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Crucifera sulforaphane (SFN) inhibits the growth of nasopharyngeal carcinoma through DNA methyltransferase 1 (DNMT1)/ Wnt inhibitory factor 1 (WIF1) axis

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Abstract

Background: Sulforaphane (SFN), a natural compound present in cruciferous vegetable, has been shown to possess anti-cancer activities. Cancer stem cell (CSC) in bulk tumor is generally considered as treatment resistant cell and involved in cancer recurrence. The effects of SFN on nasopharyngeal carcinoma (NPC) CSCs have not yet been explored.

Purpose: The present study aims to examine the anti-tumor activities of SFN on NPC cells with CSC-like properties and the underlying mechanisms.

Methods: NPC cell lines in monolayer culture, CSCs-enriched NPC tumor spheres, and also the NPC nude mice xenograft were used to study the anti-tumor activities of SFN on NPC. The population of cells expressing CSC-associated markers was evaluated using flow cytometry and ALDH activity assay. The effect of DNA methyltransferase 1 (DNMT1) on the growth of NPC cells was analyzed by using small interfering RNA (siRNA)-mediated silencing method.

Results: SFN was found to inhibit the formation of CSC-enriched NPC tumor spheres and reduce the population of cells with CSC-associated properties (SOX2 and ALDH). In the functional study, SFN was found to restore the expression of Wnt inhibitory factor 1 (WIF1) and the effect was accompanied with the downregulation of DNMT1. The functional activities of WIF1 and DNMT1 were confirmed using exogenously added recombinant WIF1 and siRNA knockdown of DNMT1. Moreover, SFN was found to inhibit the *in vivo* growth of C666-1 cells and enhance the anti-tumor effects of cisplatin.

Conclusion: Taken together, we demonstrated that SFN could suppress the growth of NPC cells via the DNMT1/WIF1 axis.

Key Words: Sulforaphane, Nasopharyngeal Carcinoma, DNMT1, WIF1

Abbreviations:

ALDH	Aldehyde Dehydrogenase
CSC	Cancer Stem Cell
DNMT1	DNA Methyltransferase 1
EBV	Epstein-Barr Virus
EGF	Epidermal Growth Factor
FGF	Fibroblast Growth Factor
IGF	Insulin-like Growth Factor
NPC	Nasopharyngeal Carcinoma
SFN	Sulforaphane
SOX2	SRY (sex determining region Y)-box 2
WIF1	Wnt Inhibitory Factor 1

1. Introduction

Nasopharyngeal carcinoma (NPC) is closely associated with latent infection of Epstein-Barr virus (EBV) and nearly all NPC tumors contain EBV (Hildesheim et al., 2002). C666-1 is a cell line consistently harboring EBV, which makes it a suitable choice for studying NPC (Cheung et al., 1999). Patients with early stage NPC can be effectively treated with radiotherapy. However, the outcome of radiotherapy alone for locally advanced NPC is not satisfactory, with 5-year survival rates between 34% and 52% (Caponigro et al., 2010). Currently, most NPC patients are treated with combination of chemotherapy and radiotherapy but treatment failures remain a big challenge for the management of NPC. Cancer stem cells (CSCs) refer to a subpopulation of cancer cells that are responsible for sustaining tumorigenesis (Eramo et al., 2008), and the present of treatment resistant CSCs in the bulk tumor has been implicated in the tumor relapse. There is increasing evidence to support the idea that eradication of CSCs in the tumor bulk is critical to the success of anti-cancer therapies. Combining drugs that can eliminate CSCs together with conventional treatment might provide a better prognosis for NPC.

Sulforaphane (SFN) is a bioactive compound derived from cruciferous vegetables. Previous studies have shown that SFN possess anti-tumor activities, such as the induction of cell cycle arrest (Chiao et al., 2002) and inhibition of CSCs (Li et al., 2013). It has also been shown that SFN can enhance the cytotoxicity of many conventional chemotherapeutic drugs (Kallifatidis et al., 2011). Currently, SFN is under various clinical trials, such as Phase II clinical trial in bladder cancer chemoprevention (NCT03517995) and lung cancer chemoprevention in former smokers (NCT03232138). However, very few studies have been performed to address the therapeutic potential of SFN in NPC. In the present study, we have examined the

impact of SFN treatment on the growth of NPC cells with CSC-associated properties. We also demonstrated the involvement of DNA methyltransferase 1 **(**DNMT1)/Wnt inhibitory factor 1 (WIF1) axis in the regulation of SFN-treated NPC cells.

2. Materials and Methods

2.1. Chemicals and antibodies

SFN was obtained from LKT laboratories (Cat. No. S8044). Cisplatin was purchased from Sigma (Cat. No. P4394). Recombinant human WIF1 (Wnt inhibitory factor 1) was purchased from R&D Systems (Cat. No. 1341-WF). Antibodies for SOX2 (Cat. No. 3579), Alexa Fluor® 647 conjugated SOX2 (Cat. No. 5067), Alexa Fluor® 647 conjugated IgG Isotype control (Cat. No. 2985) and DNMT1 (Cat. No. 5032) were obtained from Cell Signaling. Antibody against β-Actin was obtained from Sigma (Cat. No. A5316).

2.2. Cell culture

The poorly differentiated C666-1 and HONE-1 cell lines (Chai et al., 2016; Cheng et al., 2015; Glaser et al., 1989; Yao et al., 1990) were obtained from Hong Kong NPC AoE Research Tissue Bank and Cell Line Repository. The cells had been authenticated using the AmpFLSTR Identifier PCR Amplification Kit (Thermo Fisher Scientific, Inc.) (Chan et al., 2011; Cheung et al., 2013). The HONE-1 cell has also been tested for the free of HeLa-specific L1 retrotransposon insertion. C666-1 cell was grown in RPMI 1640 medium (GIBCO) supplemented with 10% fetal bovine serum (FBS, GIBCO) and antibiotics (penicillin 50 Units/ml and streptomycin 50 μg/ml; GIBCO). HONE-1 cells were cultured in DMEM medium (GIBCO) supplemented with 5% heat-inactivated FBS, 5% heat-inactivated newborn calf serum

(5%, GIBCO) and antibiotics. Cells were maintained at 37 ^{o}C in a humidified 5% CO_2 incubator.

