Neural Crest Cells and Fetal Alcohol Spectrum Disorders: Mechanisms and Potential Targets for Prevention

Shao-yu Chen \textsuperscript{1,2}\textsuperscript{*} and Maharajan Kannan\textsuperscript{1,2}\textsuperscript{*}

\textsuperscript{1} Department of Pharmacology and Toxicology, University of Louisville Health Sciences Center, Louisville, KY 40292, USA

\textsuperscript{2} University of Louisville Alcohol Research Center, Louisville, KY 40292, USA

\textsuperscript{*}To whom correspondence should be sent:
Shao-yu Chen, Ph.D., Department of Pharmacology and Toxicology, University of Louisville Health Sciences Center, Louisville, KY 40202. E-mail: shaoyu.chen@louisville.edu

Maharajan Kannan, Ph.D., Department of Pharmacology and Toxicology, University of Louisville Health Sciences Center, Louisville, KY 40202. E-mail: maharajan.bu@gmail.com

Abstract

Fetal alcohol spectrum disorders (FASD) are a group of preventable and nongenetic birth defects caused by prenatal alcohol exposure that can result in a range of cognitive, behavioral,
emotional, and functioning deficits, as well as craniofacial dysmorphology and other congenital defects. During embryonic development, neural crest cells (NCCs) play a critical role in giving rise to many cell types in the developing embryos, including those in the peripheral nervous system and craniofacial structures. Ethanol exposure during this critical period can have detrimental effects on NCCs’ induction, migration, differentiation, and survival, leading to a broad range of structural and functional abnormalities observed in individuals with FASD. This review article provides an overview of the current knowledge on the detrimental effects of ethanol on NCCs' induction, migration, differentiation, and survival. The article also examines the molecular mechanisms involved in ethanol-induced NCC dysfunction, such as oxidative stress, altered gene expression, apoptosis, epigenetic modifications, and other signaling pathways. Furthermore, the review highlights potential therapeutic strategies for preventing or mitigating the detrimental effects of ethanol on NCCs and reducing the risk of FASD. Overall, this article offers a comprehensive overview of the current understanding of the impact of ethanol on NCCs and its role in FASD, shedding light on potential avenues for future research and intervention.

**Keywords:** Neural crest cells; fetal alcohol spectrum disorders; prenatal ethanol exposure; embryos; epigenetics

**Abbreviations**

Alcohol-related birth defects; ARND - Alcohol-related neurodevelopmental disorder; ATM - Ataxia-telangiectasia mutated protein kinase; Bak - Bcl-2 antagonist killer; Bax - B cell lymphoma
2-associated X protein; Bcl-2 - B-cell lymphoma 2; Bcl-xL - B-cell lymphoma-extra large; BMP - Bone morphogenetic protein; CAM - Cell adhesion molecule; CaMKII - Calmodulin-dependent protein kinase II; CBP - CREB-binding protein; CNS - Central nervous system; E-cadherin - Epithelial cadherin; EMT - Epithelial-to-mesenchymal transition; ENS - Enteric nervous system; ERK - Extracellular-signal regulated kinases; FAS - Fetal alcohol syndrome; FASD - Fetal alcohol spectrum disorders; FGF - Fibroblast growth factor; GDNF - Glial cell-derived neurotrophic factor; GPCR - G-protein coupled receptor; GRN - Gene regulatory network; GSK3b - Glycogen synthase kinase-3b; H3 Lys-27 - Trimethylated histone 3 lysine 27; H3 Lys-4 - Trimethylated histone 3 lysine 4; JNK - c-JUN N-terminal kinases; LC3 - Ubiquitin-like autophagosome membrane light-chain 3; LEF1 - Lymphoid enhancer factor; MAPK - Mitogen-activated protein kinase; miRNA – MicroRNA; NC – Neural crest; NCCs - Neural crest cells; PAE - Prenatal alcohol exposure; NLK - Nemo-like kinase; Nrf2 - Nuclear factor (erythroid-derived 2)-like 2; NSC - Neural stem cell; PCP - Planar cell polarity; PDGF - Platelet-derived growth factor; PI3K - Phosphatidylinositol 3-kinase; PIP2 - Phosphatidylinositol-4,5-bisphosphate; PIP3 - Phosphatidylinositol-3,4,5-trisphosphate; PKC - Protein kinase C; PLC - Phospholipase-C; PNS - Peripheral nervous system; PTEN - Phosphatase and tensin homolog; PUMA - p53- upregulated modulator of apoptosis; RA - Retinoic acid; ROS – Reactive oxygen species; RTK - Receptor tyrosine kinase; SAM - S-Adenosyl-L-Methionine; SDF1 - Stromal cell-derived factor 1; Siah 1 - Seven in absentia homolog 1; SOD - Superoxide dismutase; TAK - TGF-β-activated kinase; TCF - T cell transcription factor; TFs - Transcription factors; VEGF - Vascular endothelial-derived growth factor.

