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Sulforaphane protects against ethanol-induced apoptosis in neural crest cells through restoring epithelial-mesenchymal transition by epigenetically modulating the expression of Snail1

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ABSTRACT

Ethanol-induced apoptosis in neural crest cells (NCCs), a multipotent progenitor cell population, is implicated in the Fetal Alcohol Spectrum Disorders (FASD). Studies have demonstrated that sulforaphane (SFN) can prevent ethanol-induced apoptosis in NCCs. The objective of this study is to investigate whether ethanol exposure can induce apoptosis in NCCs by inhibiting epithelial-mesenchymal transition (EMT) and whether SFN can prevent ethanol-induced apoptosis by epigenetically modulating the expression of Snail1, a key transcriptional factor that promotes EMT. We found that ethanol exposure resulted in a significant increase in apoptosis in NCCs. Co-treatment with SFN significantly reduced ethanol-induced apoptosis. Treatment with SFN also dramatically diminished ethanol-induced changes in the expression of E-cadherin and vimentin, and restored EMT in ethanol-exposed NCCs. In addition, ethanol exposure reduced the levels of trimethylation of histone H3 lysine 4 (H3K4me3) at the promoters of Snail1. SFN treatment diminished the ethanol-induced reduction of H3K4me3 at the promoter regions of the Snail1 gene, restored the expression of Snail1 and down-regulated Snail1 target gene E-cadherin. Knockdown of Snail1 significantly reduced the protective effects of SFN on ethanol-induced apoptosis. These results demonstrate that SFN can protect against ethanol-induced apoptosis by preventing ethanol-induced reduction in the levels of H3K4me3 at the promoters of Snail1, restoring the expression of Snail1 and EMT in ethanol-exposed NCCs.

1. Introduction

Fetal Alcohol Spectrum Disorder (FASD) is one of the major development defects caused by alcohol consumption by women during pregnancy. FASD is characterized by craniofacial abnormalities, mental retardation, and behavior defects [1–3]. Studies have demonstrated that ethanol-induced excessive cell death in the specific cell population is one of the major mechanisms underlying the pathogenesis of FASD [4,5]. Our studies and others have shown that ethanol can induce apoptosis in neural crest cells (NCCs) and that ethanol-induced apoptosis in NCCs contributes significantly to ethanol-induced malformations [6–8].

NCC is a multipotent and migratory progenitor cell population which originates between the neural plate and non-neural ectoderm [9]. After induction at the border of the neural plate, NCCs leave their original location through a delamination process and migrate ventrally to differentiate into a diversity of neural and non-neural cell types, including neuron, glia, craniofacial cartilage, bone and connect tissue [10–13]. NCC delamination process involves epithelium-to-mesenchyme transition (EMT). EMT is a process that orchestrates a change from an epithelial to a mesenchymal phenotype, a process which increases migratory properties, invasiveness and apoptotic resistance [14,15]. EMT is essential for both normal development and cancer invasion and metastasis [16–18]. During embryonic development, NCCs undergo an EMT and then dissociate from the neural folds and differentiate to a diversity of cell types [9,10,12]. EMT also plays a pivotal role in promoting tumor proliferation, invasion, and metastasis, exerting an anti-apoptosis effect [19,20]. Studies have shown that EMT

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conferred resistance to UV-induced apoptosis in three murine mammary epithelial cell lines [21]. Park et al. have also shown that α-mangostin can inhibit EMT and induce apoptosis in osteosarcoma cell line [22]. However, the roles of EMT in ethanol-induced apoptosis in NCCs and in the pathogenesis of FASD remain to be defined.

One of the well-known transcriptional factors that regulate EMT is Snail1 [23–25]. Snail1 promotes EMT primarily through the directly repressing E-cadherin, an EMT suppressing factor [23,26,27]. Expression of Snail1 can be regulated by many mechanisms, including epigenetic regulation. Epigenetic modification generally includes DNA methylation and histone modification, which includes histone acetylation and methylation [28]. Among the histone methylation, the triple methyl modification on the fourth lysine of histone 3 (H3K4me3) typically facilitates the activation of gene transcription, whereas the triple methyl modification on the twenty-seventh lysine of histone 3 (H3K27me3) usually represses gene transcription [29]. Studies have shown that H3K4me3 modification is involved in neurodevelopmental disorders [30–32] and that Snail1 gene expression was regulated by histone methylation on its promoter region during the EMT of the prostate cancer cells [33].

Sulforaphane (SFN) is a vegetable-derived isothiocyanate that is abundant in cruciferous vegetables such as broccoli. Our previous studies have shown that SFN exerts an anti-apoptotic effect through up-regulating antioxidant gene Nrf-2 in NCCs [34]. More recently, SFN had been reported to act as an inhibitor of histone deacetylase (HDAC) and DNA methyltransferase (DNMT), two key enzymes involved in histone deacetylation and DNA methylation, respectively, to cause epigenetic modification of genes in varied types of cells, including the genes involved in EMT in cancer cells [35–37]. Our recent studies have also shown that SFN can prevent ethanol-induced apoptosis in NCCs by diminishing ethanol-induced reduction of histone acetylation at the promoter of the anti-apoptotic gene, Bcl2 [38].

In the present study, we tested the hypothesis that SFN can protect against ethanol-induced apoptosis by restoring EMT through epigenetically modulating the expression of Snail1 in NCCs. We found that treatment with SFN significantly diminished ethanol-induced changes in the expression of E-cadherin, and restored EMT in NCCs. We also found that ethanol exposure significantly reduced the levels of H3K4me3 at the promoters of Snail1. SFN treatment diminished the ethanol-induced reduction of H3K4me3 at the promoter regions of the Snail1 gene, restored Snail1 gene expression and EMT, and reduced apoptosis in NCCs exposed to ethanol. Knockdown of Snail1 significantly diminished the protective effects of SFN on ethanol-induced apoptosis. These results demonstrate that ethanol exposure can induce apoptosis in NCCs by inhibiting EMT and that SFN can protect against ethanol-induced apoptosis by epigenetically regulating the expression of Snail1 and restoring EMT.

