Published in final edited form as:

Neurotoxicol Teratol. 2014 ; 46: 26–31. doi:10.1016/j.ntt.2014.08.006.

Involvement of seven in absentia homolog-1 in ethanol-induced apoptosis in neural crest cells

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Abstract

Ethanol-induced apoptosis in selected cell populations is a major component of pathogenesis underlying ethanol-induced teratogenesis. However, there is a fundamental gap in understanding how ethanol leads to apoptosis in embryos. In this study, we investigate the role of seven in absentia homolog-1 (Siah1) protein, an E3 ubiquitin ligase, in ethanol-induced apoptosis. Using an *in vitro* model of neural crest cell (NCC), JoMa1.3 cells, we found that exposure to 100 mM ethanol resulted in a significant increase in Siah1 mRNA expression in NCCs, an ethanol-sensitive cell population implicated in Fetal Alcohol Spectrum Disorders (FASD). Treatment with 100 mM ethanol for 24 hours also significantly increased the protein expression of Siah1 in JoMa1.3 cells. The nuclear translocation and accumulation of Siah1 was evidenced in the cells exposed to ethanol. In addition, we have found that the inhibition of Siah1 function with siRNA prevents ethanol-induced increase in Siah1 protein expression and nuclear translocation in NCCs. Down-regulation of Siah1 by siRNA also greatly diminished ethanol-induced cell death and caspase-3 activation, indicating that inhibition of Siah1 can attenuate ethanol-induced apoptosis. These results strongly suggest that Siah1 plays an important role in ethanol-induced apoptosis in NCCs.

Keywords

Apoptosis; Ethanol; Fetal Alcohol Spectrum Disorders; Neural Crest Cells; Siah1

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Conflict of interest statement

The authors declare no conflict of interest.

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1. Introduction

Fetal Alcohol Spectrum Disorder (FASD) is a term which refers to a range of structural and functional birth defects caused by prenatal exposure to alcohol, with full-blown Fetal Alcohol Syndrome (FAS) representing the severe end of the spectrum. The defects caused by prenatal ethanol exposure include physical, behavioral, mental, and learning disabilities. FASD is considered to be the leading preventable causes of mental retardation (Abel *et al.*, 1986; Burd, 2004).

Accumulated evidences support that excessive cell deaths in selected cell populations is one of the major components of the pathogenesis underlying ethanol-induced teratogenesis. Studies have shown that ethanol exposure resulted in excessive cell death in specific regions of the brain of gestational day 8 to 9 mouse embryos (Dunty *et al.*, 2001; Kotch *et al.*, 1992b). These results have been confirmed by the studies in chick (Cartwright *et al.*, 1995a; Cartwright *et al.*, 1995b). In addition, ethanol-induced cell death has been found in postmitotic neurons in the hypothalamus (De *et al.*, 1994), cerebral cortex (Olney, 2003; Young *et al.*, 2003), cerebellum (Goodlett *et al.*, 1997; Tran *et al.*, 2005), and associated brain-stem structures (Napper *et al.*, 1995). Using the TUNEL technique, it has been shown that ethanol-induced cell death in early embryos is apoptotic (Chen *et al.*, 2004; Chen *et al.*, 2001; Dunty *et al.*, 2001). These results have been confirmed by studies that have shown that ethanol-induced cell death in early embryos can be prevented by compounds that inhibit caspase activation (Cartwright *et al.*, 1998).

Among the sensitive cell populations that are vulnerable to ethanol-induced apoptosis is neural crest cells (NCCs) (Cartwright *et al.*, 1995b; Chen *et al.*, 1996; Chen *et al.*, 2000; Kotch *et al.*, 1992b; Sulik *et al.*, 1981). The NCC is a multipotent cell population that can give rise to a diversity of neural and nonneural cell types, such as melanocytes, neurons, glial cells, endocrine cells as well as mesenchymal cells (Delfino-Machin *et al.*, 2007; Hall, 2008; Teng *et al.*, 2006). Cranial neural crest cells can differentiate into a wide variety of structures, including the connective tissues, cartilage, and skeletal structures of the head and facial features (Smith, 1997).