1. Introduction

Alcohol consumption contributes to many disorders and diseases and increases the risk of health problems, including cancers, brain damage, and liver disease. Prenatal alcohol exposure
(PAE) is known to cause a broad range of adverse health implications to the developing fetus, including congenital defects and cognitive, behavioral, emotional, and functioning deficits, collectively referred to as fetal alcohol spectrum disorders (FASD) (Lange et al., 2017; Sulik, 2005). FASD is the most prevalent nongenetic birth defect in Western countries and a leading known cause of mental retardation and craniofacial dysmorphology (Lange et al., 2017; Wozniak et al., 2019). The degree and variability of ethanol-induced malformations are influenced by ethanol level, the developmental stage at the time of exposure, and the frequency and extent of the exposure (Dunty Jr et al., 2001; Kotch & Sulik, 1992; Muralidharan et al., 2013; Sulik et al., 1981). The prevalence of FASD varies by country, estimated at 0.77% in the global population and 2.0 - 5.0% in Europe and North America (Lange et al., 2017; May et al., 2018; Wozniak et al., 2019).

FASD can be categorized into fetal alcohol syndrome (FAS), alcohol-related birth defects (ARBD), alcohol-related neurodevelopmental disorder (ARND), and neurobehavioral disorder associated with prenatal alcohol exposure (ND-PAE). FAS is the most severe type of FASD, including a combination of growth retardation, neurological impairments, and craniofacial dysmorphology (Sulik, 2005; Wozniak et al., 2019).

While alcohol consumption during pregnancy can have severe effects, not all embryonic cells are equally affected (Dunty Jr et al., 2001; Kotch & Sulik, 1992; Smith, 1997). The vulnerability of particular cell types to ethanol-induced cytotoxicity is one of several variables contributing to FASD (Dunty Jr et al., 2001). Among the embryonic cell populations that are vulnerable to ethanol-induced adverse effects are neural crest cells (NCCs) (Cartwright & Smith, 1995; Chen & Sulik, 1996, 2000; Kotch & Sulik, 1992). The NCCs are a multipotent cell population that can give rise to a diversity of cell types and differentiate into various structures, such as cartilage, connective tissues, and skeletal structures of the head and facial features (Delfino-Machin et al.,
Abnormal NCC development can result in neurocristopathies, a group of developmental defects, including craniofacial defects, hearing disorders and heart abnormalities (Vega-Lopez et al., 2018). Various studies have shown that ethanol exposure can alter craniofacial development by interfering with several processes of cranial NCC development, including induction, migration, differentiation, and survival. Although early reviews have offered a broad outline of ethanol-induced apoptosis in NCCs and its relation to FASD (Smith, Garic, Berres, et al., 2014; Smith, Garic, Flentke, et al., 2014), there is still a significant knowledge gap regarding the crucial role of NCCs in the pathogenesis of FASD and how prenatal ethanol exposure affects the induction, migration, differentiation, and survival of NCCs, resulting in FASD. Reviewing the latest research advancements on the detrimental effects of ethanol on NCC development and their link with FASD can provide valuable insights into the underlying mechanisms of FASD and guide future research directions, leading to the development of effective strategies for the prevention and intervention of FASD.