2. Materials and methods

2.1. Cell culture and treatment

NCCs (JoMa1.3 cells) were cultured on culture dishes coated with fibronectin as previously described [34]. NCCs were pretreated with or without 1 μM SFN (LST Laboratories, St. Paul, MN) for 24 h, followed by concurrent exposure to 1 μM SFN and 50 or 100 mM ethanol. The stable ethanol levels were maintained by placing the cell culture dishes or plates in a plastic desiccator containing ethanol in distilled water, as described previously [39].

2.2. Quantitative real-time PCR

For quantitative real-time PCR analysis, total RNA was isolated from control and treated NCCs using a QIAGEN RNeasy mini kit (QIAGEN, Valencia, CA) according to the manufacturer's instruction. Total RNA was reverse transcribed using a QuantiTect Reverse Transcription Kit (QIAGEN, Valencia, CA) following the manufacturer's instruction. Quantitative RT-PCR was performed on a Rotor-Gene 6000 Real-time PCR system (Corbett LifeScience, Mannheim, Germany). The following primer pairs were used for this analysis: Snail1 forward: 5′-GCCCTGC ATCTGTAAGGTGT-3′, reverse: 3′-CCGGGCATTGACCTCATTCT-5′; E-cadherin forward: 5′-CATCGCCTACACCATCGTCA-3′, reverse: 3′-CCG GGCATTGACCTCATTCT-5′; β-Actin forward: 5′-CCATCCTGCGTCTGG ACCTG-3′, reverse: 3′-GTAACAGTCCGCCTAGAAGC-5′. The primers were synthesized by Integrated DNA Technologies, Inc. (IDT, Coralville, IA, USA). All assays were carried out in triplicate. Relative quantitative analysis was performed by comparing the threshold cycle number for target genes and a reference β-Actin mRNA.

2.3. Western blotting

Control and treated NCCs were washed twice with iced PBS and then lysed in cold RIPA lysis buffer with 1 mM PMSF and protease cocktail inhibitors. Whole cell lysates were centrifuged at 12,000 ×*g* for 10 min at 4 °C, and the supernatants were used for Western blot. The protein concentration in each sample was measured by using the BCA Protein Assay Reagent Kit (Pierce, Thermo Scientific, Waltham, MA). The protein levels of Histone H3, acetyl-Histone H3, cleaved caspase-3, E-cadherin, vimentin, and β–Actin were analyzed with the following antibodies: anti-acetyl-Histone H3 rabbit pAb (06-799; Millipore, Temecula, CA). anti-Histone H3 rabbit pAb (06-755; Millipore, Temecula, CA), anti-cleaved caspase-3 rabbit mAb (Cell Signaling, Beverly, MA, USA), anti-β–Actin mouse mAb (Santa Cruz, Santa Cruz, CA), anti-E-cadherin rabbit mAb (Cell Signaling, Beverly, MA, USA), anti-vimentin rabbit mAb (Cell Signaling, Beverly, MA, USA), respectively. Western blot was performed by standard protocols, and the densitometry of the blot band was analyzed by ImageJ software (National Institute of Health, USA). All Western blot analyses were performed in triplicate.

2.4. ChIP-qRT-PCR analysis

ChIP assay was performed using a ChIP assay kit (Millipore, Temecula, CA) according to the manufacturer's instruction. Briefly, NCCs from control and treated groups were collected and cross-linked with 1% formaldehyde for 10 min at 37 °C, and subjected to digest with SDS lysis buffer, then sonicated to shear DNA to the length between 200 and 1000 bp using a Qsonica Q125 sonicator (QSonica, Newtown, CT). The chromatin samples were diluted with ChIP dilution buffer and immunoprecipitated using 1 μg of H3K4me3 rabbit pAb antibody (Abcam, Cambridge, MA). Mouse monoclonal IgG (Santa Cruz, Santa Cruz, CA) was used as a negative control. Protein A agarose beads were then added to the mixture and incubated. The beads were washed, and DNA was eluted and purified for real-time PCR assay. For DNA purification, the DNA was first treated with proteinase K to remove protein and reverse the cross-links and was then purified by ChIP DNA clean & Concentrator Kits (ZYMO Research, Irvine, CA). Quantitative real-time PCR was performed on bound and input DNAs with the following primer pairs: Snail1 P1 forward: 5′- GGCATCCCTGGGTAGTGTTTT-3′, reverse: 3′-GCATGTTGGCCAGAGCGAC-5′; P2 forward: 5′- GAGCCCA AGCGGAATCTCAG-3′, reverse: 3′-GCATGTTGGCCAGAGCGAC-5′, P3 forward: 5′- CACCTGCTCCGGTCTCAG-3′, reverse: 3′-GCATGTTGGCC AGAGCGAC-5′, P4 forward: 5′- CAACAGTACGGTCACGCCC-3′, reverse: 3′-GCATGTTGGCCAGAGCGAC-5′, P5 forward: 5′- GCCTTGACAAAGG GGCGT-3′, reverse: 3′- GTCAAAGACACCCTCGGTGG-5′.

