

## RESEARCH PAPER

# Sulforaphane protects against ethanol-induced oxidative stress and apoptosis in neural crest cells by the induction of Nrf2-mediated antioxidant response

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## BACKGROUND AND PURPOSE

Nuclear factor erythroid 2-related factor (Nrf2) is a transcription factor that up-regulates a diverse array of antioxidant genes and protects cells from oxidative damage. This study is designed to determine whether D-L-sulforaphane (SFN) can protect neural crest cells (NCCs), an ethanol-sensitive cell population implicated in fetal alcohol spectrum disorders, against ethanol-induced apoptosis and whether protective effects of SFN are mediated by the induction of Nrf2-mediated antioxidant response.

## EXPERIMENTAL APPROACH

Control, SFN-treated or Nrf2-siRNA transfected NCCs were exposed to ethanol. Nrf2 activation, the expression and activities of Nrf2 downstream antioxidant proteins, reactive oxygen species generation and apoptosis were determined in control and ethanol-exposed NCCs.

## KEY RESULTS

Exposure of NCCs to SFN alone significantly increased Nrf2 activation and the expression of Nrf2 downstream antioxidants as well as the activities of the antioxidant enzymes. Treatment of NCCs with SFN along with ethanol significantly decreased ethanol-induced oxidative stress and apoptosis. In contrast, knockdown of Nrf2 by siRNA significantly increased the sensitivity of NCCs to ethanol-induced oxidative stress and apoptosis. Suppression of Nrf2 signalling in NCCs also significantly diminished SFN-mediated antioxidant response and abolished the protective effects of SFN on ethanol-induced oxidative stress and apoptosis.

## CONCLUSIONS AND IMPLICATIONS

These results demonstrated that Nrf2-mediated antioxidant response plays an important role in the susceptibility of NCCs to ethanol-induced oxidative stress and apoptosis and that the protection of SFN against ethanol-induced oxidative stress and apoptosis in NCCs is mediated by the induction of Nrf2 signalling.

## Abbreviations

ARE, antioxidant response element; D3T, 3H-1,2-dithiole-3-thione; DCHFDA, 2',7'-dichlorodihydrofluorescein diacetate; FASD, fetal alcohol spectrum disorder; MTS, 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt; NCC, neural crest cell; NQO1, NAD(P)H:quinone oxidoreductase 1; Nrf2, nuclear factor erythroid 2-related factor; ROS, reactive oxygen species; SFN, D-L-sulforaphane; SOD, superoxide dismutase

## Introduction

Fetal alcohol spectrum disorder (FASD) is one of the most common permanent birth defects caused by maternal consumption of alcohol during pregnancy. The effects of prenatal ethanol exposure include physical, mental, behavioural and/or learning disabilities with possible lifelong implications. Prenatal alcohol exposure is considered to be the leading known non-genetic cause of mental retardation (Burd, 2004).

The pathogenesis of FASD is a complex, multifactorial process that includes the vulnerability of selected cell populations to ethanol-induced apoptosis (Kotch and Sulik, 1992; Cartwright and Smith, 1995; Smith, 1997; Dunty *et al.*, 2001). Among the vulnerable cell populations are neural crest cells (NCCs) (Sulik *et al.*, 1981; Kotch and Sulik, 1992; Cartwright and Smith, 1995; Chen and Sulik, 1996; Chen and Sulik, 2000). The NCC is a multipotent progenitor cell population that can give rise to a diversity of neural and non-neural cell types, such as melanocytes, neurons, glial cells, endocrine cells, as well as mesenchymal cells that form craniofacial cartilages, bones, dermis, adipose tissue and vascular smooth muscle cells (Teng and Labosky, 2006; Hall, 2008). Studies have demonstrated that NCCs are vulnerable to ethanol-induced cytotoxicity. Ethanol can diminish NCC population by the induction of apoptosis, which appears to contribute heavily to subsequent abnormalities (Sulik *et al.*, 1981; Kotch and Sulik, 1992; Cartwright and Smith, 1995; Chen and Sulik, 1996; Smith, 1997).

Previous studies have revealed that oxidative stress plays an important role in ethanol-induced apoptosis and dysmorphology (Kotch *et al.*, 1995; Chen and Sulik, 1996; Henderson *et al.*, 1999; Chen and Sulik, 2000; Dong *et al.*, 2008). Reactive oxygen species (ROS) generation has been observed in ethanol-exposed cultured cells, including NCCs (Chen and Sulik, 1996; Chen and Sulik, 2000; Yan *et al.*, 2010), as well as in mouse embryos exposed to ethanol both *in vitro* and *in vivo* (Kotch *et al.*, 1995; Dong *et al.*, 2008). In addition, superoxide dismutase (SOD) has been shown to diminish ethanol-induced superoxide anion generation, cell death and the incidence of neural tube defects in cultured mouse embryos (Kotch *et al.*, 1995). Studies have also shown that maternal treatment with an SOD and catalase mimetic, EUK-134, reduced ethanol-induced apoptosis in selected cell populations in the developing limb buds and diminished subsequent limb defects in mouse embryos (Chen *et al.*, 2004). More recently, it has been demonstrated that nuclear factor erythroid 2-related factor 2 (Nrf2) signalling is involved in ethanol-induced apoptosis in NCCs and in mouse embryos (Dong *et al.*, 2008; Yan *et al.*, 2010; Chen, 2012).

Nrf2 is a CNC (cap 'n' collar) bZIP (basic region leucine zipper) group of transcription factors which is broadly expressed in a variety of tissues (Kensler *et al.*, 2007). Quiescent Nrf2 localizes in the cytoplasm and is rapidly turned over through a specific ubiquitin-26S proteasome pathway controlled by the Keap1/Cul3-independent ubiquitin ligase (E3) (Li and Kong, 2009). Nrf2 is activated in response to a range of oxidative and electrophilic stimuli including ROS, heavy metals and certain disease processes (Nguyen *et al.*, 2009). Upon activation, Nrf2 mediates antioxidant response by the induction of a broad range of genes including phase 2

enzymes, such as NAD(P)H:quinone oxidoreductase 1 (NQO1) and heme oxygenase-1, and antioxidant proteins, such as SOD and catalase (Kensler *et al.*, 2007; Nguyen *et al.*, 2009). Both genetic and biochemical studies have implicated the Nrf2 signalling pathway in the defence against a wide range of chemical toxicity, cancer and chronic diseases in which oxidative stress is involved (Kensler *et al.*, 2007). Recent studies have also shown that maternal ethanol treatment increased Nrf2 protein levels and Nrf2-antioxidant response element (ARE) binding in mouse embryos. Up-regulation of Nrf2 signalling by the Nrf2 inducer, 3H-1,2-dithiole-3-thione (D3T), significantly decreased ethanol-induced ROS generation and apoptosis in mouse embryos. These results demonstrate that Nrf2 signalling is involved in the induction of an antioxidant response in ethanol-exposed mouse embryos (Dong *et al.*, 2008). This premise has also been supported by studies that have shown that Nrf2-dependent maintenance of GSH homeostasis in cerebral cortical neurons is critical for preventing ethanol-induced oxidative stress and apoptosis (Narasimhan *et al.*, 2011), and that resveratrol can protect the cerebellar granule neurons against ethanol-induced cell death in a rat model of FASD by the induction of Nrf2 (Kumar *et al.*, 2011).