This review aims to provide an overview of the current knowledge on the adverse effects of ethanol on NCCs' induction, migration, differentiation, and survival. The article also examines the molecular mechanisms involved in ethanol-induced NCC dysfunction, such as oxidative stress, altered gene expression, apoptosis, epigenetic modifications, and other signaling pathways. Furthermore, the review highlights potential therapeutic strategies for preventing or mitigating the detrimental effects of ethanol on NCCs and reducing the risk of FASD.

2. The role of neural crest cells in early embryonic development

NCCs are multipotent and migratory stem cells that play a crucial role in vertebrate development. They possess a unique developmental ability along the anterior-posterior axis found
only in vertebrates (Soldatov et al., 2019). The developmental process of NCCs involves several major steps, including induction, migration, and differentiation. NCCs are formed within

![Diagram of NCC formation during neurulation.](image)

**Figure 1.** Schematic diagram illustrating the formation of NCCs during neurulation. (A) The neural plate border separates from the neural plate and ectoderm. (B) The middle region of the neural plate invaginates and forms the neural groove. The neural plate borders elevate to give rise to the neural crest. (C) The closure of the neural tube disconnects the neural crest from the epidermis. (D) The NCCs delaminate from the region between the dorsal neural tube and overlying ectoderm and migrate to various embryonic tissues.

the dorsal region of the closing neural folds (Hall, 2008). As depicted in Figure 1, during the neurulation stage of embryo development, which typically occurs at around three weeks post-conception in humans, embryonic day 7.5 (E7.5) in mice, stage 8 in chickens, and 12.0 hours post-
fertilization (hpf) in zebrafish, the neural plate borders form at the interface between the neural plate and the ectoderm. Subsequently, the middle region of the neural plate invaginates and forms the neural groove, while the neural plate borders elevate to give rise to the neural crest. When the neural tube is closed, the neural crest disconnects from the epidermis. The NCCs then delaminate from the region between the dorsal neural tube and overlying ectoderm and migrate into various embryonic tissues, where they give rise to a remarkable array of cell types, including neurons and glial cells of the peripheral nervous system, melanocytes, cartilage, and connective tissue (Le Douarin et al., 2004; Schock et al., 2023). In humans, NCCs can contribute to up to 47 different cell types, including sensory and autonomic neurons, fibroblasts, and smooth muscle cells (Cooper & Tsakiridis, 2022; Vickaryous & Hall, 2006).

The pioneering quail-chick chimera experiments conducted by Le Douarin provided valuable insights into the migration pathways and derivatives of NCCs. These experiments involved transplanting quail neural crest primordia from specific neural tube regions into corresponding positions within developing chick embryos. Tracking the contributions of quail-derived NCCs was initially accomplished through histological visualization of the distinctive quail cell nucleolus. More recently, a quail-specific antibody has been used for this purpose. The quail-chick chimera approach has proven to be highly informative and has played a significant role in uncovering the contributions of the NCCs to the development of the vertebrate body (Le Douarin, 1973; Le Douarin & Dupin, 2018).

NCCs can be divided into specific subpopulations: cranial NCCs, vagal NCCs, trunk NCCs, and sacral NCCs (Cooper & Tsakiridis, 2022). The cranial NCCs are the most anterior and diverse population that migrates into mandibular, hyoid, and branchial streams during craniofacial
development (Teng & Labosky, 2006; Yang et al., 2021). The cranial NCCs can differentiate into sensory neurons, melanocytes, and cells responsible for craniofacial structures such as bone, cartilage, and connective tissues (Hall, 2008). The vagal NCCs contribute to Schwann cells, melanocytes, and the neurons and glia of the enteric nervous system (ENS) (Gandhi & Bronner, 2018). The trunk NCC derivatives give rise to sympathoadrenal (SA) cells, which in turn form chromaffin cells, melanocytes, Schwann cells, and sympathetic ganglia (Huang et al., 2016), while the sacral NCCs contribute to the ENS and sympathetic nervous system (Martik & Bronner, 2017). After reaching their final destinations, NCCs undergo morphological changes and differentiate into their final derivatives (Hall, 2008).

