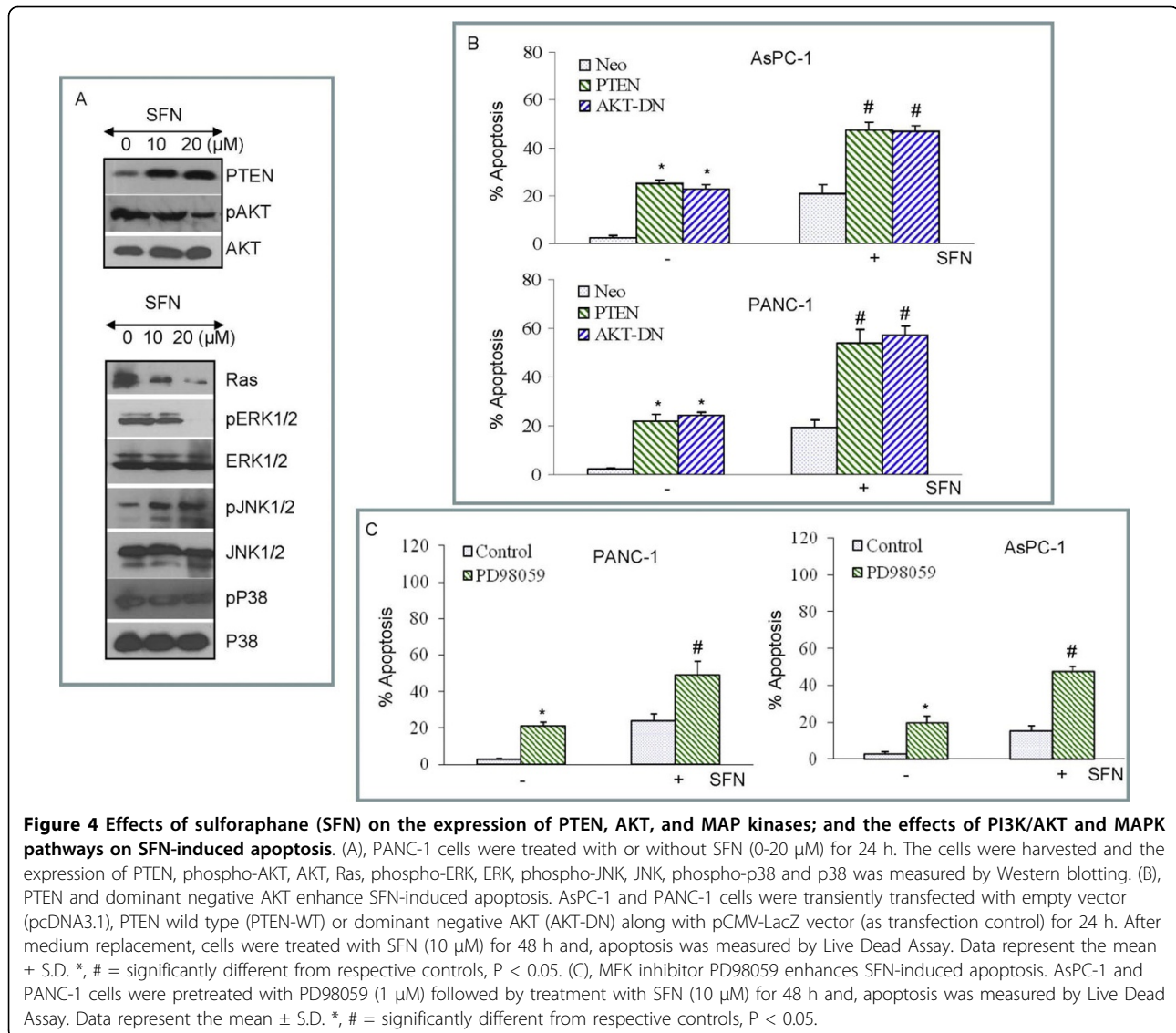
Sulforaphane induces p21^{WAF1/CIP1}, and p27^{KIP1} and inhibits cyclin D1