A number of studies have demonstrated that NCCs are particularly vulnerable to alcohol-induced apoptosis (Cartwright *et al.*, 1995a; Chen *et al.*, 1996; Chen *et al.*, 2013a; Chen *et al.*, 2013b; Kotch *et al.*, 1992b; Sulik *et al.*, 1988). Excessive cell death in mouse embryos in the regions enriched with NCCs indicated that alcohol directly damaged this cell population (Sulik *et al.*, 1988). While the embryos have capacity to repair damage, studies have shown that ethanol-induced apoptosis in NCCs contributes heavily to subsequent abnormalities (Cartwright *et al.*, 1995a; Kotch *et al.*, 1992a; Smith, 1997; Sulik *et al.*, 1981). Although there are numbers of mechanisms that have been proposed for ethanol-induced apoptosis in NCCs, the molecular pathways directly involved in ethanol-induced apoptosis in NCCs is not clear. Understanding of the molecular mechanisms underlying ethanol-induced apoptosis in NCCs can provide insight into the pathogenesis of FASD and may suggest approaches for the development of rational FASD prevention strategies.

The *Drosophila* seven in absentia (Sina) protein and its human homolog seven in absentia homolog (Siah) are members of a highly conserved family of E3 ubiquitin ligases (Carthew *et al.*, 1990). The members of this family contain an N-terminal RING domain that binds E2 proteins (Carthew *et al.*, 1990; Hu *et al.*, 1999; Lorick *et al.*, 1999). The Siah is widely expressed in developing mouse embryos and in adult tissues (Della *et al.*, 1993). Siah ligases regulate the ubiquitination and proteasomal degradation of several proteins that are important in a variety of signaling pathways, including transcriptional regulators (Tiedt *et al.*, 2001; Zhang *et al.*, 1998), membrane receptors (Liani *et al.*, 2004; Winter *et al.*, 2008), a microtubule-associated motor protein (Linares-Cruz *et al.*, 1998), and other proteins (Tan *et al.*, 2014). Studies have suggested that Siah plays important roles in a variety of biological processes including cell proliferation, migration, axon guidance, and development (Gutierrez *et al.*, 2006; Hu *et al.*, 1999; Lee *et al.*, 2008; Leung *et al.*, 2014; Lorick *et al.*, 1999; Xu *et al.*, 2006b). In addition, Siah plays a critical role in the induction of apoptosis and tumor suppression (Nemani *et al.*, 1996).

Although it is clear that Siah1 plays a critical role in several cell death paradigms and tumor suppression, the potential roles of this signaling pathway in ethanol-induced apoptosis and teratogenesis have not been investigated. In this study, we investigated the role of Siah1 in ethanol-induced apoptosis in JoMa1.3 cells, an *in vitro* model of NCCs. We found that treatment with ethanol significantly increased the mRNA and protein expression of Siah1 in JoMa1.3 cells. Ethanol exposure also resulted in the nuclear translocation of Siah1. Inhibition of Siah1 function with siRNA prevented ethanol-induced increase in Siah1 expression and nuclear accumulation. Down-regulation of Siah1 by siRNA also significantly diminished ethanol-induced cell death as well as the cleavage and activity of caspase-3, indicating that inhibition of Siah1 can attenuate ethanol-induced apoptosis. These results demonstrated for the first time that Siah1 plays an important role in ethanol-induced apoptosis in NCCs.

2. Materials and methods

2.1. Cell culture and ethanol treatment

NCCs (JoMa1.3 cells) were cultured as described previously (Chen *et al.*, 2013a). Briefly, cells were grown on cell culture dishes coated with fibronectin and maintained in Dulbecco's modified Eagle's medium (DMEM): Ham's F12 (1:1) at 37°C in 5% CO₂/95% air. For ethanol treatment, NCCs were treated with 100 mM ethanol for 6, 12 or 24 hours as described previously. Stable ethanol levels were maintained by placing the cell culture plates in a plastic desiccator containing 100 mM ethanol in distilled water (Yan *et al.*, 2010).