The induction, migration, differentiation, and survival of NCCs involve intricate molecular and cellular events, making them a highly complex and regulated process. This process is regulated by an orchestrated gene regulatory network (GRN) in which each step is temporally and spatially regulated by transcription factors (TFs), signaling molecules, and epigenetic regulators (Bhattacharya et al., 2020; Cooper & Tsakiridis, 2022; Simoes-Costa & Bronner, 2015). These molecules interact with each other through a series of feedback loops and activate downstream gene batteries (Martik & Bronner, 2017). Disruption of the gene regulatory network responsible for regulating NCC development by prenatal ethanol exposure leads to impaired induction, migration, differentiation, and survival of NCCs.

As mentioned above, cranial NCCs migrate laterally and ventrally to contribute to the formation of various facial structures, such as the facial primordia, branchial arches, cranial nerves, and melanocytes. Extensive research conducted on human populations and animal models has revealed that the impact of ethanol on NCC populations is not uniform. Specifically, cranial NCCs are more
susceptible to the effects of ethanol than trunk NCCs (Ahlgren et al., 2002; Rovasio and Battiato, 2002; Czarnobaj et al., 2014), which aligns with the observation that tissues derived from trunk NCCs are generally less affected by ethanol than those derived from cranial NCCs. Remarkably, individuals with FASD, particularly FAS, often exhibit a pattern of facial anomalies, such as a flattened midface, micrognathia, smooth philtrum, thin upper lip, and short palpebral fissures (Klingenberg et al., 2010). Many of these affected structures, including the upper and lower jaw, nose, ears, orbital bone, and forehead, are derived from cranial NCCs (Smith et al., 2014), underscoring their critical role in the pathogenesis of FASD. Due to the significant contribution of cranial NCCs to FASD and the majority of studies investigating the effects of ethanol on NCCs being conducted on cranial NCCs, this article primarily discusses the impact of ethanol on cranial NCCs, unless otherwise specified.

3. Effects of ethanol exposure on the induction and specification of neural crest cells in early embryonic development

NCC induction is a complex process that initiates at the early gastrula stage and lasts until the neural tube closure. During neuroectoderm development, transcriptional pathways are activated, which specify neural stem cell (NSC) subdomains along the dorsoventral axis in the neuroepithelium (Gandhi & Bronner, 2018). This induction occurs at the border of the neural folds between the neuroepithelium and ectoderm and the underlying paraxial and intermediate mesoderm (Teng & Labosky, 2006). In response to early inductive signals, neural plate border cells express a set of transcription factors (TFs) known as neural plate border specifiers, including Dlx5, Tfap2, Zic1, Msx1/2, Pax3/7, Hes4, Snail 1/2, Prdm1, Mafb, and Gdf7 (Liu & Cheung, 2016; Seal & Monsoro-Burq, 2020; Soldatov et al., 2019). These factors regulate each other and
cooperate with NC signaling pathways to specify NC progenitors within the neural plate border by activating the expression of NC specifier genes such as Snail, FoxD3, and SoxE family TFs (Liu & Cheung, 2016; Simoes-Costa et al., 2014). MSX1 also plays a critical role as an activator of NC specification (Simoes-Costa et al., 2014). Overall, this complex process of NC induction and specification involves the intricate interplay of multiple signaling pathways and transcriptional factors.

Induction and specification are critical processes in NCC development. Although studies investigating the effects of ethanol on NCC induction and specification are limited, a transcriptome profiling study revealed that exposure to ethanol during a stage targeted for NC induction did not lead to significant changes in the expression levels of key genes involved in NC induction, such as Snai1, Snai2, Foxd3, Wnt6, Wnt1, and Foxd3, in the neural folds of chick embryos (Berres et al., 2017). This suggests that ethanol-induced reduction in NCs may occur through mechanisms other than altered fate determination. Moreover, ethanol exposure did not affect NC induction in Xenopus embryos, as revealed by the unaltered expression of neural plate border genes Msx1, Pax3, and Zic1, and neural crest marker gene Slug (Shi et al., 2014). In contrast, Flentke and coworkers (Flentke et al., 2019) reported a decrease in the expression of NC inducers such as Id1, Bmp4, Shh, HoxA1, and Fgf8 after ethanol exposure in chick NC progenitors. Therefore, additional studies are necessary to gain a clear understanding of the effects of ethanol on the initial stages of NCC development and its role in ethanol-induced craniofacial dysmorphology.