D,L-sulforaphane (SFN) is a chemical that is abundant in broccoli sprouts (Keum *et al.*, 2005). Compelling evidence indicates that SFN-rich broccoli sprouts and other sources of dietary SFN trigger the induction of phase 2 detoxifying genes and antioxidant enzymes through activation of Nrf2 signalling and can aid in preventing cancer and other diseases (Dinkova-Kostova *et al.*, 2002). It has been demonstrated that SFN reacts with thiol groups of Keap1 to form thionoacyl adducts and that the modification of Keap1 releases Nrf2 from its sequestration and activates Nrf2 (Hong *et al.*, 2005). While being a well-recognized and effective strategy in cancer chemoprevention (Cheung and Kong, 2010; Wang and Shan, 2012), the use of SFN to enhance cellular defences through the up-regulation of endogenous antioxidants in embryonic cells has not been investigated.

With a great deal of evidence indicating that NCCs are particularly vulnerable to ethanol-induced apoptosis and that ROS plays a major role in alcohol's teratogenesis, along with recognition that antioxidant capabilities are minimal in embryos, strategies directed towards up-regulation of endogenous antioxidant activity in this particular cell population provide a novel and promising approach for reducing alcohol's teratogenic impact. To this end, using an NCC cell line, JoMa 1.3 cells, the current study was designed to test the hypothesis that SFN, a naturally occurring isothiocyanate, can provide protection against ethanol-induced oxidative stress and apoptosis in NCCs by the induction of Nrf2-mediated antioxidant response. This is, to our knowledge, the first study attempting to prevent ethanol-induced apoptosis in embryonic cells through the use of bioactive compounds naturally present in vegetables. The results from this study demonstrated that Nrf2-mediated antioxidant response plays an important role in the susceptibility of NCCs to ethanol-induced oxidative stress and apoptosis and that the protection of SFN against ethanol-induced oxidative stress and apoptosis in NCCs is mediated by the induction of Nrf2 signalling. These findings support the premise that Nrf2 is a key molecular target for diminishing alcohol's teratogenesis

and provide a note of optimism for the development of clinically applicable antioxidant strategies for the treatment or prevention of FASD.

## Methods

### *Cell culture, treatments and siRNA transfection*

NCCs (JoMa1.3 cells) were provided by Dr. Schorle and cultured as described previously by Maurer *et al.* (2007). Briefly, cells were grown on cell culture dishes coated with fibronectin and maintained in DMEM: Ham's F12 (1:1) at 37°C in 5% CO<sub>2</sub>/95% air. For dose–response analysis, NCCs were treated with 0.25, 0.5, 1, 2 or 4 μM SFN (LKT Laboratories, St. Paul, MN, USA) alone for 24 h, followed by 24 h of concurrent exposure to SFN and 50, 100 or 200 mM ethanol. For time–response study, NCCs were treated with 1 μM SFN alone for 24 h, followed by 24, 48 or 72 h of concurrent exposure to 1 μM SFN and 100 mM ethanol. For the majority of this study, NCCs were treated with 1 μM SFN alone for 24 h, followed by 24 h of concurrent exposure to SFN and 100 mM ethanol. Stable ethanol levels were maintained by placing the cell culture plates in a plastic desiccator containing 50, 100 or 200 mM ethanol, respectively, in distilled water as described previously (Yan *et al.*, 2010). For siRNA inhibition studies, NCCs were transfected with MISSION® Nrf2-siRNA or negative control siRNA (Sigma-Aldrich, St Louis, MO, USA) at a final concentration of 40 nM in the presence of N-TER™ Nanoparticle siRNA Transfection System (Sigma-Aldrich), following the manufacturer's instructions. The cells were harvested 48 h after transfection for mRNA and protein extraction and additional analysis.

### *DNA transfection and reporter gene assays*

For luciferase reporter gene assays, NCCs were transfected with pNQO1 ARE-luciferase plasmid (a generous gift from Dr. John D. Hayes) (Nioi *et al.*, 2003) and the pRL-TK Renilla control plasmid (Promega, Madison, WI, USA) using Lipofectamine 2000 reagent (Life Technologies Inc., Gaithersburg, MD, USA), according to the manufacturer's instructions. Transfected cells treated with ethanol or/and SFN were lysed, and Renilla and firefly luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega) with a lumat LB 9507 ultra-sensitive tube luminometer (Berthold Technologies, Oak Ridge, TN, USA). The firefly luciferase activities were normalized to Renilla luciferase enzyme activity.

### *Quantitative real-time PCR*

To determine the mRNA expression of Nrf2 downstream target genes, total RNAs were isolated from NCCs using the QIAGEN RNeasy mini kit (QIAGEN, Valencia, CA, USA), according to the manufacturer's instructions. The reverse transcription reaction was carried out by incubation of 500 ng of total RNA with a reaction mixture containing 4 μL of transcriptase reaction buffer (5×), 2 μL of dNTPs (contains 10 mM each dNTP), 10 U of reverse transcriptase, 20 U of RNase inhibitor and 1200 pmol random hexamer primer. Quantitative RT-PCR was carried out using a standard proto-

col on a Rotor-Gene 6000 Real-Time system (Corbett Life Science, Sydney, Australia) with the FastStart Universal SYBR Green Master qPCR kit (Roche Diagnostics, Mannheim, Germany). The following primer pairs were used for this analysis: SOD1: forward: 5'-TAACTGAAGGCCAGCATGGG-3'; reverse: 5'-CATGGACCACCATTGTACGG-3'; catalase: forward: 5'-CACTCAGGTGCGGACATTCT-3'; reverse: 5'-TCCGGAGTGGGAGAATCCAT-3'; β-actin: forward: 5'-AGCCTTCC TTCTTGGGTATGGAATC-3'; reverse: 5'-GGAGCAATGATCTT GATCTTCATGG-3'. These primers were designed using Primer3 and synthesized by Integrated DNA Technologies, Inc. (IDT, Coralville, IA, USA). All real-time PCR assays were performed in triplicate. Relative quantitative analysis was carried out by comparing threshold cycle number for target genes and a reference β-actin mRNA.