2.3. Cell viability analysis

C666-1 cells (5×10^5 cells/well) were seeded in 35 mm culture dishes for three days and then treated with SFN (10 - 20 μM) or appropriately diluted DMSO (Control) for another 2 to 4 days. HONE-1 cells (4×10^4 cells/well) were incubated in 35 mm culture dishes for overnight and then subjected to the same SFN treatments. Number of viable cells was determined under inverted microscope using trypan blue exclusion staining method.

2.4. Tumor spheres formation assay

Tumor spheres formation assay was used for the enrichment of CSCs from bulk tumor cells (Lo et al., 2013). NPC cells (1×10^4 cells/well) were seeded in DMEM/F12 (Invitrogen) supplemented with EGF (20 ng/ml), FGF (20 ng/ml), and IGF (20 ng/ml) in 6-well ultra-low attachment plate (Corning). The cultures were then incubated in a humidified chamber at 37 ^{o}C for 7 days. The cultures were fed with fresh growth factors every 2 to 3 days. The images of all tumor spheres were captured under an inverted microscope and those with a diameter greater than 20 μm were analyzed by ImageJ software.

2.5. ALDH activity assay

NPC cells were treated with 20 μM SFN for two days. After the treatment, aldehyde dehydrogenase (ALDH) activities of the cells were studied using ALDEFLUOR^{TM} Kit (Stemcell technologies, Cat. No.01700). The fluorescent signals

were subsequently measured using a flow cytometer. The ALDH inhibitor diethylaminobenzaldehyde (DEAB) was used as a negative control for the assessment of background fluorescence.

2.6. Flow cytometric analysis of SOX2^{Hi} population

SFN (20 μM) - treated NPC cells were harvested, fixed with 1.6% paraformaldehyde, washed with PBS, permeabilized in ice-cold methanol, and finally washed and adjusted to a concentration of 1×10^6 cells/ml in 0.5% bovine serum albumin (BSA). A mixture of 100 μl of the cells with either Alexa Fluor® 647 conjugated SOX2 antibody or Alexa Fluor® 647 conjugated IgG isotype control antibody was then incubated in dark for 30 minutes. After incubation, the cells were washed twice with 0.5% BSA. Finally, the cells were resuspended in 200 μl PBS and the fluorescence signals were analyzed using a flow cytometer.

2.7. Real-time PCR analysis

Total cellular RNA was extracted using TRIzol Reagent (Invitrogen) according to the manufacturer's protocol, and then reverse transcribed to complementary DNA (cDNA) using M-MLV reverse transcriptase (Invitrogen) and quantified by real-time PCR using Power SYBR Green Master Mix (Life Technologies). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control. SOX2 primers: sense 5'-CAGACTTCACATGTCCCAGC-3'; antisense 5'-GGCAGTGTGCCGTTAATGG-3' (Arima et al., 2012). WIF1 primers: sense 5'-CACTGTGGTAGTGGCATTTAAACAATA-3'; antisense 5'-GCCAATGCAAAAAGTTCATACATT-3'. GAPDH primers: sense 5'-GAAGGTGAAGGTCGGAGTC-3'; antisense

5'-GAAGATGGTGATGGGATTTC-3' (Lo et al., 2013). $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression of target transcripts.

2.8. Western blot analysis

NPC cells were lysed by lysis buffer (250 mM Tris (pH 8), 1% NP-40 and 150 mM NaCl containing 1% phosphatase inhibitors cocktail and 0.25% protease inhibitors cocktail). Equal amounts of protein from the cell lysates were resolved in SDS-polyacrylamide gel, and the separated proteins were transferred to PVDF membranes. After blocking with 5% non-fat dry milk, the membrane was incubated with primary and the corresponding secondary antibodies. Signals from the protein bands were developed by incubating the membrane with the Western Blot substrate (Labfrontier Co. Ltd.). β-actin was used as the internal control. Band intensities were analyzed by using ImageJ software.

2.9. Cell transfection

NPC cells were seeded onto fibronectin pre-coated culture plates. Transient transfection was performed using 50 nM DNMT1 siRNA (si-DNMT1) (Dharmacon, Cat. No. L-004605-00-0005) in the presence of Lipofectamine Reagent 2000 (Invitrogen). Non-targeting siRNA (si-Control) (Dharmacon, Cat. No. D-001810-01-20) was used as a negative control. After 72 hours of transfection, the cells were harvested for further analysis.

2.10. Nude mice tumorigenicity assay

Four- to six-week-old BALB/c nu/nu mice were supplied and housed by the Department of Clinical Oncology of Queen Elizabeth Hospital Hong Kong. All

procedures were conducted under license from the Hong Kong Department of Health. C666-1 cells (1×10^7 cells in 0.2 ml RPMI medium) were subcutaneously (s.c.) injected into the right flank of the nude mouse. When the tumor volume reached 70 - 100 mm^3, mice were randomly allocated into various groups. SFN was dissolved in DMSO and subsequently diluted in PBS prior to injection. Cisplatin was dissolved in Dimethylformamide (DMF) and diluted in sodium chloride solution prior to injection. Drugs were administrated by intraperitoneal (i.p.) injection. Tumor volume in mm^3 (length x width x height) and the body weight of the animals were measured twice a week.

2.11. Drug combination study

The *in vivo* combination effect of SFN and cisplatin was calculated using the Zheng-Jun Jin method (Jin, 2004). The method provides a q value which was calculated from the equation, $q = E_{a+b}/(E_a+E_b-E_a \times E_b)$, where E_a, E_b, and E_{a+b} indicate the inhibitory rates of SFN, cisplatin, and the combination of the two drugs, respectively. E_{a+b} is the observed value of inhibitory effect of two drugs and $E_a+E_b-E_a \times E_b$ is the mathematical expectation value of inhibitory effect of two drugs. $q < 0.85$, $0.85 < q < 1.15$, and $q > 1.15$ indicate antagonism, additivity， and synergy, respectively.

2.12. Statistical analysis

Student t-test in SPSS 11 software was used to determine the level of significance. *p*-value smaller than 0.05 was considered as significant.