4. Impact of ethanol exposure on neural crest cell migration

Following the induction, NCCs undergo significant changes in gene regulation, enabling them to engage in epithelial-mesenchymal transition (EMT). This process involves major structural
changes, including remodeling of adhesive properties, cytoskeletal rearrangements, polarity, and acquisition of migratory behavior (Gandhi & Bronner, 2018; Szabo & Mayor, 2018). During this morphological transition, NCCs delaminate from the neural tube and neuroepithelium, invade surrounding tissues, migrate spatially towards peripheral regions of the embryo, and differentiate into various cell types (Schock et al., 2023). The conserved distribution pattern and directional migration of NCCs are regulated by a balance between intrinsic and extracellular signals and by crosstalk between cells and the extracellular matrix (ECM) (Szabo & Mayor, 2018).

Ethanol exposure can disrupt the migration of NCCs (Czarnobaj et al., 2014; Fan et al., 2022; Rovasio & Battiato, 2002; Zhang et al., 2017). A study has shown that *Xenopus laevis* cranial NCC cultures exposed to a low ethanol concentration exhibited reduced numbers of migrating cells, delayed dispersion, and decreased displacement distances (Czarnobaj et al., 2014). Moreover, *in vitro* ethanol exposure resulted in decreased directional movement and shorter travel distances of chick cephalic and trunk NCCs (Rovasio & Battiato, 2002). Ethanol exposure was also found to impair the migration of chick cranial NCCs by disrupting the actin cytoskeleton (Oyedele & Kramer, 2013). Real-time tracking of NCC migration in ethanol-exposed zebrafish embryos demonstrated a loss of left-right symmetry and shorter migration distance (Boric et al., 2013). A recent study has further shown that ethanol exposure inhibited NCC migration both *in vitro* and *in vivo* in zebrafish embryos (Fan et al., 2022).

4.1. Ethanol exposure impairs neural crest cell migration by disrupting the epithelial-to-mesenchymal transition

EMT is a critical process in NCC migration and is regulated by several transcription factors, such as Snail/Slug, Foxd3, Sox9/10, Ets1, and LSox5. Snail, a family of zinc finger transcriptional
repressors, has been shown to induce the expression of Slug, Foxd3, and Ets1 and is known to downregulate the expression of epithelial cadherin (E-cadherin) and cell adhesion molecules, claudins and occludins, which are crucial for cell-cell adhesion (Kalluri & Weinberg, 2009). E-cadherin expression is regulated spatially and temporally, and its downregulation is one of the main initiation events of EMT (Cano et al., 2000). Snail also enhances the expression of mesenchymal markers, such as Vimentin and matrix metallopeptidase 2 (MMP-2). Vimentin is a type III intermediate filament expressed in developing organisms (Pattabiraman et al., 2020). The loss of epithelial markers and the increase of mesenchymal markers are the hallmarks of EMT (Liu et al., 2015).