### *Western blotting*

Western blotting was performed as described previously (Dong *et al.*, 2011). Briefly, NCCs were washed with PBS and then lysed in pre-cold RIPA (Cell Signaling, Beverly, MA, USA) with 1 mM freshly prepared phenylmethylsulfonyl fluoride (Sigma-Aldrich) and protease cocktail inhibitors (Roche Applied, Indianapolis, IN, USA). Cell lysates were then 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 determined using bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA) following the manufacturer's instructions. Western blots were performed by standard protocols. The levels of Nrf2, SOD1, catalase and caspase-3 were analysed with the following antibodies respectively: rabbit polyclonal anti-Nrf2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit polyclonal anti-superoxide dismutase (SOD) antibody (Abcam, Cambridge, MA, USA), rabbit polyclonal anti-catalase antibody (Abcam) and rabbit monoclonal anti-cleaved caspase-3 antibody (Cell Signaling). The membranes were then developed on a Kodak X-OMAT 2000A imaging system (Kodak, Rochester, NY, USA) and the intensity of the protein band was analysed using the Adobe Photoshop CS software (Adobe Systems, San Jose, CA, USA). All Western blot analyses were performed in triplicate.

### *Determination of antioxidant enzyme activities*

The activities of two antioxidant enzymes, SOD and catalase, were determined as described previously (Yan *et al.*, 2010). Total SOD activity was determined using an SOD Assay Kit (Dojindo Molecular Technologies, Inc., Gaithersburg, MD, USA), according to the manufacturer's protocol, and was measured using a SpectraMax M5 Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). The activity of catalase was determined using an Amplex Red Catalase Assay Kit (Molecular Probes, Eugene, OR, USA) according to the manufacturer's instruction and was measured using a SpectraMax M5 Microplate Reader.

### *Detection of intracellular ROS generation*

Intracellular ROS generation was evaluated using a 2',7'-dichlorodihydrofluorescein diacetate (DCHFDA) assay as described previously (Yan *et al.*, 2010). Briefly, control and treated NCCs were washed with HBSS without phenol red

and lysed in pre-cold RIPA. Cell lysates were centrifuged at  $12\,000\times g$  for 10 min at  $4^{\circ}\text{C}$ . The supernatant, which was equivalent to  $40\ \mu\text{g}$  of protein, was transferred to a 96-well microplate and incubated with  $20\ \mu\text{M}$  DCHFDA (Molecular Probes) at  $37^{\circ}\text{C}$  for 30 min. The fluorescence intensity was determined using a SpectraMax M5 Microplate Reader with a fluorescence excitation at 485 nm and emission at 538 nm. The ROS levels in experimental groups were expressed as fold change over control.

### Analysis of cell viability and apoptosis

Cell viability was measured using an MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt) assay kit (Promega), following the manufacturer's protocol. Apoptosis was determined by the analysis of caspase-3 cleavage and activity, and phosphatidylserine externalization. Caspase-3 cleavage was determined by Western blot as described previously (Dong *et al.*, 2008). Caspase-3 activity was determined by using homogeneous luminescent Caspase-Glo™ 3/7 Assay (Promega), following the manufacturer's instructions. The phosphatidylserine externalization was determined by flow cytometry using an Annexin V FITC Apoptosis Detection Kit (BD Bioscience, San Jose, CA, USA) as described previously (Yan *et al.*, 2010).

### Statistical analysis

Statistical analyses were performed using GraphPad Prism software (GraphPad Software, San Diego, CA, USA). All data are expressed as means  $\pm$  SEM of three separate experiments. Comparisons between groups were analysed by a one-way ANOVA. Multiple comparison post-tests between groups were conducted by using Bonferroni's test. Differences between groups were considered significant at  $P < 0.05$ .

## Results

### SFN treatment significantly reduced ethanol-induced cell death in NCCs

To determine whether SFN can reduce ethanol-induced cell death in NCCs, JoMa 1.3 cells were treated with ethanol or/and SFN at different concentrations for 24 h or treated with 100 mM ethanol or/and  $1\ \mu\text{M}$  SFN for 24, 48 or 72 h. JoMa 1.3 cells were chosen as a model for the proposed studies because (i) they are NCCs derived from mouse embryos; (ii) this cell line expresses early NCC markers and can be instructed to differentiate into neurons, glia, smooth muscle cells, melanocyte and chondrocytes (Maurer *et al.*, 2007); and (iii) this cell line represents a powerful tool for studying the mechanisms of NCC development (Murphy *et al.*, 2011) and has been used to elucidate the role of miRNAs in NCC development (Cordes *et al.*, 2009; Sheehy *et al.*, 2010). As shown in Figure 1A–C, while SFN at 0.5, 1 or  $2\ \mu\text{M}$  and at 0.25, 0.5, 1 or  $2\ \mu\text{M}$  significantly reduced cell death in NCCs exposed to 50 and 100 mM ethanol, respectively, SFN at the concentrations up to  $4\ \mu\text{M}$  did not significantly diminish cell death in NCCs treated with 200 mM ethanol. The maximum reduction in ethanol-induced cell death was observed in the cells treated with  $1\ \mu\text{M}$  SFN. In

addition, time–response analyses illustrated that while  $1\ \mu\text{M}$  SFN can significantly reduce cell death in NCCs exposed to 100 mM ethanol for up to 72 h, the maximum protection was observed in NCCs exposed to ethanol for 24 h (Figure 1D–F). These results indicate that SFN can potently prevent ethanol-induced cell death in NCCs. Based on these observations,  $1\ \mu\text{M}$  SFN, 100 mM ethanol and the 24 h culture period were utilized for the subsequent experiments.

### Ethanol exposure and SFN treatment significantly increased Nrf2 protein expression in NCCs

To determine whether treatment with ethanol or SFN can increase Nrf2 protein expression in NCCs, the levels of Nrf2 protein were examined in NCCs exposed to ethanol or SFN alone or in combination by Western blot analysis. As shown in Figure 2A, exposure to 50, 100 or 200 mM ethanol for 24 h resulted in a moderate increase in Nrf2 expression. Treatment with  $1\ \mu\text{M}$  SFN alone significantly increased the levels of Nrf2 protein in NCCs. The combination of the SFN treatment and 100 mM ethanol exposure resulted in a significantly greater increase in Nrf2 protein expression as compared to the group treated with ethanol alone. However, induction of Nrf2 expression by ethanol or SFN alone or in combination was dramatically decreased in NCCs in which Nrf2 expression was inhibited by siRNA, indicating that Nrf2 signalling in NCCs can be efficiently knocked down by siRNA (Figure 2B).