2.2. Siah1 siRNA transfection

Siah1 siRNA transfection was performed as described previously (Chen *et al.*, 2013b). Briefly, JoMa1.3 cells were transfected with MISSION® Siah1-siRNA or negative control siRNA (Sigma-Aldrich, St Louis, MO) at a final concentration of 40 nM in the presence of N-TER™ Nanoparticle siRNA Transfection System (Sigma-Aldrich, St Louis, MO), following the manufacturer's instructions. The cells were harvested 48 hours after transfection for experiments.

2.3. Immunofluorescence analysis

Cells grown on coverslips were treated with or without 100 mM ethanol for 24 hours. Following the treatment, cells were rinsed with ice-cold PBS and fixed with 4% paraformaldehyde for 15 min at room temperature followed by permeabilization with PBS containing 0.25% Triton X-100 for 10 min. Then the cells were washed in PBS three times for 5 min each, blocked with 1% BSA in PBST for 60 min and then subjected to immunofluorescence staining with goat polyclonal Siah1 antibody (Santa Cruz, Santa Cruz, CA) (dilution 1:100) in 1% BSA in PBST in a humidified chamber overnight at 4 °C. The cells were then washed with cold PBS three times for 5 min each, and incubated with Alexa Fluor® 594 chicken anti-goat IgG (H+L) secondary antibody (Invitrogen, Carlsbad, CA) (dilution 1:250) in 1% BSA in PBST for 1 hour at room temperature in the dark. The cells were washed three times with PBST for 5 min each in the dark and then counterstained with 0.2 µg/ml DAPI (DNA stain) for 1 min. After washing with PBS three times for 5 min each, the cells were examined by fluorescence microscopy (Olympus IX71).

2.4. Subcellular fractionation

Cells from control and ethanol treated groups were collected and the nuclear fractions of NCCs were generated using the TransFactor extraction kit (Clontech, Mountain View, CA) following the manufacturer's protocol. Briefly, cells were rinsed twice with ice-cold PBS before they were scraped in ice-cold PBS. Cells were resuspended in lysis buffer, incubated for 15 min., and centrifuged for 5 min. at 450 X g. The cell pellet was resuspended in lysis buffer, homogenized with 15 strokes of a homogenizer, and centrifuged at 10,000 X g for 20 min. Then the crude nuclear pellet was resuspended in buffer and disrupted with 15 strokes of a homogenizer. The nuclear suspension was shaken gently for 30 min at 4°C and the supernatant was collected by centrifuging at 20,000 X g for 5 min. This fraction is the nuclear extract. The protein concentrations were determined using Pierce® BCA protein assay. Fraction purity was assessed using nuclear marker histone 2B and cytosolic marker IκB-α (Santa Cruz, Santa Cruz, CA). Siah1 nuclear accumulation was analyzed by determining the Siah1 protein levels in nuclear fractions using Western blot.

2.5. Quantitative real-time PCR

The mRNA expression of Siah1 was analyzed using quantitative real-time PCR. Total RNAs were isolated from control and treated NCCs using the QIAGEN RNeasy mini kit (QIAGEN, Valencia, CA, USA). Quantitative RT-PCR was carried out using the FastStart Universal SYBR Green Master qPCR kit (Roche Diagnostics, Mannheim, Germany) on a Rotor-Gene 6000 Real-Time system (Corbett Life Science, Sydney, Australia). The primer pairs used for this analysis are: Siah1: forward: 5'-ACTCTGCCACACACCGAAAAGG-3'; reverse: 5'-ATGACGGCATCCAAGGAGCCTT-3'. These primer pairs were designed using Primer3 and synthesized by Integrated DNA Technologies, Inc. (IDT, Coralville, IA, USA). All quantitative RT-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.