3. Results

3.1. Effects of SFN on the cell viability of NPC cells

The growth of NPC cells was evaluated using the trypan blue exclusion assay to determine the number of viable cells after SFN treatment. Results showed that the proliferation of both NPC cell lines was inhibited by SFN in a dose- and time-dependent manner (Fig. 1).

3.2. SFN significantly reduced the numbers of NPC tumor spheres

The tumor spheres formation assay is frequently used to enrich cells with CSC-like properties under the 3-dimensional (3D) culturing condition (Lo et al., 2013). The number and diameter of the tumor spheres derived from NPC cells after SFN treatments were quantified. Results in Figure 2A and 2B showed that both the number and the size of the tumor spheres of NPC cells were reduced dose-dependently. To further determine whether SFN-pretreated NPC cells are able to produce tumor spheres, C666-1 cells in 2-dimensional (2D) culture were pre-treated with SFN (5 – 20 μM) for 2 days, the SFN pre-treated cells were then allowed to grow under the conditions of tumor spheres cultures. Results in Figure 2C clearly showed that the ability of pre-treated cells to form tumor spheres was reduced in a dose-dependent manner.

3.3. SFN reduced the expression of the NPC CSC-associated phenotypes

It has previously been shown in NPC that the expression of SOX2 and high ALDH activity is associated with the cells with CSC-like characteristics (Lun et al., 2012; Wu et al., 2013a; Yu et al., 2013). As shown in Figure 3, the percentage of C666-1 (Fig. 3A) and HONE-1 (Fig. 3B) cells with high ALDH activity was reduced

after SFN treatment. Results in Figure 4 showed that SFN treatment significantly reduced the gene (Fig. 4A) and protein (Fig. 4B) expression of SOX2. The population of SOX2 positively stained NPC cells (Fig. 4C, 4D) was also reduced. Taken together, SFN was found to reduce the population of NPC cells with CSC-associated characteristics.

3.4. Involvement of DNMT1/WIF1 axis in SFN-mediated suppression of the growth of tumor spheres

Epigenetic deregulation of cell signaling in NPC is well documented, and DNMT has been implicated as an important regulator in several cell signaling pathways (Li et al., 2011). The Wnt inhibitory factor 1 (WIF1), a negative regulator of the CSC-associated Wnt signaling pathway, has also been frequently found to be silent in NPC tumor (Fendri et al., 2010). Accumulating evidence indicates that the chemopreventive effect of SFN might be partly due to the ability of SFN to modify the epigenetic mechanism (Atwell et al., 2015). In the present study, we sought to determine whether DNMT and WIF1 are involved in SFN-mediated tumor suppression. First of all, SFN was found to dose-dependently reduce the protein expression of DNMT1 (Fig. 5A). In the functional study, the impact of siRNA-mediated knockdown of DNMT1 on the growth of tumor spheres was examined. Results in Figure 5B showed that DNMT1 siRNA could reduce the DNMT1 protein expression and the effect was accompanied with the reduction in the growth of tumor spheres (Fig. 5C). WIF1, an endogenous Wnt antagonist, was frequently found to be epigenetically silent in NPC (Chan et al., 2007; Lin et al., 2006). Next, we determined whether the SFN or DNMT1 siRNA treatment could restore the expression of WIF1 mRNA. Results clearly showed that SFN or DNMT1

siRNA treatment could restore the expression of WIF1 in NPC cells (Fig. 5D and 5E). Finally, we determine the functional role of WIF1 on the growth of the tumor spheres by adding recombinant human WIF1 protein into the NPC cell cultures. Results in Figure 5F clearly showed that the tumor spheres-forming capacity of C666-1 cells was inhibited by WIF1. Taken together, these findings suggested that SFN could restore the expression of WIF1, and DNMT1/WIF1 axis is involved in SFN-mediated suppression of the growth of tumor spheres in NPC.

3.5. The in vivo anti-tumor activity of SFN

Cisplatin-based chemoradiotherapy is commonly used in the treatment of NPC (Yu et al., 2018). We studied the effect of SFN, alone or in combination with cisplatin, on the growth of NPC tumor. NPC bearing mice (10 mice per group) were treated with SFN (60 mg/kg, four times per week), cisplatin (3 mg/kg, twice per week), or a combination of SFN (60 mg/kg) and cisplatin (3 mg/kg) for 3 weeks. Results from Figure 6 showed that the average tumor volume was 868 mm^3 for the untreated control group, 622 mm^3 for the SFN treatment group (E_a = 28.3% inhibition), 518 mm^3 for the cisplatin treatment group (E_b = 40.3% inhibition), and 386 mm^3 for the combination group (E_{a+b} = 55.5% inhibition). The combination *q* value was calculated to be 0.970 and the combination effect of SFN and cisplatin in this *in vivo* study was considered to be additive. This observation indicated that SFN could further enhance the inhibitory effect of cisplatin on the tumor growth of NPC.

4. Discussion

SFN, a natural phytochemical derived from broccoli/broccoli sprouts, has been shown

to exert anti-tumor activities in different types of cancer (Chiao et al., 2002; Li et al., 2010). SFN has also been shown to inhibit the self-renewal of CSCs in pancreatic (Li et al., 2013), prostate (Labsch et al., 2014), and breast cancer (Li et al., 2010). However, to our knowledge, only two publications so far showed the anti-tumor effect of SFN on NPC, and the effect was associated with the inhibition of EBV reactivation (Wu et al., 2013b) and the inhibition of signal transduction and activator of transcription (STAT) signaling (Li et al., 2018). In the present study, we demonstrated for the first time that SFN could inhibit the growth of NPC cells with CSC-associated characteristics.