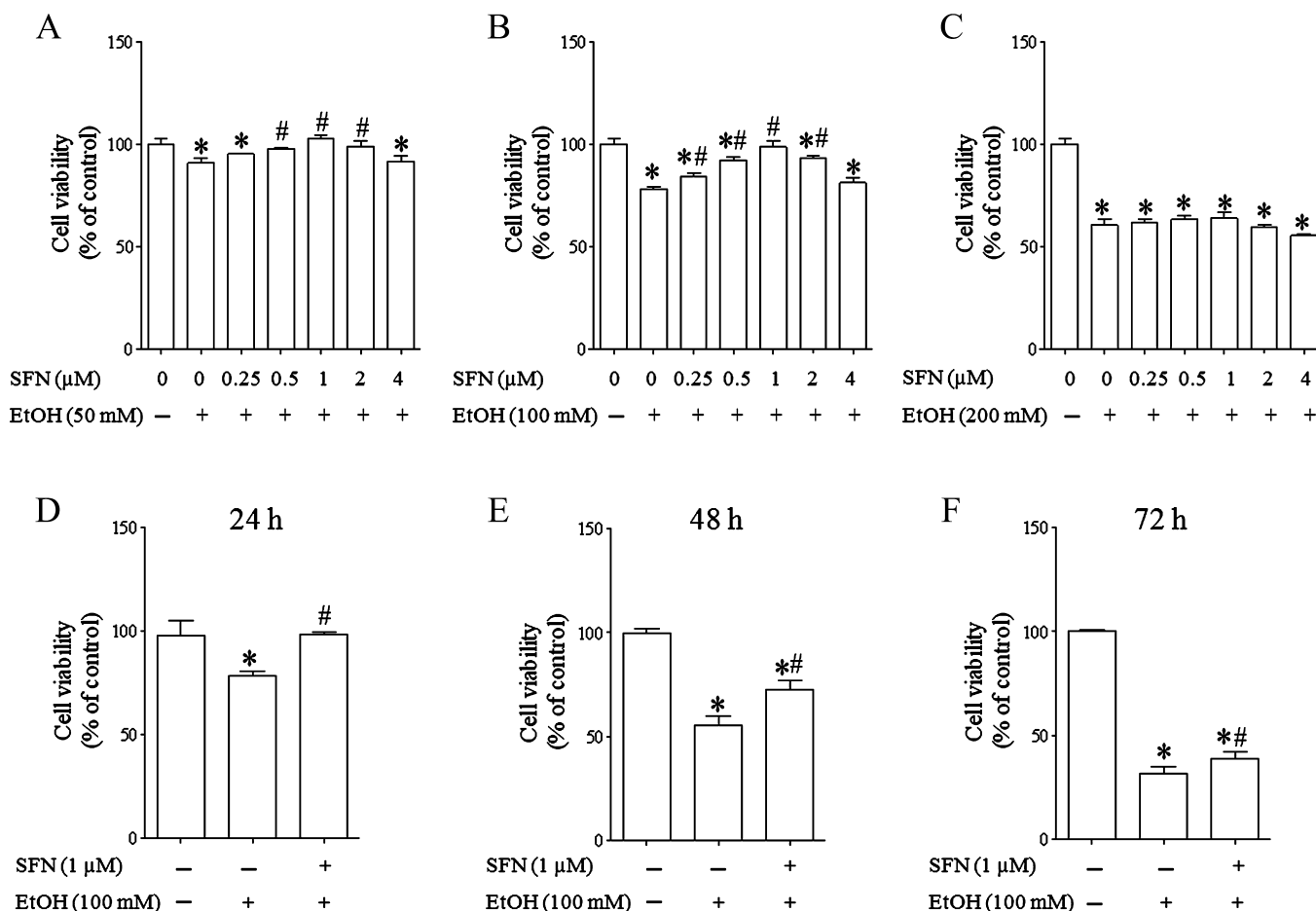
### Ethanol exposure and SFN treatment significantly increased the ARE promoter activity in NCCs

Nrf2 activates transcription of its target genes through binding specifically to ARE found in the gene promoters (Ohtsuji *et al.*, 2008; Nguyen *et al.*, 2009). To determine whether treatment with ethanol and SFN can activate Nrf2, we examined the ARE promoter activity in NCCs exposed to ethanol and SFN. ARE-luciferase reporter assay showed an increased ARE promoter activity in NCCs exposed to ethanol alone. Treatment with SFN alone or in combination with ethanol resulted in a significantly greater increase in the ARE promoter activity as compared to the group treated with ethanol alone. However, ethanol- or SFN-induced increase in ARE promoter activity was significantly decreased in NCCs transfected with Nrf2-siRNA (Figure 3). These data demonstrate that, in addition to increasing Nrf2 protein expression, ethanol and SFN can activate ARE activity in NCCs.

### SFN treatment significantly increased Nrf2 transcriptional activity in control and ethanol-exposed NCCs

To further confirm that ethanol and SFN can increase Nrf2 transcriptional activity in NCCs, the effects of ethanol and SFN on mRNA expression of Nrf2 target genes, SOD1 and catalase, were determined in NCCs. We found that ethanol exposure resulted in a moderate increase in mRNA expression of SOD1 and catalase. mRNA expression of these two genes was significantly increased in NCCs treated with SFN alone or in combination with ethanol. However, these effects were not observed in Nrf2-siRNA transfected NCCs exposed to ethanol and SFN (Figure 4).





**Figure 1**

SFN treatment significantly reduced ethanol-induced cell death in NCCs. (A–C) NCCs were treated with 50, 100 or 200 mM ethanol alone or co-treated with SFN at indicated concentrations for 24 h. (D–F) NCCs were treated with 100 mM ethanol alone or in combination with 1  $\mu$ M SFN for 24, 48 or 72 h. Ethanol-induced cell death was determined by MTS assay as described in the *Method* section. Data are expressed as percentage of control and represent the mean  $\pm$  SEM of three separate experiments. \* $P$  < 0.05 versus control. # $P$  < 0.05 versus EtOH.