PI3K/AKT signaling pathway may be involved in the control of the cell cycle progression most likely through mechanisms involving the activation of FOXO transcription factors [82]. We next examined the effects of SFN on cell cycle regulatory genes. SFN induced the expression cell cycle inhibitors p21^{WAF1/CIP1} and p27^{KIP1}, and inhibited the expression of cyclin D1 in PANC-1 cells (Fig. 5). These data suggest that SFN causes growth arrest by regulating expression of cell cycle genes.

Overexpression of FOXO transcription factors inhibits cell viability and enhances FOXO transcriptional activity in pancreatic cancer cells

In order to examine whether FOXO transcription factors affect the ability of SFN to inhibit cell viability, pancreatic cancer cells were transfected with FOXO1, FOXO3a or FOXO4 (Fig. 6A and 6B). FOXO expression plasmids and FOXO-luciferase construct (pGL3-6X DBE) have previously been described [101]. Overexpression of FOXO1, FOXO3a, and FOXO4 inhibited cell viability in PANC-1 and AsPC-1 cells. The inhibitory effects of SFN on cell viability were further enhanced when pancreatic cancer cells were transfected with FOXO1, FOXO3a, and FOXO4. These data suggest that FOXO transcription factors can enhance the antiproliferative effects of SFN.

We next examined whether SFN induces transcriptional activation of FOXO in the presence or absence phosphorylation deficient triple mutants of FOXO proteins (FOXO1-TM, FOXO3a-TM, or FOXO4-TM). PANC-1 and AsPC-1 cells were transfected with wild type FOXO promoter linked to a luciferase reporter gene in the presence or absence of plasmids expressing FOXO1-TM, FOXO3a-TM, or FOXO4-TM (Fig. 6C



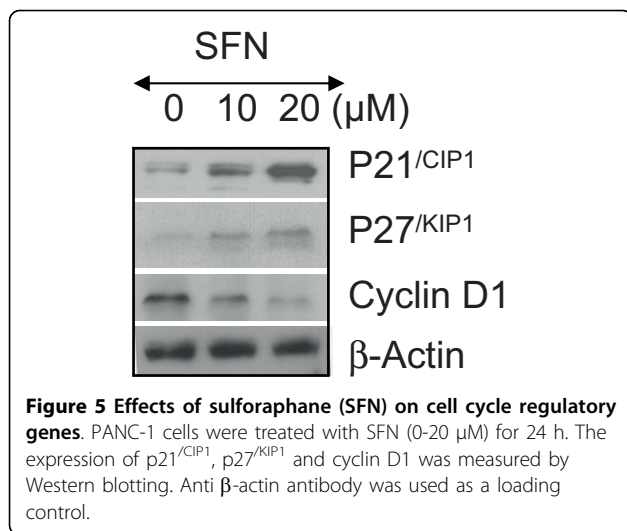
and 6D). After transfection, cells were treated with SFN for 24 h, and luciferase activity was measured. Transfection of cells with plasmids expressing FOXO1-TM, FOXO3a-TM, or FOXO4-TM induced FOXO transcriptional activity compared with the empty vector (control). SFN-induced FOXO transcriptional activity was further enhanced in the presence of FOXO1-TM, FOXO3a-TM, and FOXO4-TM. These data indicate that FOXO transcription factor may play a major role in mediating biological effects of SFN in pancreatic cancer cells.