Snail1 is a transcription factor that plays a significant role in the induction of EMT by inhibiting E-cadherin (Cano et al., 2000). The expression of Snail1 can be regulated by epigenetic mechanisms, including histone H3 lysine methylation (Li et al., 2019). The tri-methylation of histone H3 lysine regulates gene expression. For instance, H3K4me3, H3K79me3, and H3K36me3 are generally associated with transcriptional activation, and H3K27me3 can repress transcription (Barski et al., 2007). Ethanol exposure has been shown to reduce the levels of H3K4me3 at the Snail1 promoter and decrease the expression of Snail1, leading to an increase in E-cadherin and reduction in Vimentin, thereby inhibiting EMT in NCCs (Li et al., 2019). In addition, a recent study has also investigated the role of miRNA in the impairment of EMT and migration of NCCs after ethanol exposure (Fan et al., 2022). The study found that exposure to ethanol significantly inhibited EMT and migration of NCCs by increasing the expression of miR-34a and subsequent inhibition of Snail1, leading to an increase in the expression of E-cadherin and a reduction in the expression of Vimentin. The downregulation of miR-34a diminished ethanol-induced inhibition of Snail1, upregulation of E-cadherin, and reduction of Vimentin, and reduced
ethanol-induced inhibition of NCC migration, suggesting that ethanol-induced upregulation of miR-34a and subsequent downregulation of Snail1 is the major cause of the inhibition of NCC migration. In contrast, it was reported that transient ethanol exposure induced calcium transient, which in turn mediated the upregulation of Snai2. Surprisingly, while Snai2 was upregulated, transient ethanol exposure increased the expression of E-cadherin rather than downregulating it as expected and enhanced NCC migration in chick embryos (Flentke et al., 2019). The authors of this study acknowledged that these findings are inconsistent with the well-known ability of ethanol to inhibit NCC migration, and they have proposed some potential speculations to explain the unexpected results. These findings highlight the complexity of ethanol's effects on NCC migration, and further investigation is necessary to understand better the underlying mechanisms of ethanol-induced impairment of NCC migration.

4.2. Ethanol disrupts neural crest cell migration by impairing the chemotactic mechanisms

The migration of NCCs is influenced by various chemotactic factors, including vascular endothelial-derived growth factor (VEGF), platelet-derived growth factor (PDGF), fibroblast growth factors (FGFs), glial cell-derived neurotrophic factor (GDNF), stromal cell-derived factor 1 (SDF1), semaphorin/neuropilin, RGM-a/neogenin, and complement fragment c3a (McLennan et al., 2015; Shellard & Mayor, 2016; Tolosa et al., 2016). The chemotactic mechanisms directing NCC migration are critical for their spatiotemporal orientation towards specific regions of the embryo (Shellard & Mayor, 2016). For example, cranial NCCs originating from the prosencephalon, mesencephalon, and rhombencephalon follow specific migratory pathways into the craniofacial region. Meanwhile, a subgroup of NCCs migrate to the periocular mesenchyme and critically contribute to the developing eye (Eason et al., 2017). Mesencephalic NCCs, on the
other hand, migrate toward the optic vesicle in response to Sonic hedgehog (Shh) concentration gradients in a Patched (Ptc)/Smoothened (Smo)-dependent manner (Tolosa et al., 2016). Shh is a highly conserved paracrine factor that acts as a critical checkpoint in craniofacial development (Ahlgren et al., 2002; Tolosa et al., 2016).

Ahlgren and colleagues investigated the effects of ethanol on Shh signaling and cranial NCC development. They observed that ethanol treatment in chick embryos reduced the expression of Shh and its downstream targets, including Patched, Gli, Gli2, and Gli3, but had moderate impacts on Fgf-8 transcripts and no noticeable changes in Wnt1 or BMP7 expression. The administration of Shh prevented ethanol-induced cranial NCC mortality and related craniofacial defects (Ahlgren et al., 2002). These findings suggest that ethanol-induced craniofacial abnormalities are partly caused by the depletion of Shh and subsequent NCC death. Tolosa and coworkers also conducted a study to investigate the impact of ethanol on Shh signaling and NCC migration. They observed that exposure to a teratogenic concentration of ethanol decreased the chemotactic index and orientation and repulsive migration of NCCs along the Shh gradient. The study also found that ethanol had a higher cytotoxic effect and reduced the proliferative capacity of NCCs in the absence of Shh (Tolosa et al., 2016). The observed decrease in the chemotactic index, orientation, and repulsive migration of NCCs along the Shh gradient suggests that ethanol interferes with normal guidance cues provided by Shh. However, ethanol may also disrupt chemotaxis by interfering with the ability of NCCs to respond to the chemotactic signals. Further research is necessary to fully elucidate the underlying molecular mechanisms by which ethanol impairs the chemotactic response of NCCs. Nonetheless, these findings suggest that the chemotactic mechanism plays a crucial role in the directional migration of NCCs and that ethanol exposure may disrupt this mechanism, leading to aberrant NCC migration patterns.
4.3. Ethanol impairs neural crest cell migration by homocysteine dysregulation