2.5. Snail1 siRNA transfection

For Snail1 siRNA transfection, NCCs were transfected with Snail1 siRNA (SMART pool: OB-TARGET plus human Snail1) (GE Healthcare Dharmacon, Lafayette, CO) or scramble control siRNA (IDT, Coralville, IA) in a final concentration of 25 nM by using Lipofectamine™2000

(Thermo Fisher, Waltham, MA), according to the manufacturer's instructions. The cells were harvested 24 h after transfection for experiments.

2.6. Analysis of apoptosis

Apoptosis was determined by the analysis of cleavage of caspase-3 and the flow cytometric analysis of Annexin V staining. Caspase-3 cleavage was determined by Western blot as described previously [34]. The number of apoptotic cells was determined by flow cytometry using a FITC Annexin V apoptosis detection kit (BD Bioscience, Franklin Lakes, NJ, USA), following the manufacturer's instruction. Briefly, control and treated NCCs were collected, washed twice with PBS, re-suspended in binding buffer and then incubated with Annexin V and PI for 15 min. The apoptotic cells were detected using a FACSCalibur flow cytometer (BD Bioscience, Franklin Lakes, NJ, USA).

2.7. Statistical analysis

Statistical analyses were performed using GraphPad Prism software (GraphPad Software, San Diego, CA, USA). All data were expressed as mean ± SEM of at least three independent experiments. Comparisons between groups were analyzed by one-way ANOVA. Multiple comparison post-tests were conducted by using Bonferroni's test. Differences between groups were considered significant at $p < 0.05$.

3. Results

3.1. Ethanol exposure induced apoptosis in NCCs

To determine whether ethanol exposure can induce apoptosis in NCCs, NCCs were exposed to 50 or 100 mM ethanol, and ethanol-induced apoptosis was analyzed by analysis of caspase-3 activation and Annexin V staining. As shown in Fig. 1A, ethanol exposure resulted in significant increases in caspase-3 activation in a dose-dependent manner, indicating that ethanol treatment can induce apoptosis in NCCs. This result was confirmed by the results from the flow cytometric analysis of Annexin V staining, which have shown that exposure of NCCs to 50 or 100 mM ethanol caused a substantial increase in the number of early apoptotic NCCs (Fig. 1B).

3.2. SFN diminished ethanol-induced inhibition of EMT in NCCs

To determine whether ethanol exposure can induce apoptosis in NCCs by inhibiting EMT and whether SFN can protect NCCs against apoptosis through restoring EMT, NCCs were cultured with 1 μM SFN alone for 24 h, followed by 24 h of concurrent exposure to 1 μM SFN and 50 mM ethanol. As shown in Fig. 2, ethanol treatment significantly inhibited EMT in NCCs, as indicated by the increased expression of E-cadherin, an EMT-suppressing marker, and a decreased expression of vimentin, an EMT-promoting marker. Treatment with SFN significantly diminished ethanol-induced changes in the expression of E-cadherin and vimentin, and restored EMT in NCCs. These results indicate that ethanol exposure can inhibit EMT in NCCs, which can be prevented by SFN.

3.3. SFN treatment diminished the ethanol-induced reduction in the H3K4me3 enrichment at the promoter regions of the Snail1 gene in NCCs

Snail1 is a key transcriptional repressor of E-cadherin and plays an important role in the regulation of EMT [23,26,27]. To determine the mechanisms by which ethanol inhibits EMT and SFN diminishes the ethanol-induced inhibition of EMT in NCCs, we next determine whether ethanol and SFN can modulate EMT through epigenetically regulating the expression of Snail1. We first determined whether ethanol exposure can reduce the H3K4 trimethylation (H3K4me3), an epigenetic modification which is associated with the activation of transcription of genes [29,40]. As shown in Fig. 3A, exposure to 50 mM ethanol resulted in a significant decrease in the levels of H3K4me3 in NCCs. Co-treatment with SFN and ethanol significantly increased the H3K4me3 expression in NCCs, indicating that ethanol-induced reduction of H3K4 trimethylation in NCCs can be diminished by SFN. In addition, the