Inhibition of PI3K/AKT and MEK/ERK pathways synergistically/additively induces FOXO transcriptional activity and apoptosis in the presence or absence of sulforaphane

Since inhibition of PI3K/AKT and MEK/ERK pathways induce apoptosis in pancreatic cancer cells, we sought to

examine whether these pathways act together to regulate SFN-induced apoptosis. AKT inhibitor (AKT Inh-IV) and MEK1/2 inhibitor (PD98059) synergistically/additively induced apoptosis in PANC-1 and AsPC-1 cells (Fig. 7A and 7B). AKT inhibitor and PD98059 alone enhanced SFN-induced apoptosis. Interestingly, the combination of AKT inhibitor and PD98059 with SFN induced more apoptosis than AKT inhibitor plus SFN or PD98059 plus SFN. These data suggest that inhibition of PI3K/AKT and MEK/ERK pathways act synergistically/additively to regulate apoptosis in the absence or presence of SFN.

Since inhibition of PI3K/AKT and MEK/ERK pathways synergistically/additively induces apoptosis in pancreatic cancer cells, we sought to examine whether inhibition of these two pathways act together to regulate FOXO activity. AKT inhibitor (AKT Inh-IV) and

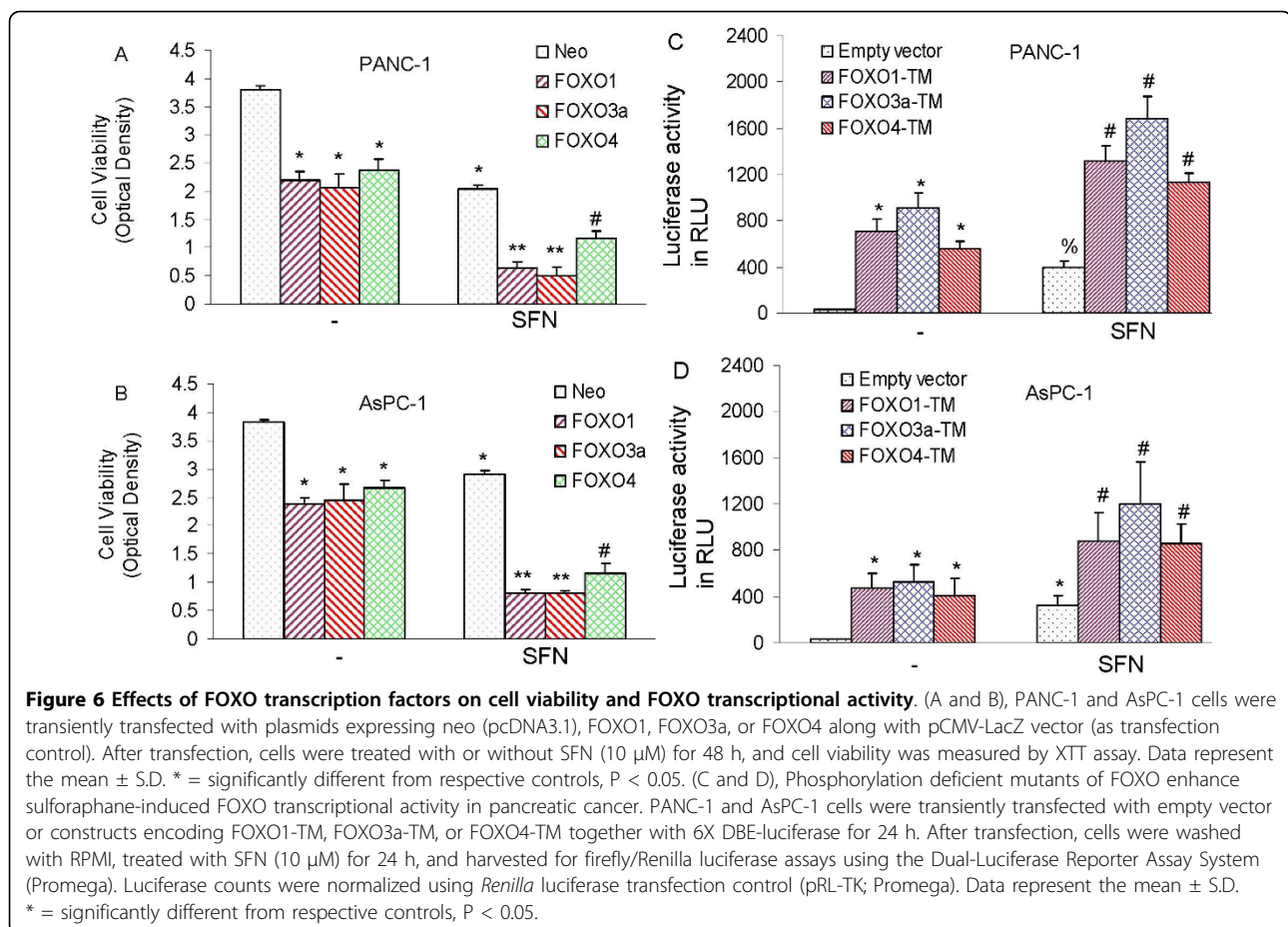


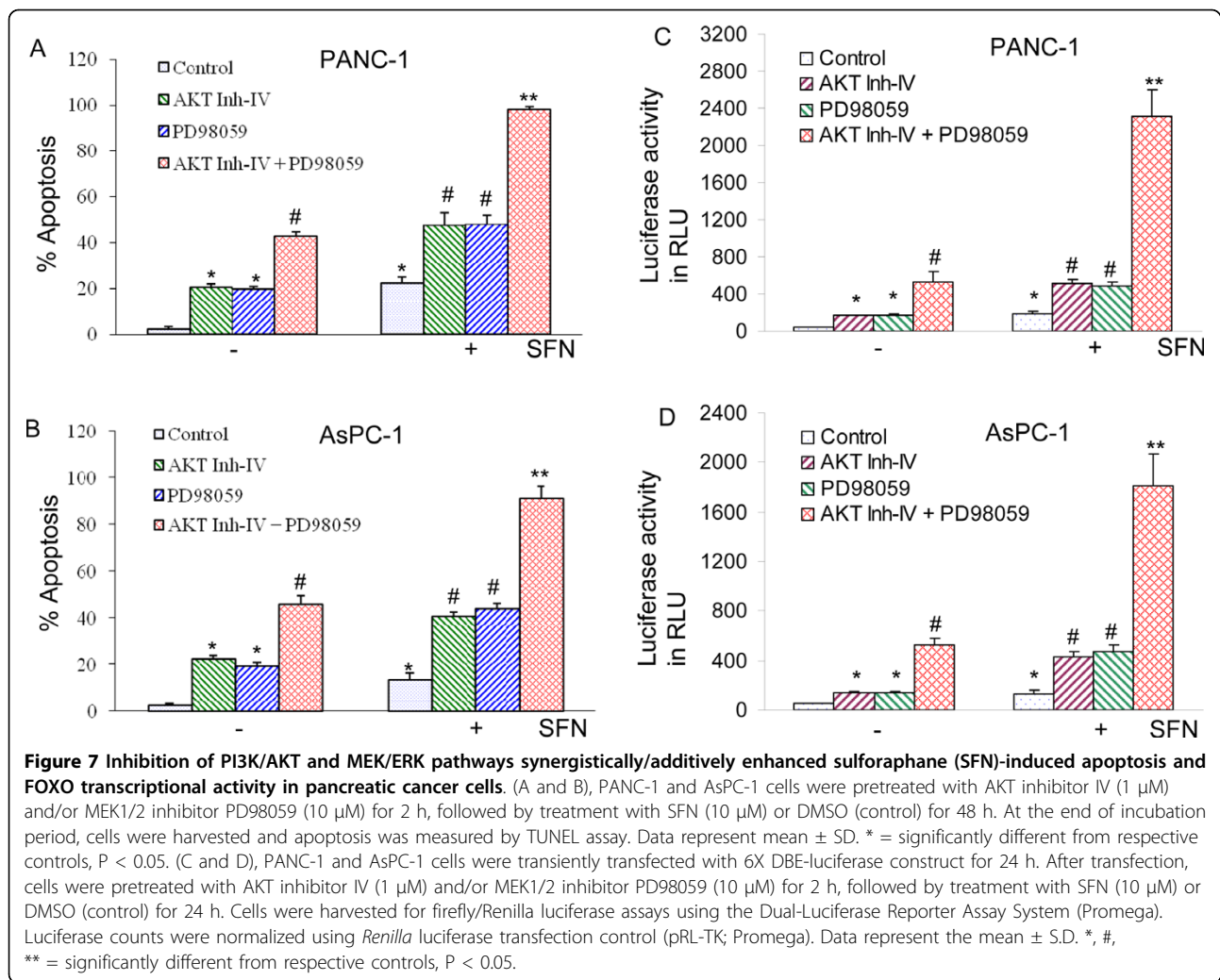
MEK1/2 inhibitor (PD98059) synergistically induced FOXO transcriptional activity in AsPC-1 and PANC-1 cells (Fig. 7C and 7D). AKT inhibitor or PD98059 enhanced SFN-induced FOXO transcriptional activity. Interestingly, the combination of AKT Inh-IV and