Alcohol consumption is known to decrease folic acid uptake and increase homocysteine accumulation, which can lead to oxidative stress, DNA damage, apoptosis, and developmental defects (Bleich et al., 2000; Halsted et al., 2002; Stickel et al., 2000). Folic acid plays a critical role in regulating the migration of NCCs, as demonstrated by a study showing that knockdown of the reduced folate carrier (RFC), a membrane-bound receptor that facilitates the transport of reduced folate into cells, inhibited the ventral migration of NCCs, leading to their failure to segregate into distinct streams in Xenopus embryos. This migration defect was rescued by co-injection of the Xenopus RFC mRNA, suggesting that folic acid plays a critical role in regulating the directional movement of NCCs during embryonic development (Li et al., 2011). Folic acid deficiency can lead to elevated levels of homocysteine due to reduced breakdown of homocysteine. It has been shown that ethanol exposure resulted in elevated homocysteine levels during robust NCC emigration at stage 27 in Xenopus embryos. With an in situ hybridization assay of Twist expression, a study investigated the effects of ethanol and elevated homocysteine on NCC migration and found that Twist-positive NCCs were absent in the most ventral region of the branchial arch in ethanol-exposed embryos. However, microinjection of 5-methyltetrahydrofolate (5-MTHF), the most bioactive form of folic acid, reduced ethanol-induced increases in homocysteine and alleviated the effects of ethanol on NCC migration in Xenopus embryos (Shi et al., 2014). These findings suggest that ethanol-induced impairment in NCC migration may result from ethanol-induced folic acid deficiency and subsequent elevation of homocysteine levels.
Figure 2. Schematic representation of the mechanisms underlying ethanol-induced impairment of NCC migration. Upward and downward vertical red arrows indicate upregulation and repression, respectively, in ethanol-exposed NCCs.

Overall, these findings highlight the multifaceted effects of ethanol on NCC migration, including disruption of EMT, impairment of chemotactic mechanisms, and dysregulation of homocysteine. Understanding these mechanisms provides valuable insights into the underlying causes of craniofacial abnormalities associated with prenatal ethanol exposure. The molecular pathways involved in ethanol-induced impairment of NCC migration are shown in Figure 2.

5. Ethanol-induced impairment of neural crest cell differentiation during development

The differentiation of NCCs involves a sequence of cell fate decisions (Schock et al., 2023; Soldatov et al., 2019). During NCC differentiation, the multipotent NCCs undergo a series of molecular and morphological changes and acquire specific cell fates to differentiate into various
cell types, including neurons, glia, melanocytes, cartilage, and bone cells. This process is regulated by specific signaling molecules, such as bone morphogenetic proteins (BMPs), Wnt, fibroblast growth factors (FGFs), and sonic hedgehog (SHH) (Schock et al., 2023). For instance, BMPs promote the differentiation of sensory neurons and glial cells (Schneider et al., 1999), while Wnt signaling is essential for the formation of melanocytes and peripheral neurons (Bhatt et al., 2013). FGF signaling plays a critical role in promoting the fate of NCCs towards a skeletogenic type (Li et al., 2010). The timing and duration of signaling molecule exposure and the crosstalk between different signaling pathways are essential factors that determine the fate of NCCs and their differentiation into specific cell types. A recent study has demonstrated that ethanol exposure significantly inhibited the expression of a type II collagen gene Col2a1a, one of the chondrocyte markers, in ocular and craniofacial NCCs and disrupted eye and jaw development in zebrafish embryos (Williams & Bohnsack, 2022). It has also been shown that microRNAs (miRNAs) are also involved in ethanol-induced impairments in NCC differentiation.