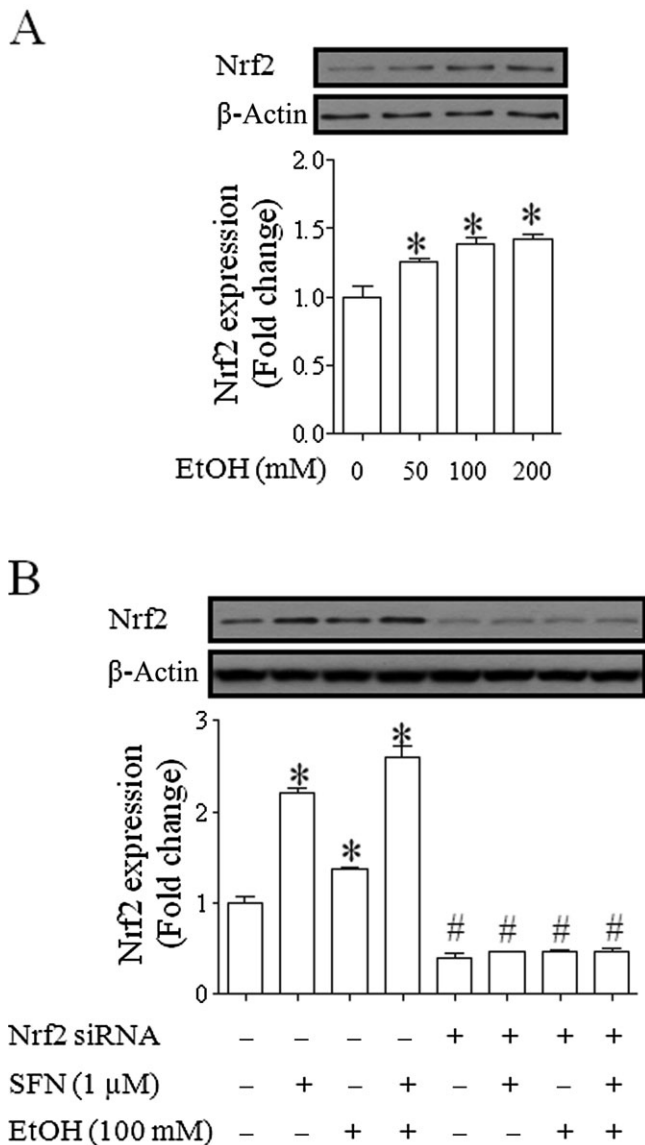
### *Treatment with SFN significantly increased the expression of antioxidant proteins and their catalytic activity in control and ethanol-exposed NCCs*

To examine whether increases in mRNA expression of Nrf2 target genes in NCCs exposed to ethanol and SFN are accompanied by over-expression of Nrf2 downstream antioxidant proteins, the expression of antioxidant proteins, SOD1 and catalase, was determined by Western blot. As shown in Figure 5, exposure of NCCs to ethanol alone resulted in a moderate increase in the protein expression of SOD1 and catalase. Treatment with SFN alone or in combination with ethanol resulted in a significantly greater increase in the protein expression of SOD1 and catalase, confirming the corresponding changes in major antioxidant proteins that accompany mRNA induction. In contrast, ethanol- or SFN-induced up-regulation of these two antioxidant proteins was significantly decreased in NCCs transfected with Nrf2-siRNA, demonstrating that SFN-induced up-regulation of antioxidant proteins in ethanol-exposed NCCs is Nrf2-dependent.

To determine the corresponding changes in the activities of major antioxidant enzymes that accompany protein induction in NCCs, the activities of SOD and catalase were determined in the NCCs exposed to ethanol and SFN. While treatment with ethanol or SFN alone significantly increased the activities of SOD and catalase, treatment with SFN in combination with ethanol resulted in significantly greater increases in the activities of these antioxidant enzymes. However, knockdown of Nrf2 expression by siRNA significantly reduced ethanol- and SFN-induced increases in the activities of SOD and catalase in NCCs (Figure 6), indicating that SFN-induced antioxidant response in ethanol-exposed NCCs is mediated by Nrf2 signalling.

### *ROS generation was significantly decreased in ethanol-exposed NCCs treated with SFN but increased in ethanol-exposed NCCs transfected with Nrf2-siRNA*

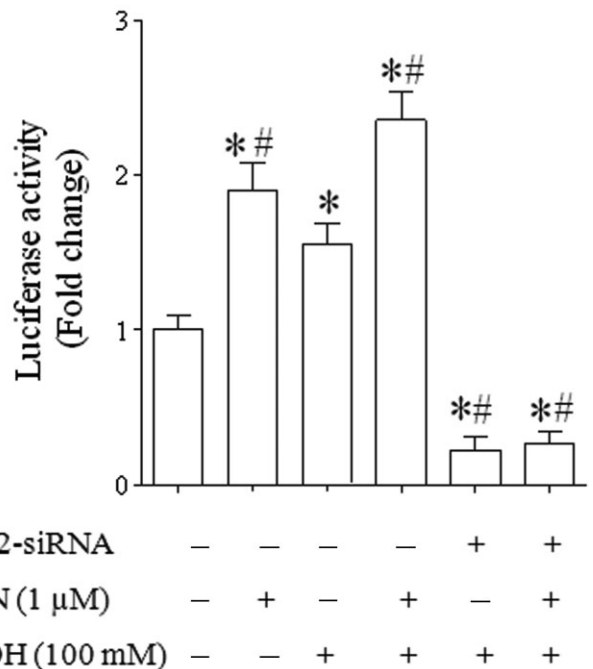
To determine whether SFN-induced activation of Nrf2 and induction of Nrf2 downstream antioxidant proteins can



**Figure 2**

Ethanol exposure and SFN treatment significantly increased Nrf2 protein expression in NCCs. (A) NCCs were treated with 50, 100 or 200 mM ethanol for 24 h. (B) NCCs transfected with control or Nrf2-siRNA were treated with 100 mM ethanol or 1 μM SFN alone or co-treated with ethanol and SFN. Protein expression of Nrf2 was determined by Western blot. Data are expressed as fold change over control and represent the mean ± SEM of three separate experiments. (A) \**P* < 0.05 significant difference between control and treatment groups. (B) \**P* < 0.05 significant difference between control and treatment groups in NCCs transfected with control siRNA. #*P* < 0.05 compared with corresponding treatment in control siRNA group.

reduce ethanol-induced ROS generation in NCCs, a DCHFDA assay was used to analyse the ROS generation in NCCs transfected with control or Nrf2-siRNA. Consistent with our previous studies, ethanol exposure resulted in a significant increase in ROS generation. Exposure of NCCs transfected with Nrf2-siRNA to ethanol resulted in a significantly greater