2.6. Western blotting

Western blotting was performed as described previously (Chen *et al.*, 2013b). The levels of Siah1 and the cleavage of caspase-3 were analyzed with SIAH1 rabbit polyclonal antibody (Abcam, Cambridge, MA) (Schonhoff *et al.*, 2006) and rabbit monoclonal anti-cleaved caspase-3 antibody (Cell Signaling, Beverly, MA), respectively. Mouse monoclonal anti β -actin antibody (Santa Cruz, Santa Cruz, CA) was used as a loading control. The membranes were then developed on a Kodak X-OMAT 2000A imaging system (Kodak, Rochester, NY) and the intensity of the protein band was analyzed using the Adobe Photoshop CS software (Adobe Systems, San Jose, CA). All Western blot analyses were performed in triplicate.

2.7. 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, Madison, WI) following the manufacturer's protocol. Apoptosis was determined by the analysis of caspase-3 cleavage and activity as well as TUNEL assay. Caspase-3 cleavage and activity were determined by Western blot and by using homogeneous luminescent Caspase-Glo™ 3/7 Assay (Promega, Madison, WI), respectively, as described previously (Chen *et al.*, 2013b). TUNEL assay was conducted by using a TiterTACS In Situ Detection Kit (Trevigen, Gaithersbury, MD), according to the manufacturer's protocol.

2.8. Statistical analysis

Statistical analyses were performed by using StatView software (SAS Institute Inc.). All data are expressed as mean $\pm$ SEM of three separate experiments. Statistical comparisons between groups were analyzed by One-way ANOVA. Multiple comparison post-tests between groups were conducted by using Bonferroni's test. Differences between groups were considered significant at $p < 0.05$.

3. Results

3.1. Ethanol exposure resulted in a significant increase in mRNA expression of Siah1 in NCCs

To determine whether ethanol exposure can result in the changes in Siah1 transcription, mRNA expression of Siah1 was examined in JoMa 1.3 cells exposed to 100 mM ethanol for 6, 12 or 24 hours. JoMa 1.3 cell is an *in vitro* model of NCCs that is widely used for studying the mechanisms of NCC development (Cordes *et al.*, 2009; Makeyev *et al.*, 2012; Murphy *et al.*, 2011; Sheehy *et al.*, 2010) and neural crest-derived cancer (Schulte *et al.*, 2013). This ethanol concentration was selected for this study because previous studies have indicated that acute ethanol administration that produces peak maternal blood ethanol concentrations of 400–500 mg/dl (approximately 85–105 mM) is required for inducing apoptosis and major malformations in early mouse embryos (Dunty *et al.*, 2001; Kotch *et al.*, 1992a; Sulik *et al.*, 1981). This ethanol concentration, while it is relatively high, is not beyond that which can be observed in chronic alcoholics (Adachi *et al.*, 1991). As shown in Fig. 1, treatment with 100 mM ethanol for 6, 12 or 24 hours significantly increased the mRNA expression of Siah1 in JoMa1.3 cells. The most dramatic increase in Siah1 mRNA expression was observed in cells exposed to ethanol for 12 hours.

3.2. Treatment with ethanol significantly increased total Siah1 protein levels in NCCs

To examine the effects of ethanol on Siah1 protein expression in NCCs, Siah1 protein levels were determined in cells exposed to 100 mM ethanol for 24 hours. As shown in Fig. 2, Western blot analysis revealed a significant increase in total Siah1 protein levels in cells exposed to ethanol for 24 hours. These results indicated that ethanol exposure can significantly increase Siah1 protein levels in NCCs.