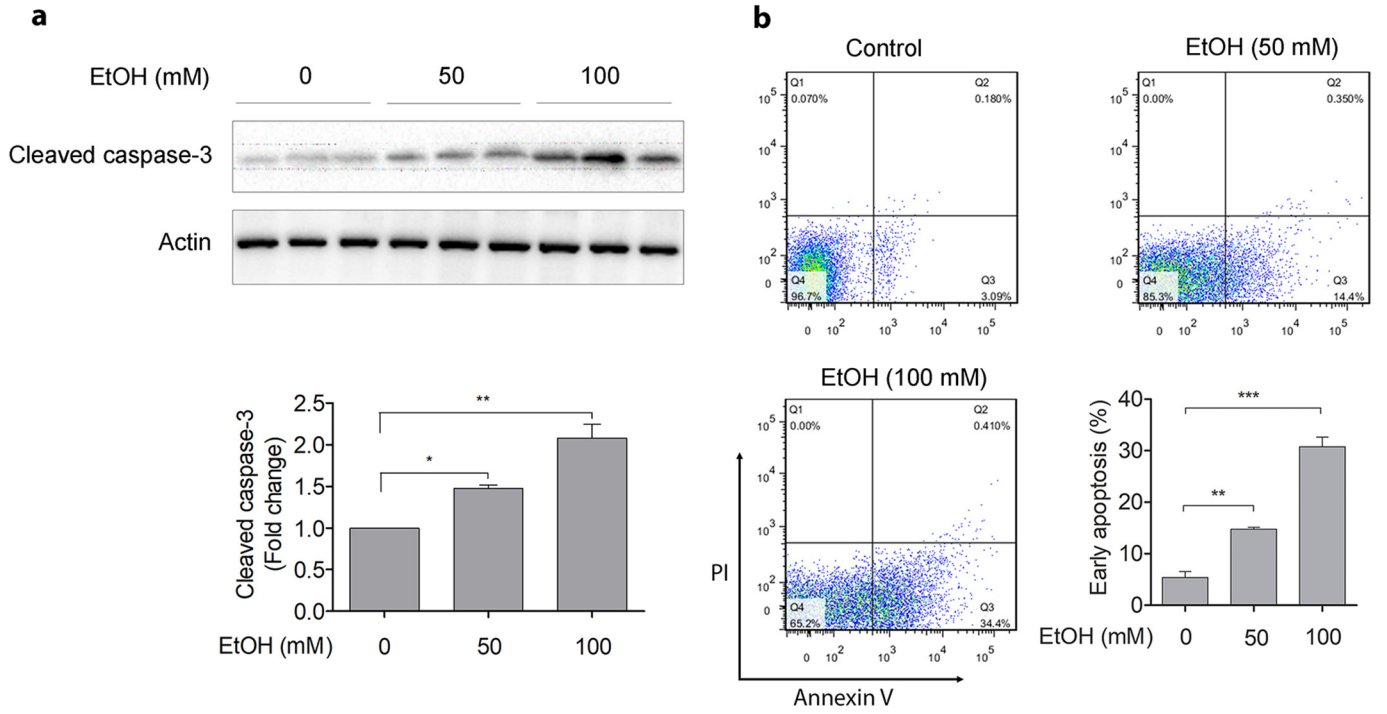

Fig. 1. Ethanol exposure induced apoptosis in NCCs. NCCs were exposed to 50 or 100 mM ethanol for 24 h. Apoptosis was determined by the analysis of caspase-3 cleavage by Western blot (A, B) or flow cytometry with Annexin V-FITC apoptosis detection kit (B). Data are expressed as fold change over control (A) or percentage (B) and represent the mean ± SEM of three separated experiments. *$p < 0.05$, **$p < 0.01$.

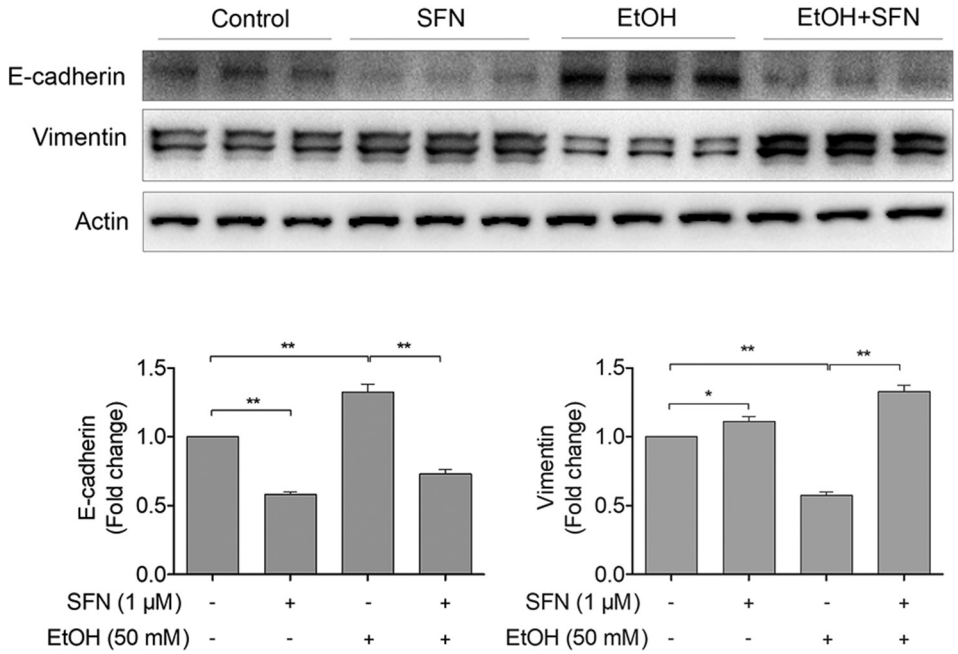

Fig. 2. SFN diminished ethanol-induced EMT inhibition in NCCs. NCCs were pre-treated with 1 μM SFN for 24 h and followed by 24 h of concurrent exposure to SFN and ethanol. The expression of EMT-suppressing factor E-cadherin and EMT-promoting factor vimentin was determined by Western blot. Data are expressed as fold change over control and represent the mean ± SEM of three separate experiments. $^{*}p < 0.05$, $^{**}p < 0.01$.

ChIP-qPCR analysis revealed that ethanol exposure resulted in a significant reduction of H3K4me3 enrichment at the promoter regions of Snail1. SFN can diminish the ethanol-induced reduction of H3K4me3 enrichment at the promoter regions of Snail1 (Fig. 3C).

3.4. SFN treatment restored the expression of Snail1 in ethanol-exposed NCCs

We next tested whether the ethanol-induced reduction of H3K4me3 enrichment at the promoter regions of Snail1 can downregulate the Snail1 and whether SFN treatment can restore the expression of Snail1 in ethanol-exposed NCCs. As expected, qRT-PCR and Western blot analysis revealed a significant decrease in the mRNA and protein expression of Snail1 in ethanol-exposed NCCs. Treatment with SFN restored the mRNA and protein expression of Snail1 in NCCs exposed to ethanol (Fig. 4). This result demonstrates that SFN can prevent ethanol-induced down-regulation of Snail1 in NCCs.