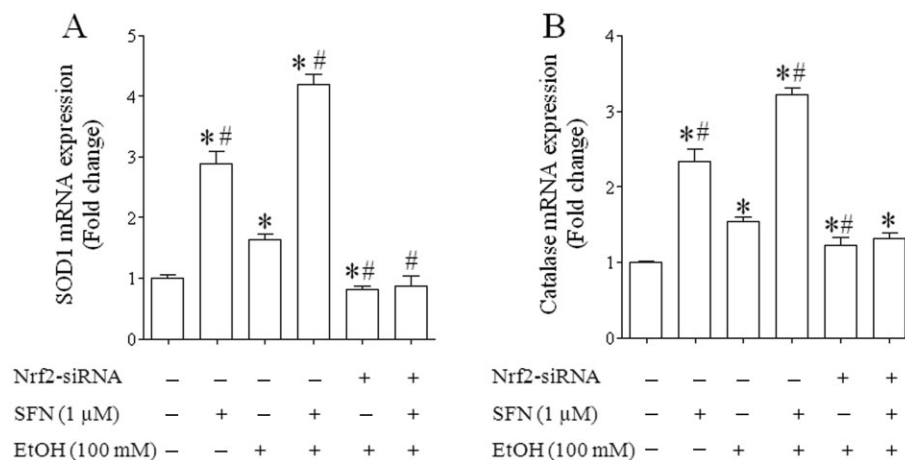
**Figure 3**

Ethanol exposure and SFN treatment significantly increased the ARE promoter activity in NCCs. NCCs transfected with control or Nrf2-siRNA and pNQO1 ARE-luciferase plasmid were treated with 100 mM ethanol or 1 μM SFN alone or co-treated with ethanol and SFN. The firefly luciferase activities were measured as described in the *Methods* section. Data are expressed as fold change over control and represent the mean ± SEM of three separate experiments. \**P* < 0.05 versus control. #*P* < 0.05 versus EtOH.

increase in ROS generation, indicating that Nrf2 signalling is important in preventing ethanol-induced oxidative stress in NCCs. Treatment with SFN significantly diminished ethanol-induced ROS in NCCs transfected with control siRNA. However, SFN was not able to reduce ethanol-induced ROS generation in NCCs transfected with Nrf2-siRNA (Figure 7).

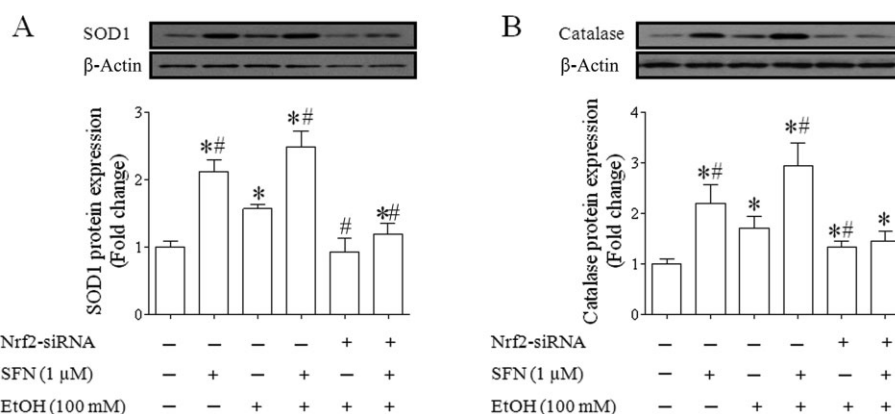
*Ethanol-induced apoptosis was significantly decreased in NCCs treated with SFN but increased in NCCs transfected with Nrf2-siRNA*

To examine the potential of SFN in preventing ethanol-induced apoptosis in NCCs, ethanol-induced apoptosis was determined in control and SFN-treated NCCs. As shown in Figure 8A, MTS analysis showed a significant increase in cell death in ethanol-exposed NCCs. While transfection of NCCs with siRNA did not increase cell death in NCCs, suppression of Nrf2 signalling by siRNA increased the sensitivity of NCCs to ethanol-induced cell death. Flow cytometric analysis of FITC Annexin V showed a very similar result (Figure 8B). Ethanol exposure also resulted in a significant increase in caspase-3 cleavage and activity (Figure 8C,D). Suppression of Nrf2 signalling by siRNA resulted in significantly greater increases in apoptosis as well as caspase-3 cleavage and activity, indicating that Nrf2 signalling is an important



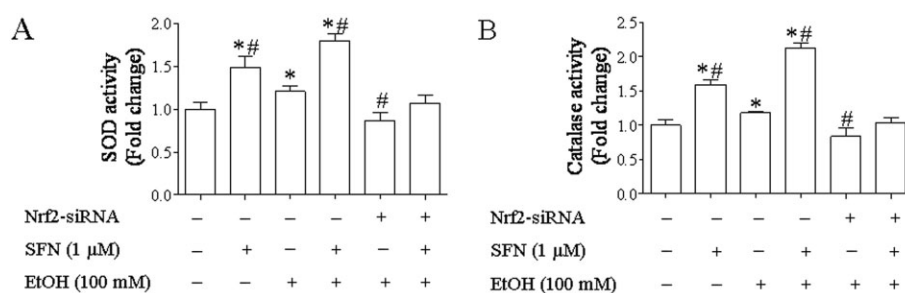
**Figure 4**

SFN treatment significantly increased Nrf2 transcriptional activity in control and ethanol-exposed NCCs. NCCs transfected with control or Nrf2-siRNA were treated with 100 mM ethanol or 1  $\mu$ M SFN alone or co-treated with ethanol and SFN. The mRNA expression of SOD (A) and catalase (B) were determined as described in the *Methods* section. Data are expressed as fold change over control and represent the mean  $\pm$  SEM of three separate experiments. \* $P$  < 0.05 versus control. # $P$  < 0.05 versus EtOH.



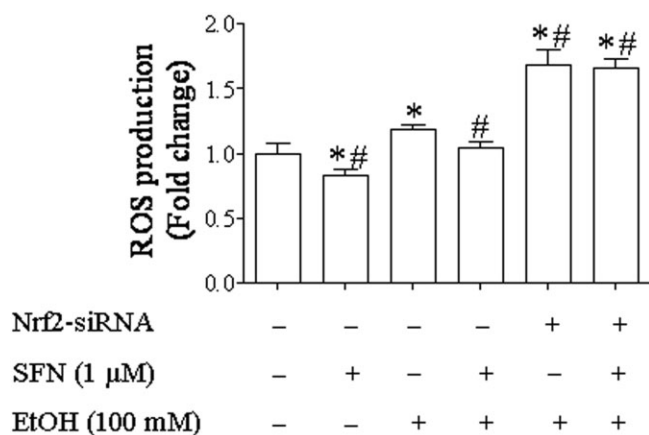
**Figure 5**

SFN treatment significantly increased the expression of antioxidant proteins in control and ethanol-exposed NCCs. NCCs transfected with control or Nrf2-siRNA were treated with 100 mM ethanol or 1  $\mu$ M SFN alone or co-treated with ethanol and SFN. The protein expression of SOD (A) and catalase (B) were determined as described in the *Methods* section. Data are expressed as fold change over control and represent the mean  $\pm$  SEM of three separate experiments. \* $P$  < 0.05 versus control. # $P$  < 0.05 versus EtOH.