3.3. Ethanol exposure significantly increased nuclear Siah1 protein levels in NCCs

It is well known that Siah1 elicits apoptosis by causing the degradation of selected nuclear proteins following Siah1 nuclear translocation (Germani *et al.*, 2003; Gutierrez *et al.*, 2006; Lee *et al.*, 2008; Xu *et al.*, 2006a). To examine whether ethanol exposure of NCCs can promote nuclear translocation and accumulation of Siah1, the levels of nuclear Siah1 protein were determined by Western blot analysis in control and ethanol-exposed JoMa1.3 cells. We found that ethanol exposure resulted in an approximately 1-fold increase in nuclear Siah1 protein levels, indicating that ethanol exposure can promote Siah1 nuclear accumulation (Fig. 3A). To confirm that ethanol exposure can trigger nuclear translocation and accumulation of Siah1, the subcellular localization of Siah1 was determined by immunofluorescence analysis in control and ethanol-exposed cells. We found that ethanol treatment resulted in a significant increase in the Siah1 protein levels in nucleus as compared to control (Fig. 3B)

3.4. Knockdown of Siah1 using siRNA diminished ethanol-induced increases in Siah1 protein expression and nuclear accumulation

To determine whether Siah1-siRNA can effectively prevent ethanol-induced increase in Siah1 protein expression and nuclear accumulation in NCCs, loss-of-function analyses were performed using JoMa1.3 cells transfected with control or Siah1-siRNA. We found that transfection with Siah1-siRNA resulted in a significant decrease in the levels of total Siah1 protein in ethanol-exposed cells (Fig. 2). Inhibition of Siah1 by Siah1-siRNA also resulted in a significant decrease in the level of nuclear Siah1 protein in cells exposed to ethanol (Fig. 3A, 3B).

3.5. Inhibition of Siah1 function with siRNA attenuates ethanol-induced apoptosis in NCCs

To determine whether Siah1 is involved in ethanol-induced cell death, cell viability was first determined in control and ethanol-exposed JoMa1.3 cells transfected with control- or Siah1-siRNA. As indicated in Fig. 4, exposure to 100 mM ethanol for 24 hours resulted in a significant decrease in cell viability, confirming the results from our previous studies (Chen *et al.*, 2013a; Chen *et al.*, 2013b). Knockdown of Siah1 by siRNA significantly inhibited the reduction of cell viability in NCCs exposed to ethanol. Ethanol exposure also significantly increased caspase-3 cleavage and activity. In addition, TUNEL analysis of apoptosis confirmed that ethanol exposure increased apoptosis in NCCs. Inhibition of Siah1 by siRNA greatly diminished ethanol-induced increase in the cleavage and activity of caspase-3 and TUNEL staining, indicated that knockdown of Siah1 can attenuate ethanol-induced apoptosis in NCCs (Fig. 5A, B, C).

4. Discussion

Both *in vitro* and *in vivo* studies have implicated apoptotic death of NCCs in ethanol-induced teratogenesis (Cartwright *et al.*, 1995a; Chen *et al.*, 2001; Dunty *et al.*, 2001; Kotch *et al.*, 1992b; Sulik *et al.*, 1988). Kotch and Sulik found that ethanol's teratogenesis and the patterns of malformation in mouse embryos can be directly correlated to the selective vulnerability of the cells at the margin of the anterior neural folds, including the NCCs (Kotch *et al.*, 1992b). Using a chick-specific antibody to NCCs, Cartwright and Smith found that ethanol exposure resulted in fewer NCC numbers in chick embryos and that the loss of NCCs resulted in the phenotype characteristic of Fetal Alcohol Syndrome (FAS) (Cartwright *et al.*, 1995a). However, the mechanisms underlying ethanol-induced apoptosis in NCCs is not completely clear. In this study, using an *in vitro* model of NCC, JoMa1.3 cells, we have shown that ethanol treatment resulted in a significant increase in mRNA expression and the total protein levels of Siah1 in NCCs. In addition, the nuclear translocation of Siah1 was evidenced in the NCCs exposed to ethanol. This is the first study that demonstrates that ethanol increased Siah1 protein levels and nuclear accumulation in NCCs.