PD98059 with SFN induced greater FOXO transcriptional activity than AKT Inh-IV plus SFN or PD98059 plus SFN. These data suggest that inhibition of PI3K/AKT and MEK/ERK pathways acts synergistically/additively to regulate FOXO transcriptional activity in the absence or presence of SFN.

Discussion

Our study demonstrates, for the first time, that cancer preventive effects of SFN are regulated through activation of FOXO transcription factors. Specifically, we have demonstrated that (i) SFN induces apoptosis through caspase-3 activation, and causes growth arrest through induction of p21 and p27 and inhibition of cyclin D1; (ii) SFN induces apoptosis through inhibition of both PI3K/AKT and MEK/ERK pathways, and activation of FOXO transcription factors; (iii) inhibition of PI3K/AKT and MEK/ERK pathways acts together to enhance the activation of FOXO transcription factors; and (iv) phosphorylation deficient mutants of FOXO proteins further enhance SFN-induced FOXO activity and apoptosis. Our data are in agreement with others who demonstrated the anticancer activity of SFN in pancreatic cancer [110-112].





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 [113-117]. FOXO transcription factors are emerging as critical transcriptional integrators among pathways regulating differentiation, proliferation, survival, and angiogenesis [118-121]. FOXO transcription factors regulate angiogenesis and postnatal neovascularization by regulation angiopoietin 2 (Ang2) and eNOS [121]. Gene expression profiling showed that FOXO1 and FOXO3a specifically regulate a nonredundant but overlapping set of angiogenesis- and vascular remodeling-related genes [121]. The FOXO1-deficient mice died around embryonic day 11 because of defects in the branchial arches and remarkably impaired vascular development of embryos and yolk sacs [118]. We have recently demonstrated that inhibition of the MEK/ERK and PI3K/AKT pathways synergistically induced FOXO transcriptional activity and inhibited angiogenesis (cell migration and capillary tube formation); these

events were further enhanced in the presence of SFN [24]. Phosphorylation deficient mutants of FOXO enhanced antiangiogenic effects of SFN by activating the FOXO transcription factor. These studies suggest that activation of FOXO transcription factor by SFN could be an important physiological process to inhibit angiogenesis which may ultimately control tumor growth.

Activation of Kras has been shown to activate both PI3K/AKT and MAPK pathways [24,101,122-124]. Oxidative stress and activation of the JNK pathway induce the nucleocytoplasmic translocation of the pancreatic transcription factor Pdx-1, which leads to pancreatic β -cell dysfunction [125,126]. Furthermore, FOXO1/FKHR plays a role as a mediator between the JNK pathway and Pdx-1 [127]. Under oxidative stress conditions, FOXO1 changed its intracellular localization from the cytoplasm to the nucleus in the pancreatic β -cell line HIT-T15. The overexpression of JNK also induced the nuclear localization of FOXO1, but in contrast, suppression of JNK reduced the oxidative stress-induced