In NPC, the $ALDH1^+$ cells had been shown to have a higher colony-formation ability and could form tumor *in vivo* more efficiently (Wu et al., 2013a). The $ALDH^+$ cells in NPC were also found to be more resistant to chemotherapy and ionizing radiation (Yu et al., 2013). In the present study, SFN was found to effectively reduce the $ALDH^+$ population in NPC cells. SOX2 is another CSC-associated marker enriched in the $ALDH^+$ NPC cell population (Wu et al., 2013a), and the expression of SOX2 in NPC cells has been shown to have a high tumor spheres forming capacity (Lun et al., 2012). In the present study, SFN was also found to reduce the population of SOX2 expressing cells. Taken together, we provided evidence that SFN could inhibit the growth of NPC cells processing CSC-associated characteristics. Therefore, SFN may be used to inhibit the growth of the treatment-resistant population of NPC cells and so to increase the treatment efficacy.

Promoter hypermethylation and silencing of the expression of tumor suppressor genes is a common event in NPC (Lung et al., 2012). The tumor suppressor WIF1, an

important negative regulator of the CSC-associated Wnt signaling, is frequently hypermethylated and silenced in NPC (Chan et al., 2007; Lin et al., 2006). The aberrant expression phenotype of WIF1 is also being used as one of the major biomarkers in NPC (Yang et al., 2015). DNMT1 is primarily responsible for the maintenance of global gene methylation (Robert et al., 2003). In the DNMT1 siRNA knockdown experiment, we firstly demonstrated the importance of DNMT1 in the formation of NPC tumor spheres. We further found that SFN, by down-regulating the expression level of DNMT1, restored the expression of WIF1 in NPC cells. Previous mechanistic studies indicate that SFN may serve as histone deacetylase inhibitor and regulate promoter methylation, however, the molecular mechanism of SFN to influence the expression of DNMT1 is still unknown (Kaufman-Szymczyk et al., 2015). Taken together, the observation of inhibition of DNMT1 expression in NPC is consistent with previous studies on other cancers such as breast (Meeran et al., 2010) and cervical cancer (Ali Khan et al., 2015). SFN-mediated restoration of WIF1 and tumor suppression is a novel observation in NPC.

Cisplatin-based chemoradiotherapy is a contemporary approach for NPC (Xu et al., 2016), but it is generally effective in early-stage of NPC (Dugbartey et al., 2016). Current hypothesis suggested that the development of drug resistance and secondary malignancies encountered in cancer cases may be attributed to the presence of CSCs. Here, we demonstrated that SFN could enhance the anti-tumor activities of cisplatin in the *in vivo* models. Our results further support previous observations that SFN could sensitize tumor cells to conventional chemotherapeutic agents (Hunakova et al., 2014; Kallifatidis et al., 2011), such as the commonly used cisplatin in NPC. All in all, our pre-clinical study provided evidence that SFN could be used as an

anti-tumor agent to enhance the conventional chemotherapies in NPC.

Conflicts of interest

None.

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Figure 1. Effects of SFN on the growth of NPC cells. The number of viable (A) C666-1 and (B) HONE-1 cells after SFN treatments was evaluated using the trypan-blue exclusion assay. Results were expressed as mean ± SD from three independent experiments. * $p < 0.05$; ** $p < 0.01$.

Figure 2. Effects of SFN on the formation of NPC tumor spheres. (A) C666-1 cells and (B) HONE-1 cells were treated with SFN or an appropriately diluted DMSO (Control) in 6-well ultra-low attachment plates for 7 days. (C) C666-1 cells were seeded in 35 mm culture dishes for 3 days and subsequently treated with SFN (5, 10 and 20 μM) or an appropriately diluted DMSO (Control) for another 2 days. After treatment, cells were then harvested and an equal amount of viable cells (10,000 cells/well) was seeded for the tumor spheres formation assay. Size distribution by measuring the spheroid diameter, representative spheroid images and total number of tumor spheres formed were presented. Scale bar = 20 μm (A), and = 50 μm (B and C). Results were expressed as mean ± SD from three independent experiments. * $p < 0.05$, ** $p < 0.01$.

Figure 3. SFN reduced the population of ALDH-positive NPC cells. (A) C666-1 cells and (B) HONE-1 cells. NPC cells were treated with 20 μM SFN or an appropriately diluted DMSO (Control) for 2 days. ALDH activity was evaluated using the ALDEFLUOR Assay kit. Quantitative measurement and representative diagrams of the ALDH-positive populations were presented. Results were expressed as mean ± SD from three independent experiments. * $p < 0.05$.

Figure 4. SFN reduced SOX2 expression in C666-1 cells. (A) The gene expression level of SOX2 mRNA in SFN-treated C666-1 cells was determined by real-time PCR analysis at 24 hours after SFN treatment. (B) The protein expression level of SOX2 was determined by Western blot at 2 days after SFN treatment. (C) Flow cytometric analysis of SOX2 expressing population at day-2 after SFN treatment. Cells stained with the isotype control antibody were used as negative controls. (D) Statistical plot of SOX2 expressing population from flow cytometric analysis. Results were expressed as mean ± SD from three independent experiments. * $p < 0.05$, ** $p < 0.01$.

Figure 5. SFN-mediated inhibition of tumor spheres formation involves DNMT1/WIF1 axis. (A) The protein expression level of DNMT1 in SFN-treated C666-1 cells at 24 hours after SFN treatment. DNMT1 siRNA treatment reduced the DNMT1 protein expression (B), and inhibited the formation of tumor spheres (C). (D) SFN treatment restored the gene expression of WIF1. The gene expression level

of WIF1 was determined by real-time PCR analysis at 24 hours after SFN treatment. (E) DNMT1 siRNA treatment restored the gene expression of WIF1. (F) Exogenously added recombinant WIF1 protein inhibited the tumor spheroid growth of C666-1 cells. Scale bar = 50 μm. Results were expressed as mean ± SD from three independent experiments. * $p < 0.05$, ** $p < 0.01$.

Figure 6. Combination effect of SFN and cisplatin on the growth of C666-1 xenograft. NPC xenograft was established as described in the Materials and Methods. The NPC bearing mice were i.p. injected with PBS (Control), 60 mg/kg SFN (four times per week), 3 mg/kg cisplatin (twice per week), or combination of the two drugs. Each group contained 10 mice. Inhibition rate was determined at the end of the experiment, and the *q* value (= 0.970) indicated an additive effect in the SFN and cisplatin combination treatment. The tumor volume (A) and the body weight (B) were measured twice per week throughout the experiment. Representative tumor-bearing mice (C) were presented. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared to the Control.