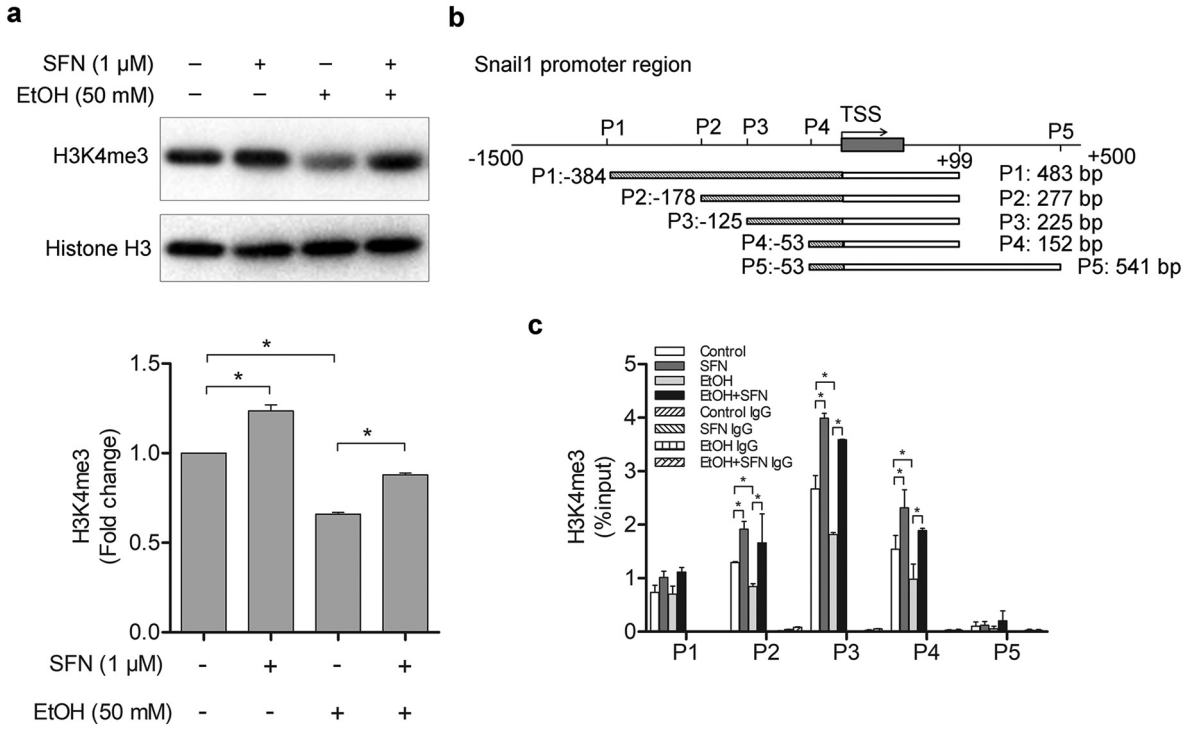

Fig. 3. SFN diminished the ethanol-induced reduction of the levels of H3K4me3 at the Snail 1 promoter. (A) The levels of H3K4me3 in control and treated NCCs were determined by Western blot. (B) Schematic depiction of the Snail1 promoter and the primer sets for ChIP-qPCR analyses. (C) H3K4me3 enrichment at corresponding sites (P1-P5) was determined by ChIP-qPCR. Data are expressed as fold change over control (A) or the percentage of input (C) and represent the mean ± SEM of three separated experiments $^{*}p < 0.05$. TSS: Transcriptional start site.

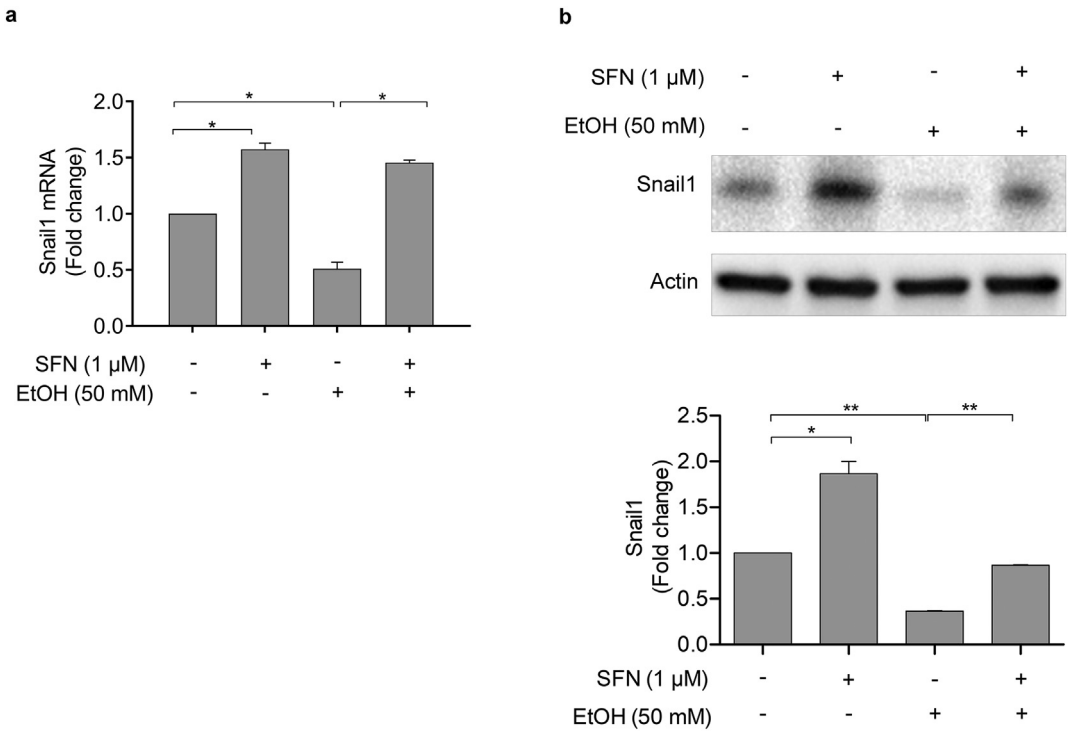

Fig. 4. SFN restored Snail1 expression in ethanol-exposed NCCs. NCCs were exposed to 1 μM SFN for 24 h, followed by concurrent exposure to 1 μM SFN and 50 mM ethanol for 24 h. The expression of Snail1 mRNA (A) and protein (B) was determined by quantitative RT-PCR and Western blot, respectively. Data are expressed as fold change over control and represent the mean ± SEM of three separated experiments. *$p < 0.05$. **$p < 0.01$.