nuclear localization of FOXO1, suggesting the involvement of the JNK pathway in FOXO1 translocation. In addition, oxidative stress or activation of the JNK pathway decreased the activity of AKT in HIT cells, leading to the decreased phosphorylation of FOXO1 following nuclear localization. Furthermore, adenovirus-mediated FOXO1 overexpression reduced the nuclear expression of Pdx-1, whereas repression of FOXO1 by FOXO1-specific small interfering RNA retained the nuclear expression of Pdx-1 under oxidative stress conditions. Activation of ERK has been shown to phosphorylate FOXO proteins, resulting in nuclear exclusion and transcriptional repression. In addition to ERK, direct phosphorylation of FOXO by AKT results in cytoplasmic retention and inactivation, inhibiting the expression of FOXO-regulated genes, which control the cell cycle, cell death, cell metabolism and oxidative stress [82,128,129]. Taken together, these studies demonstrate that dephosphorylation and activation of FOXO by inhibition of PI3K/AKT and MEK/ERK pathways has significant implication for pancreatic cancer treatment and prevention, where Kras is activated in about 90% patients.

In addition to phosphorylation, the acetylation/deacetylation of FOXO can be regulated by p300, Cbp (CREB-binding protein) and Pcaf (p300/CBP-associated factors) in response to oxidative stress or DNA binding, followed by deacetylation by class I and II histone deacetylases [130-132], including Sirt1, the NAD⁺-dependent deacetylase encoded by the ortholog of yeast longevity gene Sir2 [133]. Therefore, further studies are needed to examine the consequences of acetylation/deacetylation of FOXO transcription factors on anti-proliferative and anti-angiogenic effects of SFN.

In conclusion, we have demonstrated that SFN induces cell cycle arrest and apoptosis through regulation of FOXO transcription factors. Pharmacological and genetic inhibitions of PI3K/AKT and MEK/ERK pathways can have synergistic effects on the activation of FOXO transcription factors through dephosphorylation and nuclear retention. Thus, SFN appears to be as an attractive agent for pancreatic cancer prevention and treatment.

Methods

Reagents

Antibodies against PTEN, phospho-AKT, AKT, phospho-ERK, ERK, phospho-p38, p38, p21/CIP1, p27/KIP1, cyclin D1, and β -actin were purchased from Cell Signaling Technology, Inc. (Danvers, MA). 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). Sulforaphane was purchased

from LKT Laboratories, Inc. (St. Paul, MN). Kits for Terminal Deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling (TUNEL) and caspase-3 assays were purchased from EMD Biosciences/Calbiochem (San Diego, CA).

Cell Culture

PANC-1, MIA PaCa-2, AsPC-1 and Hs 766T cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic (Invitrogen) at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

Western Blot Analysis

Western blots were performed as we described earlier [134,135]. In brief, cells were lysed in RIPA buffer containing 1 × 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) in a Tris (20 mM), glycine (150 mM) and methanol (20%) buffer 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.

Caspase-3 Assay

Cells (3×10^4 per well) were seeded in a 96-well plate with 200 μ l culture medium. Approximately 16 h later, cells were treated with various doses of SFN to induce apoptosis. Caspase-3 activity was measured by a fluorometer as per manufacturer's instructions (EMD Biosciences).

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 Bonferoni's multiple comparison tests using PRISM statistical analysis software (GrafPad Software, Inc., San Diego, CA). Significant differences among groups were calculated at $P < 0.05$.

List of abbreviations used

ANOVA: Analysis of Variance; PTEN: Phosphatase and Tensin Homolog Deleted on Chromosome 10; RIPA: Radio-Immunoprecipitation Assay; SDS-PAGE: Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis; SFN: Sulforaphane; TBS: Tris Buffer Saline.

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Authors' contributions

SKR and SS performed the experiments. SS and RKS designed and wrote the manuscript. All the authors have read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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