Graphical Abstract

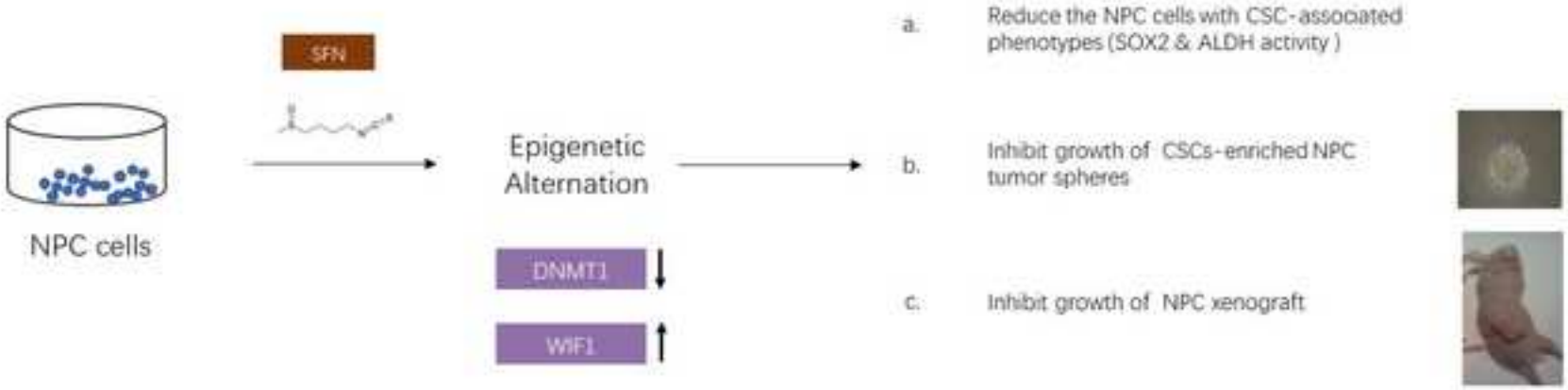

Figure 1

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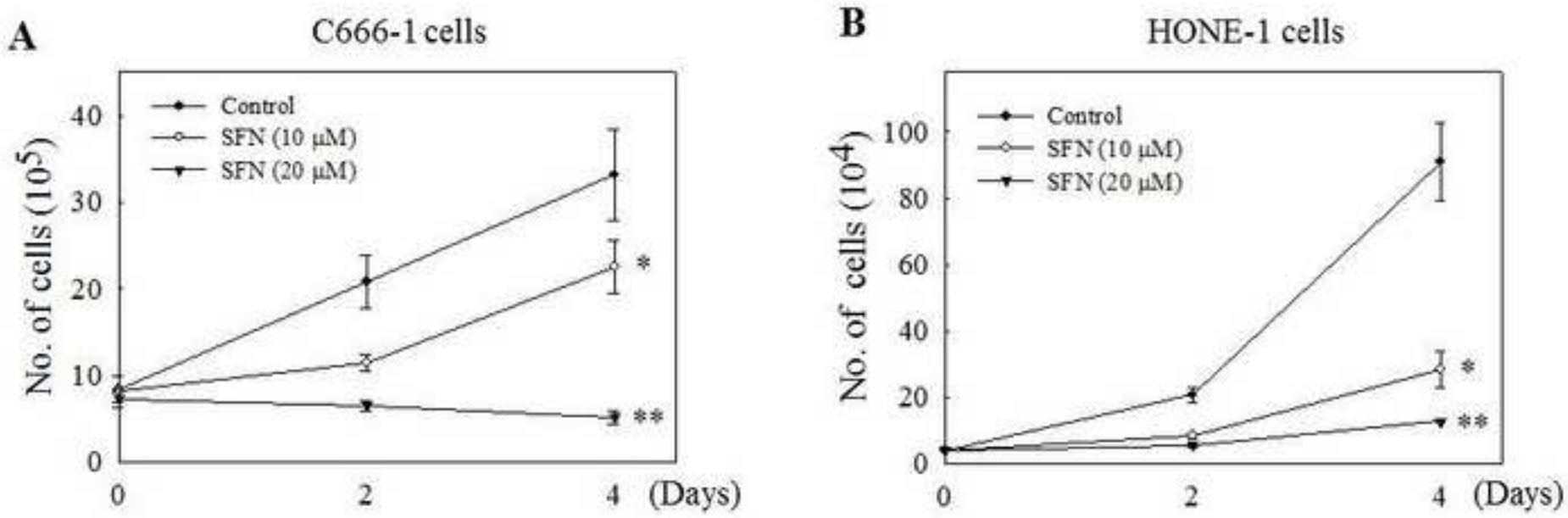