3.5. Treatment with SFN significantly decreased the up-regulation of Snail1 target gene E-cadherin in NCCs exposed to ethanol

To determine whether ethanol-induced down-regulation of Snail1 can increase the expression of E-cadherin, a Snail1 target gene and an EMT suppressing marker, in NCCs and whether SFN can decrease ethanol-induced up-regulation of E-cadherin, the mRNA expression of E-cadherin was determined in control and ethanol-exposed NCCs. As shown in Fig. 5, exposure of NCCs to ethanol resulted in a significant increase in E-cadherin expression. Treatment with SFN significantly decreased ethanol-induced up-regulation of E-cadherin in NCCs. Since down-regulation of E-cadherin is considered to be a hallmark of EMT [25,41], these results demonstrate that SFN can prevent ethanol-induced inhibition of EMT in NCCs.

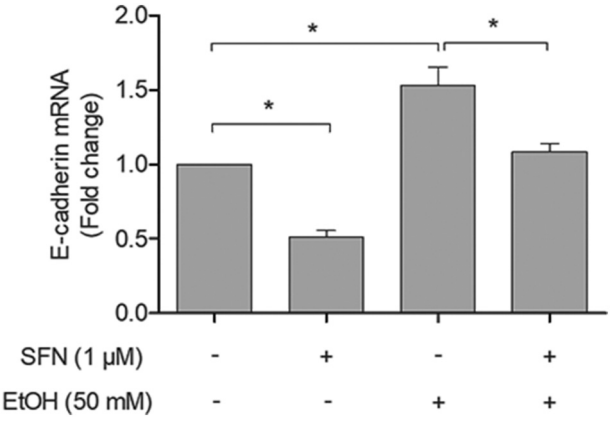

Fig. 5. SFN decreased the expression of Snail1 target gene E-cadherin in ethanol-exposed NCCs. NCCs were pre-treated with 1 μM SFN for 24 h and then exposed to 1 μM SFN and 50 mM ethanol for an additional 24 h. The expression of E-cadherin mRNA was determined by quantitative RT-PCR. Data are expressed as fold change over control and represent the mean ± SEM of three separated experiments. *$p < 0.05$.

3.1. SFN treatment significantly diminished ethanol-induced apoptosis in NCCs through up-regulation of Snail1

To determine whether SFN can prevent ethanol-induced apoptosis, caspase-3 activation and Annexin V staining were examined in control and ethanol-exposed NCCs. As shown in Fig. 6A, exposure of NCCs to ethanol resulted in a significant increase in caspase-3 activation, indicating that ethanol exposure induced apoptosis in NCCs. Treatment with SFN significantly reduced caspase-3 activation in NCCs exposed to ethanol. To further confirm that SFN can diminish ethanol-induced apoptosis through up-regulation of Snail1, apoptosis was analyzed by the flow cytometric analysis of Annexin V staining in NCCs transfected with control or Snail1 siRNA. We found that knockdown of snail1 by siRNA significantly increased ethanol-induced apoptosis as compared to the NCCs transfected with control siRNA, confirming that down-regulation of snail1 can induce apoptosis in NCCs. Down-regulation of Snail1 by siRNA also significantly diminished the protective effects of SFN on ethanol-induced apoptosis in NCCs (Fig. 6B), indicating that SFN can attenuate ethanol-induced apoptosis by modulating the expression of Snail1, further supporting our hypothesis.

4. Discussion

Apoptosis in NCCs is one of the major mechanisms underlying the pathogenesis of FASD. Recent studies have shown that SFN can epigenetically restore the expression of Bcl-2 and attenuate ethanol-induced apoptosis by enhancing histone acetylation at the Bcl-2 promoter [38]. In this study, we have shown that treatment with SFN significantly diminished ethanol-induced changes in the expression of E-cadherin and vimentin, and restored EMT in NCCs. We also found that ethanol exposure significantly reduced the levels of H3K4me3 at the promoter regions of Snail1. In addition, SFN treatment diminished the ethanol-induced reduction of H3K4me3 at the promoter regions of the