**Figure 6**

SFN treatment significantly increased the catalytic activities of antioxidant proteins in control and ethanol-exposed NCCs. NCCs transfected with control or Nrf2-siRNA were treated with 100 mM ethanol or 1  $\mu$ M SFN alone or co-treated with ethanol and SFN. The catalytic activities of SOD (A) and catalase (B) were determined as described in the *Methods* section. Data are expressed as fold change over control and represent the mean  $\pm$  SEM of three separate experiments. \* $P$  < 0.05 versus control. # $P$  < 0.05 versus EtOH.



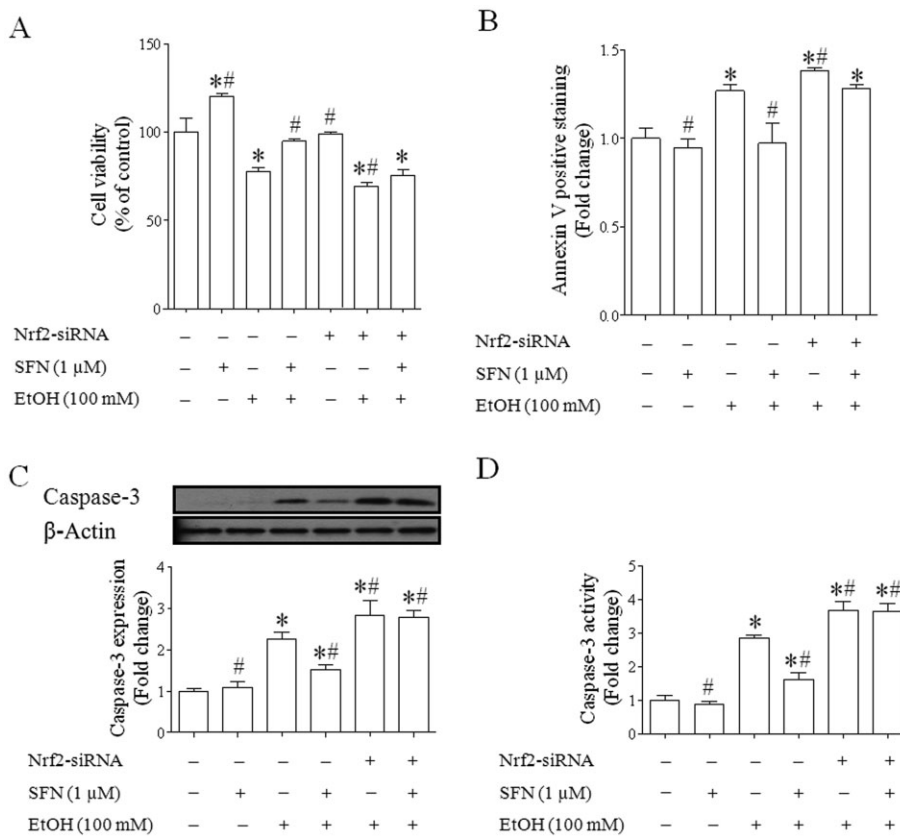
**Figure 7**

ROS generation in ethanol-exposed NCCs treated with SFN or transfected with Nrf2- siRNA. NCCs transfected with control or Nrf2-siRNA were treated with 100 mM ethanol or 1 μM SFN alone or in combination. ROS generation was determined as described in the *Methods* section. Data are expressed as fold change over control and represent the mean ± SEM of three separate experiments. \**P* < 0.05 versus control. #*P* < 0.05 versus EtOH.

determinant for the vulnerability of NCCs to ethanol-induced apoptosis. In contrast, treatment with SFN significantly reduced ethanol-induced apoptosis in NCCs. However, as expected, the protection of SFN against ethanol-induced apoptosis was lost in NCCs transfected with Nrf2-siRNA, demonstrating that SFN protects against ethanol-induced apoptosis by the induction of Nrf2-mediated antioxidant response.

## Discussion

Recent studies have demonstrated that maternal ethanol exposure resulted in a moderate increase in the expression of Nrf2 and its downstream target detoxifying and antioxidant genes. In addition, maternal D3T (an Nrf2 inducer) pretreatment significantly decreased ethanol-induced ROS generation and apoptosis in mouse embryos (Dong *et al.*, 2008). These results demonstrate that Nrf2 signalling is involved in the induction of an antioxidant response in ethanol-exposed mouse embryos. To test whether Nrf2-mediated antioxidant response can also be induced in ethanol-exposed NCCs, in this study, Nrf2 activation and the expression of Nrf2 downstream antioxidants were determined in NCCs exposed to



**Figure 8**

Ethanol-induced apoptosis in NCCs treated with SFN or transfected with Nrf2-siRNA. NCCs transfected with control or Nrf2-siRNA were treated with 100 mM ethanol or 1 μM SFN alone or in combination. Ethanol-induced apoptosis was determined by MTS assay (A), flow cytometry analysis of FITC Annexin V (B) and analysis of the cleavage and activity of caspase-3 (C, D). Data are expressed as percentage of control (A) or fold change over control (B, C, D) and represent the mean ± SEM of three separate experiments. \**P* < 0.05 versus control. #*P* < 0.05 versus EtOH.



100 mM ethanol for 24 h. This ethanol concentration and exposure regimen was chosen because previous studies have shown that acute ethanol administration that produces peak maternal blood ethanol concentrations of 400–500 mg/100 mL (approximately 85–105 mM) is needed to induce apoptosis and major malformations that are characteristics of fetal alcohol syndrome in early mouse embryos (Sulik *et al.*, 1981; Kotch and Sulik, 1992; Dunty *et al.*, 2001). While this ethanol concentration is relatively high, it is not beyond that which can be observed in chronic alcoholics (Adachi *et al.*, 1991). Studies have also shown that susceptibility to specific ethanol-induced defects is directly related to the timing of ethanol exposure and, therefore, acute ethanol exposures during critical periods of vulnerability for specific organs or cell types are desirable and essential for the studies regarding ethanol teratogenesis (Coles, 1994; Maier *et al.*, 1996; Goodlett and Johnson, 1999). The results from this study have shown that *in vitro* exposure of NCCs to ethanol increased the protein expression of Nrf2 and its downstream antioxidants, SOD and catalase, confirming that Nrf2 activation and Nrf2-mediated antioxidant response can be induced in NCCs, a cell population that is sensitive to ethanol-induced apoptosis that contributes heavily to the subsequent abnormalities (Sulik *et al.*, 1981; Kotch and Sulik, 1992; Cartwright and Smith, 1995).