Figure 2

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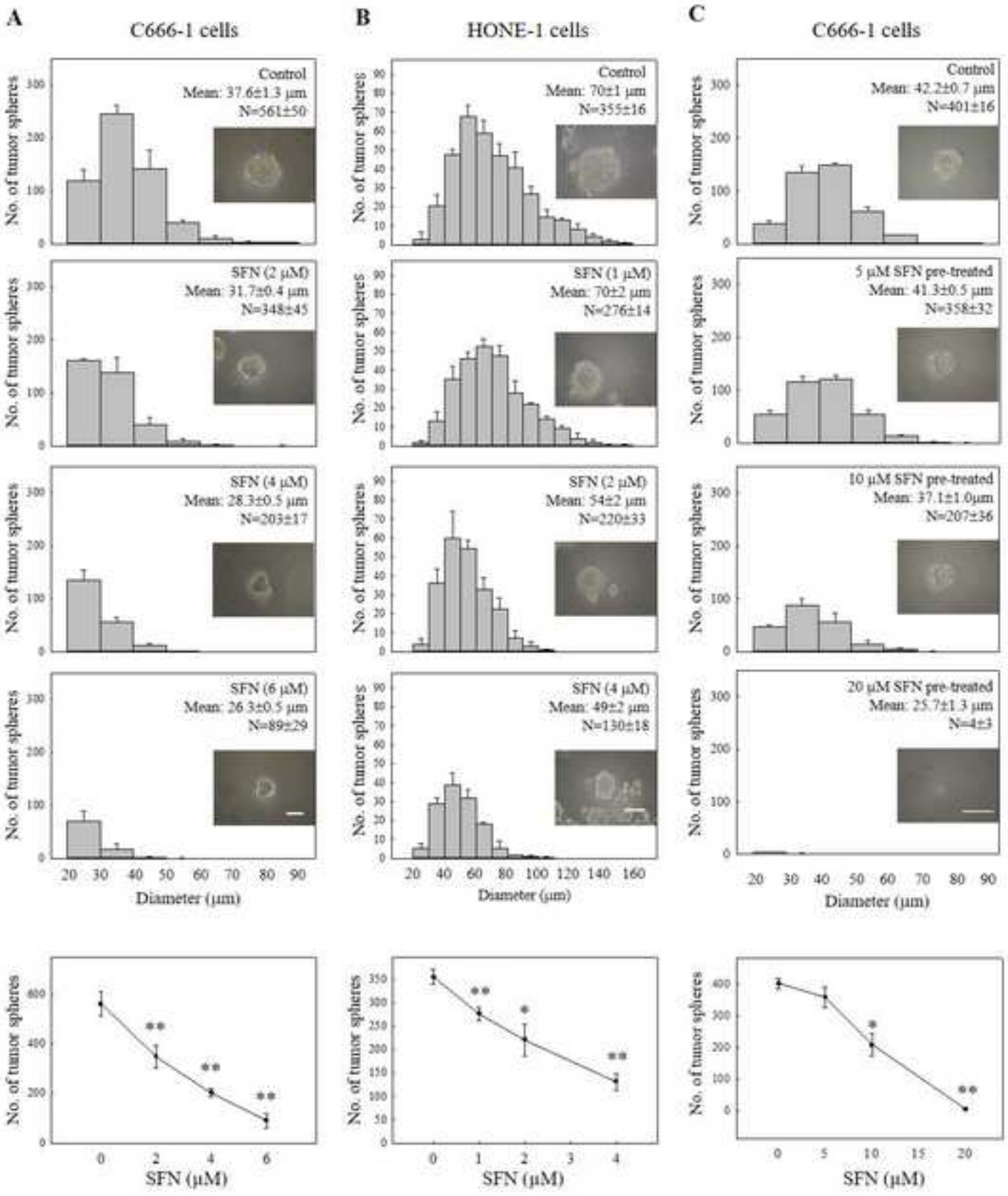

Figure 3

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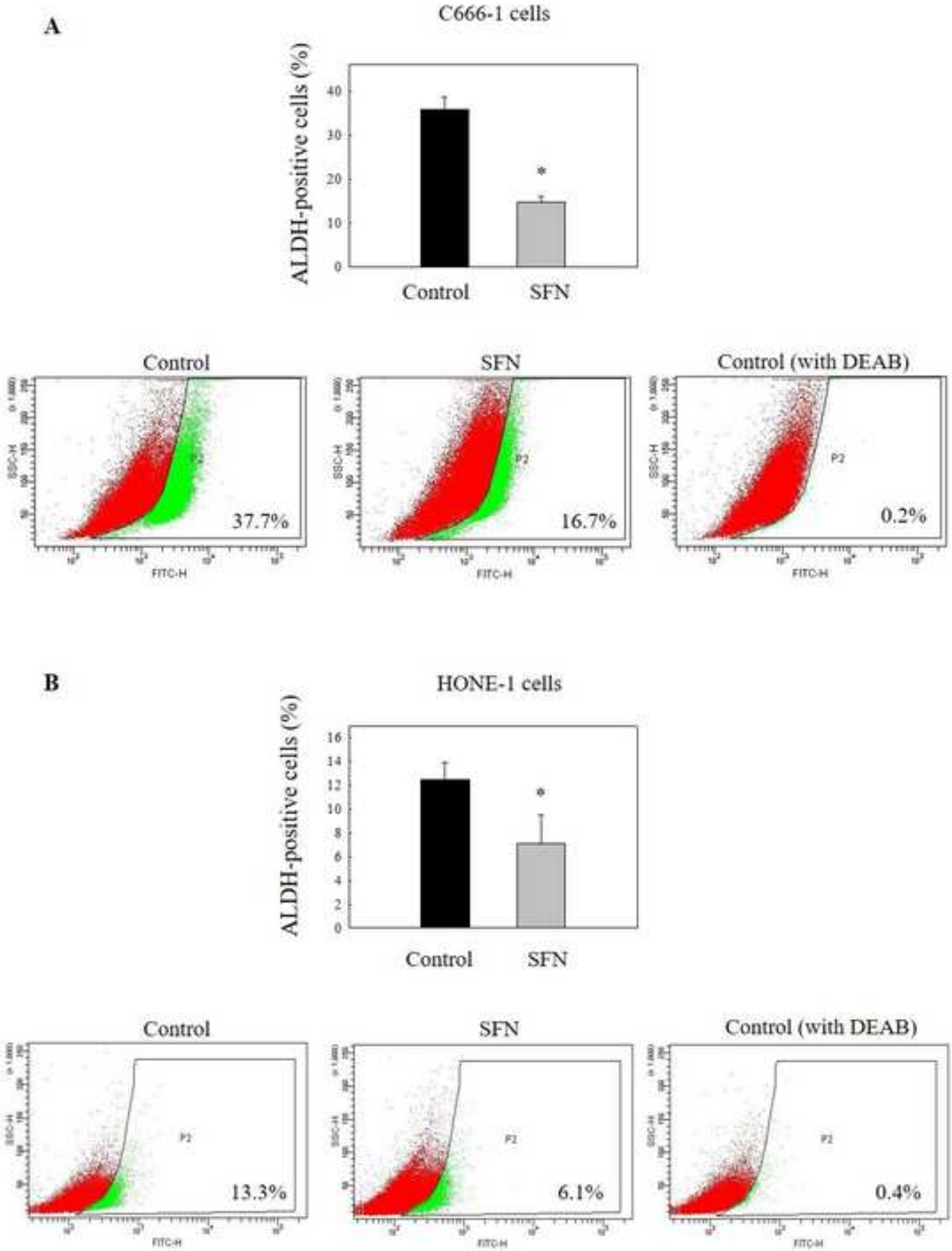