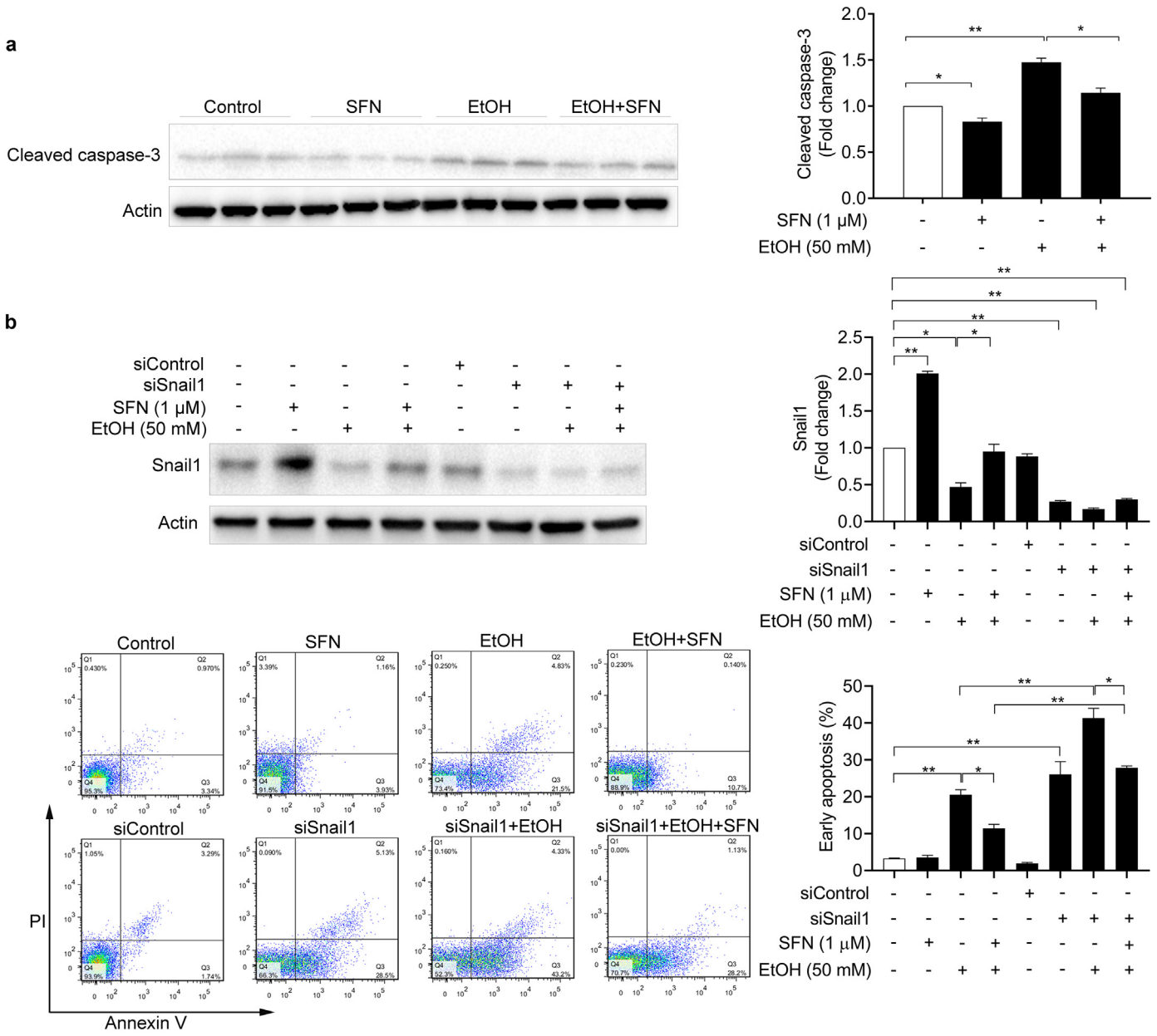

Fig. 6. SFN diminished ethanol-induced apoptosis in NCCs through up-regulation of Snail. (A) NCCs were exposed to 1 μM SFN for 24 h, followed by concurrent exposure to 1 μM SFN and 50 mM ethanol for 24 h. Apoptosis was determined by the analysis of caspase-3 cleavage using Western blot (B) NCCs transfected with control or Snail1 siRNA were exposed to 1 μM SFN for 24 h, followed by concurrent exposure to 1 μM SFN and 50 mM ethanol for 24 h. The protein expression of Snail1 was determined by Western blot. Apoptosis was determined by flow cytometry with Annexin V-FITC apoptosis detection kit. Data are expressed as fold change over control or percentage of all cells and represent the mean ± SEM of three separated experiments. *$p < 0.05$, **$p < 0.01$.

Snail1 gene, restored Snail1 gene expression and EMT, and decreased apoptosis in NCCs exposed to ethanol. We also demonstrated that SFN can diminish ethanol-induced apoptosis in NCCs, which is consistent with the results from our previous works [34,38].

It is well known that the EMT is a process which can inhibit apoptosis, promote the proliferation, migration and metastasis of tumor cells [19,20,42,43]. It has been shown that EMT conferred resistance to UV-induced apoptosis in murine mammary epithelial cell lines [21]. Studies have also shown that α-mangostin can inhibit EMT and induce apoptosis in the osteosarcoma cell line. EMT is also critical for the development of tissues and organs in the embryos [12,14,17] and plays a crucial role in the regulation of the migration of NCCs [9,10,44]. However, the involvement of EMT in the ethanol-induced apoptosis in NCCs is currently unclear. We have demonstrated that ethanol treatment significantly inhibited EMT in NCCs, as indicated by an increased expression of E-cadherin and a decreased expression of vimentin, and induced apoptosis in NCCs. Treatment with SFN significantly diminished ethanol-induced changes in the expression of E-cadherin and vimentin, restored EMT and reduced apoptosis in NCCs. These results suggested that inhibition of EMT contributes to ethanol-induced apoptosis in NCCs.

Snail1 is a member of the Snail superfamily of zinc-finger transcription factors which is involved in cell survival and differentiation [23,24,45]. Snail1 has a crucial role in the regulation of EMT through its repression of E-cadherin, an adhesion molecule mostly expressed in the surface of epithelial-like cells [16,26,45–47]. Studies have shown that ethanol treatment decreased the expression of Snail1 mRNA and inhibited EMT in B16-BL6 melanoma cells [48] and that Snail1 repressed TGF- β-induced apoptosis in hepatocytes by triggering EMT [42]. Ethanol exposure was also found to be able to down-regulate the

Snail2 in NCCs of chick embryos [49]. Consistent with these findings, we found that ethanol exposure resulted in a significant reduction in the expression of Snail1, accompanied by a dramatic increase in the expression of E-cadherin in NCCs. These results suggested that ethanol exposure can inhibit EMT in NCCs by down-regulating Snail1, leading to the up-regulation of E-cadherin.