Siah proteins possess E3 ubiquitin ligase activity that regulates the degradation of a number of proteins, including themselves by ubiquitination (Nakayama *et al.*, 2004; Tiedt *et al.*, 2001; Wheeler *et al.*, 2002). Under normal condition, Siah1 protein is maintained at a relatively low level through ubiquitin-dependent proteolysis (Hu *et al.*, 1999). However, endogenous Siah1 protein levels can be increased significantly in response to apoptotic stimulus and DNA damage (Hu *et al.*, 1997; Nemani *et al.*, 1996; Xu *et al.*, 2006b). Overexpression of Siah1 has been found to result in cell-cycle arrest and induction of apoptosis (Matsuzawa *et al.*, 2001; Relaix *et al.*, 2000; Roperch *et al.*, 1999). In this study, there was a substantial ethanol-induced elevation of Siah1 protein in NCCs, accompanying with a significantly increased apoptosis, indicating that Siah1 may be involved in ethanol-induced apoptosis in NCCs.

Siah1 has been suggested to play a role in the nuclear translocation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Hara *et al.*, 2006). The S-nitrosylation of GAPDH at the catalytic site cysteine-150 enables the binding of GAPDH to Siah1. Binding of GAPDH to Siah1, which possesses a nucleus localization signal, leads to the nuclear translocation of the GAPDH-Siah1 protein complex (Hara *et al.*, 2005; Shahani *et al.*, 2012). Nuclear GAPDH stabilizes Siah1, which in turn degrades key cellular proteins and initiates apoptosis. A recent study has shown that exposure of cells to ethanol significantly promoted GAPDH nuclear translocation and induced cell death in U-118 MG and SH-SY5Y cells (Ou *et al.*, 2009). In this study, we found that ethanol exposure promoted the nuclear translocation and accumulation of Siah1, suggesting that nuclear accumulation of Siah1 is critical for ethanol-induced apoptosis in NCCs.

Studies from other investigators have shown that knockdown of Siah1 by siRNA reduced glucose-induced GAPDH nuclear translocation and subsequent cell death (Yego *et al.*, 2010). Down-regulation of Siah1 by small interference RNA has also been found to interfere with the JNK pathway and confer protection from apoptosis induced by trophic factor deprivation and DNA damage (Xu *et al.*, 2006b). Consistent with these results, our studies

have indicated that siRNA treatment diminished ethanol-induced increase in Siah1 protein levels and reduced nuclear accumulation of Siah1. Down-regulation of Siah1 by siRNA also attenuated ethanol-induced apoptosis in NCCs, indicating that Siah1 is involved in ethanol-induced apoptosis in NCCs. However, it is noteworthy that while Siah1-siRNA can completely prevent ethanol-induced increase in Siah1 protein expression and nuclear translocation, it can only partially prevent ethanol-induced apoptosis in NCCs. Ethanol-induced apoptosis is a complex, multifactorial process. The fact that siRNA can only provide partial prevention against ethanol-induced apoptosis suggests that mechanisms other than Siah1 signaling may also be involved in ethanol-induced apoptosis. Indeed, the studies from our laboratory and others have demonstrated that oxidative stress plays an important role in ethanol-induced apoptosis in NCCs (Chen *et al.*, 1996; Chen *et al.*, 2013a; Chen *et al.*, 2013b).

In summary, our results show for the first time that ethanol exposure resulted in significant increases in mRNA and protein expression of Siah1. Treatment with ethanol also promoted nuclear translocation of Siah1 in NCCs. The ability of Siah1 siRNA to diminish the ethanol-induced increases in Siah1 protein levels and Siah1 nuclear accumulation, and attenuate apoptosis in ethanol-exposed NCCs indicates that Siah1 signaling plays a critical role in ethanol-induced apoptosis in NCCs. These findings provide new insights into the role of Siah1 in ethanol-induced apoptosis. Given the implication of apoptosis in NCCs in ethanol-induced birth defects, these results suggest that Siah1 may represent a novel therapeutic target to FASD and that pharmacological approaches that disrupt Siah1 signaling might be employed to ameliorate ethanol-induced apoptosis and birth defects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the National Institute of Health grants AA017446, AA020265 and AA021434 (S.-YC) from the National Institute on Alcohol Abuse and Alcoholism.