Figure 4

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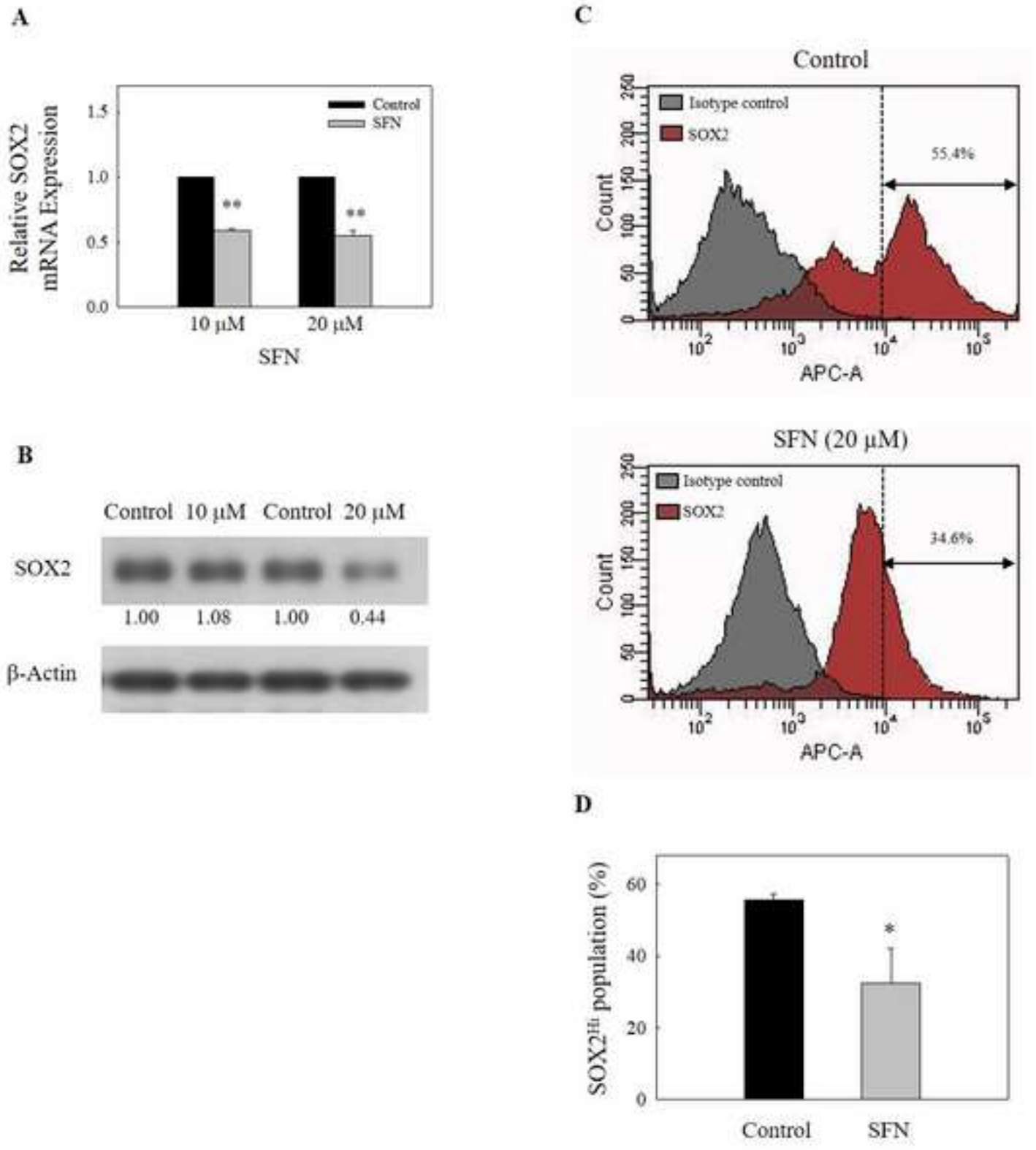

Figure 5

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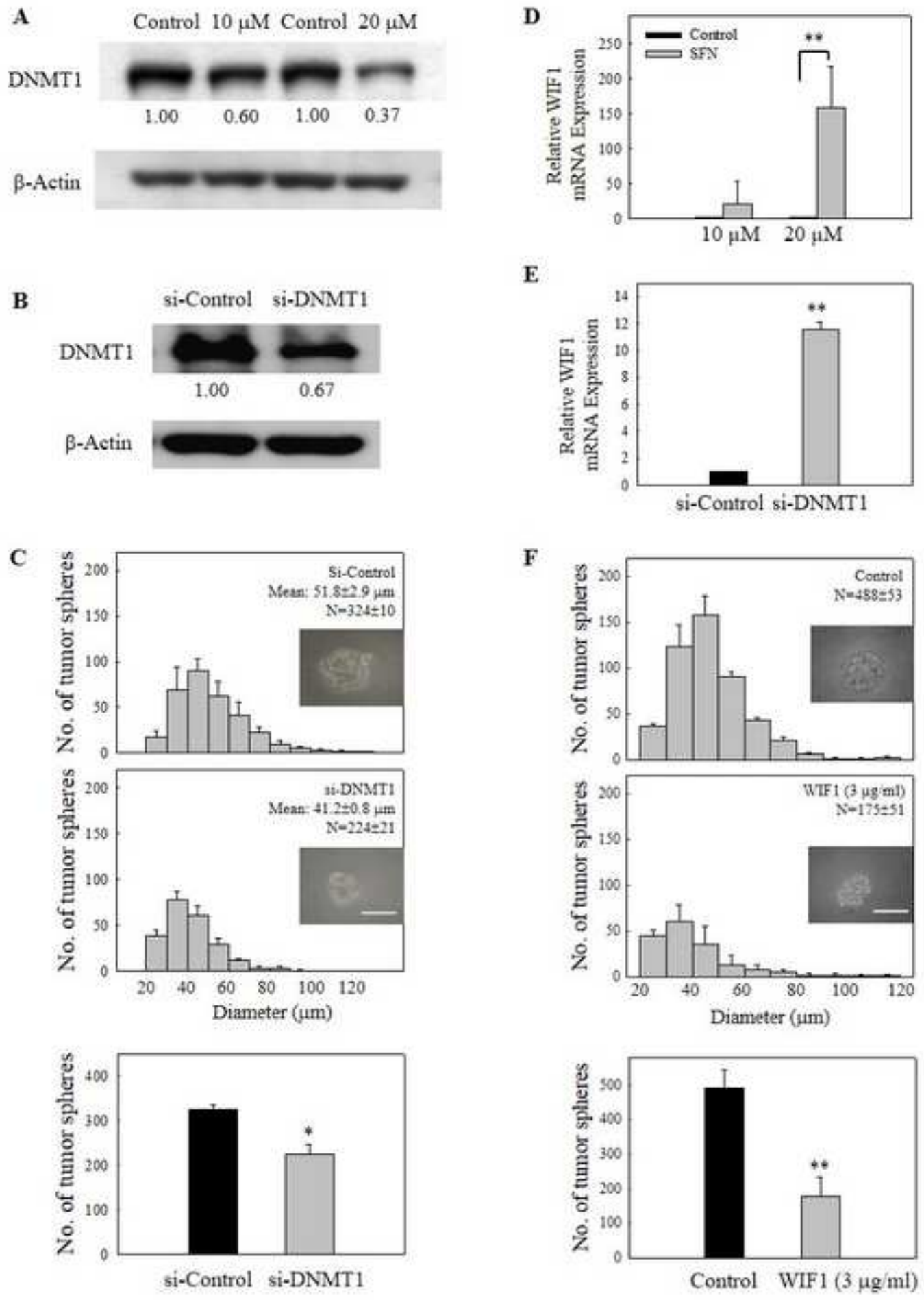