Nrf2 signalling plays a significant role in protecting cells from endogenous and exogenous stresses (Kensler *et al.*, 2007; Jin *et al.*, 2012; Lu *et al.*, 2012; Wu *et al.*, 2012). It was reported that over-expression of Nrf2 protected cells from Fas-induced apoptosis (Kotlo *et al.*, 2003). Activation of Nrf2 also protected against cell death in an *in vitro* model of ischaemia/reperfusion (Danilov *et al.*, 2009). Studies have also shown that exposure to chromium (VI) and cadmium resulted in an increased ROS production and apoptosis in mouse embryonic fibroblast cells lacking Nrf2 (He *et al.*, 2007; 2008). Loss of Nrf2 function has also been found to be associated with increased susceptibility to chemical carcinogenesis, inhalation particles, ovarian toxicants and autoimmune disease (Ramos-Gomez *et al.*, 2001; Li *et al.*, 2004; Hu *et al.*, 2006; Ma *et al.*, 2006). Consistent with these studies, we have shown that significantly greater increase in ethanol-induced ROS generation was observed in NCCs transfected with Nrf2-siRNA. In addition, suppression of Nrf2 increased the sensitivity of NCCs to ethanol-induced apoptosis. These findings suggest that Nrf2 signalling plays an important role in the susceptibility of NCCs to ethanol-induced apoptosis. This premise is further supported by the results from current studies that have shown that up-regulation of Nrf2 by SFN can increase the resistance of NCCs to ethanol-induced oxidative stress and apoptosis.

SFN is an isothiocyanate compound naturally present in high concentrations in several varieties of cruciferous vegetables such as sprouts of broccoli, cabbage and cauliflower (Soane *et al.*, 2010). Studies have shown that systemically administered SFN after the onset of focal ischaemia decreased the total brain infarct volume (Zhao *et al.*, 2007). SFN also protects cultured cortical neurons from glutamate toxicity (Kraft *et al.*, 2004). In addition, SFN can prevent 6-hydroxydopamine-induced cytotoxicity in dopaminergic neurons (Han *et al.*, 2007). More recently, studies have found that SFN has cardioprotective effects in neonatal cardiac myo-

cytes by reducing ROS generation and increasing cell viability (Angeloni *et al.*, 2009). Protective effects against post-ischaemia by SFN have also been reported in kidneys (Yoon *et al.*, 2008), astrocytes (Danilov *et al.*, 2009) and pancreatic islet cells (Solowiej *et al.*, 2004). The current results show that treatment with SFN resulted in significant increases in Nrf2 protein expression and ARE promoter activity, and increases in the mRNA and protein expression of Nrf2 target genes, SOD1 and catalase, and their enzyme activities in ethanol-exposed NCCs. SFN treatment also significantly reduced ethanol-induced oxidative stress and apoptosis in NCCs. These findings clearly demonstrate that SFN can prevent oxidative stress and apoptosis in NCCs exposed to ethanol by activating Nrf2 signalling. However, while we have demonstrated that SFN treatment increased the protein expression and activities of SOD1 and catalase in this study, it is noteworthy that it does not imply that the protective effects of SFN are mediated only by SOD1 and catalase. Other antioxidants induced by Nrf2, which can induce a wide spectrum of antioxidants, may also contribute to SFN's anti-apoptotic actions. It has been reported that SFN can activate multiple Nrf2-induced genes (Hu *et al.*, 2004). Our previous studies have also shown that a number of Nrf2 downstream antioxidant genes can be induced in early mouse embryos (Dong *et al.*, 2008).

Of greater importance for the study is the observation that suppression of Nrf2 signalling abrogated the protective activity of SFN against ethanol-induced oxidative stress and apoptosis in NCCs. Although the major mechanism by which SFN protects cells is thought to be through Nrf2-mediated induction of antioxidant and phase 2 detoxification enzymes that elevates cell defence against oxidative damage, other mechanisms of action might also account for the anti-apoptotic effects of SFN. Indeed, it has been demonstrated that there are multiple mechanisms activated in response to SFN, including suppression of NF- $\kappa$ B (Moon *et al.*, 2009), down-regulation of pro-apoptotic Bid and TNF- $\alpha$  expression, and up-regulation of anti-apoptotic Bcl-xL (Sun *et al.*, 2011). However, the present study has shown that suppression of Nrf2 signalling abolished SFN-induced antioxidant response in NCCs. Knockdown of Nrf2 also abrogated the anti-apoptotic activity of SFN in ethanol-exposed NCCs. These results demonstrated that the protection of SFN against ethanol-induced oxidative stress and apoptosis in NCCs is mainly mediated by the induction of Nrf2 signalling.

Oxidative stress is an important contributing factor in the pathogenesis of ethanol-induced birth defects (Chen *et al.*, 2004; Chen and Sulik, 1996; Dong *et al.*, 2010; Kotch *et al.*, 1995). This suggests that therapeutic strategies directed against ROS might be particularly valuable for the prevention of the ethanol-induced damage and malformations in early embryos, in which ROS are involved and the antioxidant capabilities are minimal. Therefore, either administration of exogenous antioxidants or up-regulation of endogenous antioxidant is an important therapeutic strategy to prevent ethanol-induced cell injury and subsequent malformation. The results of this study further support that up-regulation of endogenous antioxidants through the activation of Nrf2 signalling can prevent ethanol-induced oxidative stress and apoptosis. The potency of SFN in preventing ethanol-induced

oxidative stress and apoptosis in NCCs, along with the fact that SFN is a natural micronutrient found in cruciferous vegetables, including cauliflower, broccoli and cabbage (Keum *et al.*, 2005) makes SFN a promising therapeutic agent for human FASD.

In summary, the results of this study demonstrate that Nrf2-dependent antioxidant response can be induced in NCCs, an ethanol-sensitive cell population implicated in FASD, and that the loss of Nrf2 increased the sensitivity of NCCs to ethanol-induced oxidative stress and apoptosis. We have also shown that SFN-mediated Nrf2-dependent antioxidant response can prevent ethanol-induced oxidative stress and apoptosis in NCCs. These findings provide key insights into the roles of Nrf2 signalling in the induction of antioxidant response in ethanol-exposed embryonic cells and suggest that the induction of Nrf2-mediated antioxidant response by SFN is a promising safe and effective approach for the prevention of FASD.

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## Conflicts of interest

None.

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