The expression of Snail1 can be regulated by a variety of mechanisms. Studies have shown that NF-kB, HIF-1a, SMAD, STAT3, and Gli1 can directly interact with the Snail1 promoter and regulate Snail1 at the transcriptional level [26,43,45]. The expression of Snail1 can also be regulated epigenetically. In eukaryotic cells, epigenetic regulation of gene expression mainly comprises DNA modification and histone modification [29,46,47]. It has been reported that HDAC inhibitor valproic acid elevated histone acetylation to transcriptionally activate Snail1 gene expression and promote EMT in colorectal cancer cells [50]. HDAC inhibitors, Trichostatin A (TSA) and Suberoylanilide hydroxamic acid (SAHA) also induced EMT in prostate cancer cells [51]. While acetylation of histone lysine residues can increase genome accessibility, thus promoting gene transcription, methylation at lysine residues can have either activating or repressing effects on gene transcription. It is well-known that trimethylation of histone 3 at lysine 27 (H3K27me3) is associated with transcription repression while trimethylation of histone 3 at lysine 4 (H3K4me3) is associated with transcription activation [52]. H3K4m3 is highly enriched at active promoters near transcription start sites and is widely used as a histone mark to identify active gene promoters [53]. In this study, we found that ethanol exposure resulted in a significant decrease in the levels of H3K4me3 in NCCs and a significant reduction of H3K4me3 enrichment at the promoter regions of Snail1. Co-treatment with SFN and ethanol significantly increased the H3K4me3 expression in NCCs and diminished ethanol-induced reduction of H3K4me3 enrichment at the promoter regions of Snail1. SFN also restored the expression of Snail1 and EMT and reduced apoptosis in ethanol-exposed NCCs. Down-regulation of Snail1 by siRNA significantly diminished the protective effects of SFN on ethanol-induced apoptosis in NCCs. These results indicate that the ethanol can induce apoptosis in NCCs by inhibiting EMT through epigenetically down-regulating the expression of Snail1 which can be prevented by SFN.

SFN is a well-studied dietary inhibitor of HDAC [35,37]. Our recent studies have shown that SFN can epigenetically restore the expression of Bcl-2 and attenuate ethanol-induced apoptosis by enhancing histone acetylation at the Bcl-2 promoter [38]. In this study, we have shown that as an HDAC inhibitor, SFN significantly increased H3K4 methylation at the promoter regions of Snail1 and activated the transcriptional expression of Snail1, consistent with the results from other studies. For example, studies on a variety of cultured cells have shown that HDAC inhibitors induced an increase in H3K4 methylation in these cells [54]. Other studies have also shown that HDAC inhibitors increased lysine methylation on specific histone lysine residues, including H3K4me2 and H3K4me3 [55,56]. Moreover, HDAC inhibition by VPA increased histone H3K4 methylation in rat cortical neurons and astrocytes [56]. SFN has also been shown to increase H3K4 methylation in prostate cancer cells [57]. However, the mechanisms by which SFN increases H3K4Me3 enrichment at the promoter regions of Snail1 is not clear. One possibility is that SFN-induced increase in the histone acetylation may influence histone methylation by altering expression and activity of the H3K4 methyltransferase or H3K4 demethylase. Studies have shown that H3 peptides or HDAC inhibitors can increase the activity of the H3K4 methyltransferase MLL4 and that HDAC inhibitors can also decrease the activity of the H3K4 demethylase KDM1A(LSD1), KDM5A (JARID1A) or KDM5B (PLU1) [58–62]. Elucidation of the mechanistic link between SFN-induced HDAC inhibition and H3K4me3 enrichment at the promoter regions of Snail1 is currently under investigation in our laboratory.

In summary, our studies indicate that ethanol exposure can inhibit EMT through down-regulation of Snail1 by decreasing H3K4M3 enrichment at the promoter regions of Snail1 and increase apoptosis in NCCs. SFN treatment can diminish the ethanol-induced reduction in the H3K4M3 enrichment at the promoter regions of Snail1, restore mRNA expression of Snail1 and EMT in NCCs exposed to ethanol. However, down-regulation of Snail1 by siRNA significantly diminished the protective effects of SFN on ethanol-induced apoptosis in NCCs. These findings demonstrate that the disruption of EMT contributes to ethanol-induced apoptosis in NCCs and that SFN can prevent ethanol-induced apoptosis by restoring EMT though epigenetically regulating the expression of EMT-related genes, suggesting that elucidation of Snail1's role in EMT and ethanol-induced apoptosis in NCCs may provide critical insight into the pathogenesis of FASD.

List of abbreviations

DNMT	DNA methyltransferase
EMT	epithelial-mesenchymal transition
FASD	Fetal Alcohol Spectrum Disorders
H3K4me3	trimethylation of histone H3 lysine4
HDAC	histone deacetylase
NCC	neural crest cell
SFN	sulforaphane

Authors' contributions

YHL and SYC conceptualized and designed the experiments and participated in data interpretation and manuscript preparation. YHL, FQY and JL performed the experiments and participated in data analysis. LHL, TW and WKF participated in data interpretation and discussion. All authors reviewed the manuscript.

Transparency document

The Transparency document associated with this article can be found, in online version.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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