Figure 6

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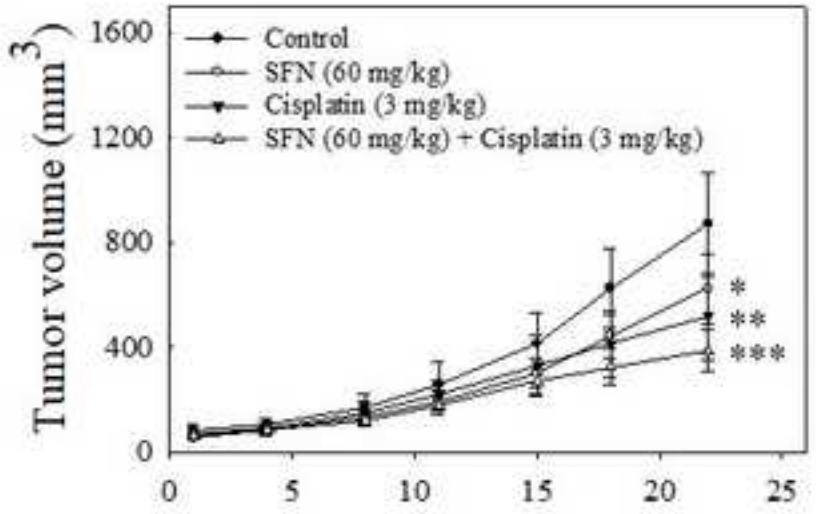
A



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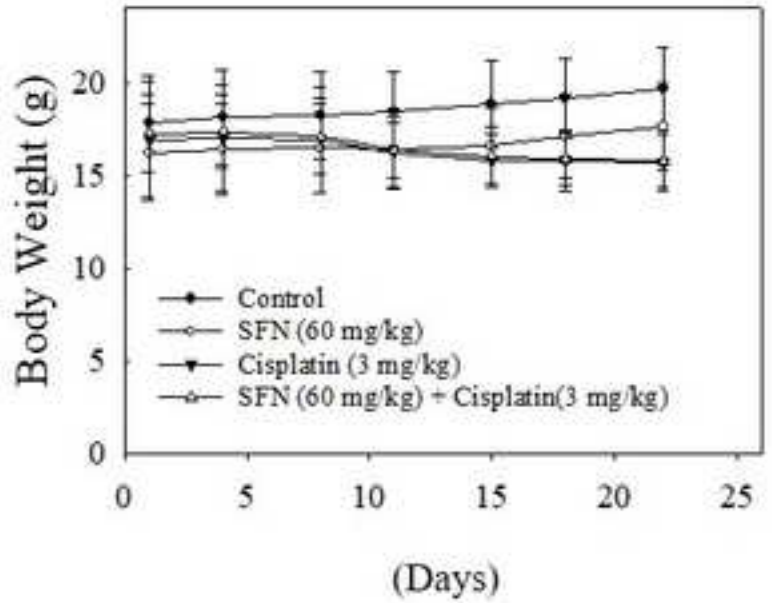

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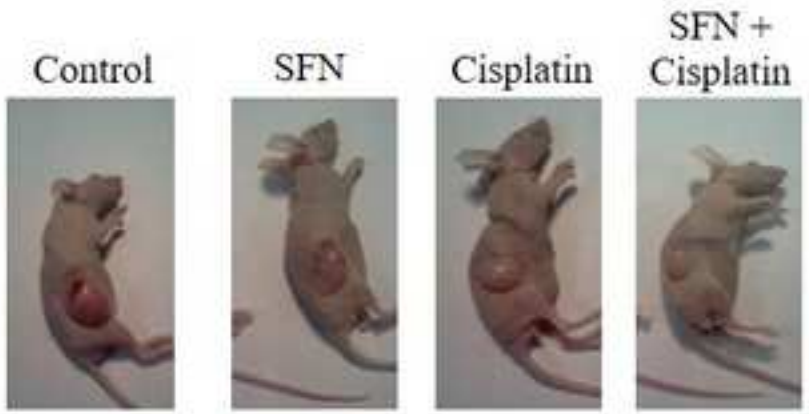

Group	Inhibition rate
SFN (E_a)	0.283
Cisplatin (E_b)	0.403
SFN + Cisplatin (E_{a+b})	0.555

q value = 0.970