Abbreviations

FASD	Fetal Alcohol Spectrum Disorder
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
MTS	(3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H- tetrazolium salt)
NCC	Neural crest cell
PUMA	p53 upregulated modulator of apoptosis
Siah1	seven in absentia homolog-1
XIAP	inhibitor of apoptosis protein

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Highlights

- Ethanol exposure significantly increased Siah1 mRNA expression in NCCs
- Ethanol increased the protein expression and nuclear translocation of Siah1 in NCCs
- Siah1-siRNA reduced the expression and nuclear translocation of Siah1
- Down-regulation of Siah1 diminished caspase-3 activation and apoptosis in NCCs
- Siah1 signaling is involved in ethanol-induced apoptosis in NCCs

Figure 1.

Ethanol exposure resulted in a significant increase in Siah1 mRNA expression in NCCs. NCCs were treated with 100 mM ethanol for 0, 6, 12 or 24 hours. mRNA expression of Siah1 was determined by quantitative real-time PCR. Data are expressed as fold change over control and represent the Mean $\pm$ SEM of three separate experiments. * $p < 0.05$ vs. control.

Figure 2.

Ethanol exposure resulted in a significant increase in Siah1 protein levels in NCCs. Transfection with Siah1-siRNA significantly reduced Siah1 protein levels in ethanol-exposed NCCs. NCCs transfected with control or Siah1-siRNA were treated with 100 mM ethanol for 24 hours. Protein expression of Siah1 was determined by Western blot. Data are expressed as fold change over control and represent the Mean $\pm$ SEM of three separate experiments. * $p < 0.05$ vs. control. # $p < 0.05$ vs. EtOH

Figure 3.

Treatment with ethanol significantly increased nuclear Siah1 protein levels in NCCs. (A) NCCs transfected with control or Siah1-siRNA were treated with 100 mM ethanol for 24 hours. Nuclear Siah1 protein levels were determined by Western blot. Data are expressed as fold change over control and represent the Mean $\pm$ SEM of three separate experiments. * $p < 0.05$ vs. control. # $p < 0.05$ vs. EtOH. (B) Immunofluorescence analysis of Siah1 in control (a, b, c), ethanol-exposed NCCs transfected with control siRNA (d, e, f), and ethanol-exposed NCCs transfected with Siah1-siRNA (g, h, i). Siah1 was detected by goat polyclonal Siah1 antibody (red) as described in Materials and Methods. Nuclei were stained with DAPI (blue).

Figure 4.

Siah1-siRNA significantly reduced ethanol-induced cell death in NCCs. NCCs transfected with control or Siah1-siRNA were treated with 100 mM ethanol for 24 hours. Ethanol-induced cell death was determined by MTS assay. Data are expressed as percentage of control and represent the mean $\pm$ SEM of three separate experiments. * $p < 0.05$ vs. control. # $p < 0.05$ vs. EtOH.

Figure 5.

Knockdown of Siah1 by siRNA resulted in a significant reduction in the cleavage and activity of caspase-3 (A, B) and apoptosis (C) in NCCs exposed to ethanol. NCCs transfected with control or Siah1-siRNA were treated with 100 mM ethanol for 24 hours. Caspase-3 cleavage was determined by Western blot. Caspase-3 activity was determined as described in the methods. TUNEL assay was conducted by using a TiterTACS In Situ Detection Kit. Data are expressed as fold change over control and represent the Mean $\pm$ SEM of three separate experiments. * $p < 0.05$ vs. control. # $p < 0.05$ vs. EtOH