miRNAs are small, single-stranded RNA molecules approximately 22 nucleotides in length that play a crucial role in gene regulation and various biological processes, including embryogenesis, cell differentiation, organogenesis, growth, apoptosis, stem cell maintenance, evolution, and disease (Antonaci & Wheeler, 2022; Vidigal & Ventura, 2015). A recent prospective cohort study has demonstrated that miRNAs, including miR-421, miR-128-3p, and miR-30c-5p, derived from umbilical cord collected at birth can accurately predict the severity of neonatal opioid withdrawal syndrome and the need for pharmacological treatment, and identify infants at risk of prolonged hospitalization, highlighting the potential of miRNAs as biomarkers for risk assessment in infants born to women with drug abuse, including opioid and alcohol abuse (Mahnke et al., 2022). A study has also shown that ethanol exposure can significantly increase
miR-140-3p and miR-140-5p in neural stem cells-derived extracellular vesicles and that dysregulated miRNA content of extracellular vesicles contributed to ethanol-induced aberrant neural progenitor cell growth and maturation (Tseng et al., 2019). miRNAs also play a crucial role in neural crest formation (Weiner, 2018) and are important targets of ethanol during embryonic development. Increasing evidence suggests that miRNAs play a crucial role in the ethanol-induced impairment of NCCs and the etiology of FASD (Chen et al., 2015; Fan et al., 2022; Fan et al., 2019; Yuan et al., 2020; Zhao et al., 2021). Specifically, a study has found that miR-34a mediated ethanol-induced impairment of neural differentiation of NCCs by targeting autophagy (Fan et al., 2019).

Autophagy is a highly coordinated and evolutionarily conserved mechanism that breaks down and recycles old and damaged cellular components (Ichimiya et al., 2020). During autophagy, ubiquitin-like autophagosomal membrane light-chain 3 (LC3), a ubiquitous soluble protein, undergoes a conversion from a cytosolic version (LC3I) to a phosphatidylethanolamine-conjugated version (LC3II) that is transported to autophagosomal membranes (Saha et al., 2018). The amount of LC3II is associated with the quantity of autophagosomes, and a higher LC3II/I ratio is considered an indicator of autophagy (Fader & Colombo, 2009). Among the autophagy-related proteins, Atg9a, a multi-spanning membrane protein, plays a vital role in assembling autophagosomes and subsequently activating autophagy (Imai et al., 2016). In addition, p62 is a multifunctional scaffold protein that interacts with the LC3 autophagy regulator in the autophagic degradation pathway (Ichimiya et al., 2020).

Autophagy is a critical process for normal neural differentiation and apoptotic cell clearance (Aburto et al., 2012). It plays an important role in both development and differentiation, as
inhibition of autophagy-related genes leads to neurodegeneration and developmental defects (Sharma et al., 2022). It has been demonstrated that the accumulation of the protein p62 impaired neural stem cell (NSC) differentiation by increasing reactive oxygen species (ROS) accumulation and decreasing SOD1 (Wang et al., 2016). Studies have also shown that the coordinated regulation of β-catenin by autophagy and BMP signaling determined the fate of cranial NCCs during craniofacial development (Yang et al., 2021). In a recent study, Fan and coworkers investigated the impact of ethanol exposure on the miRNAs and autophagy pathways in the neural differentiation of NCCs. Their findings showed that exposure to ethanol significantly reduced autophagy, as evidenced by a decreased LC3II/I ratio and increased expression of p62 protein, and inhibited neural differentiation of NCCs. Additionally, the knockdown of p62 restored the expression of neurogenesis genes neurofilament and Mash1 in ethanol-exposed NCCs, indicating that ethanol exposure can inhibit neural differentiation of NCCs by inhibiting autophagy. The study also found that ethanol exposure significantly increases miR-34a expression in NCCs. miR-34a inhibition restored the expression of miR-34a direct target Atg9a and significantly decreased ethanol-induced inhibition of autophagy in NCCs. Moreover, down-regulation of miR-34a prevented ethanol-induced inhibition of neural differentiation of NCCs (Fan et al., 2019). These results demonstrate that miR-34a mediates the ethanol-induced inhibition of neural differentiation of NCCs by targeting Atg9a.

These findings highlight the detrimental effects of ethanol on NCC differentiation and provide mechanistic insights into the underlying processes involved, particularly in the role of miRNAs and autophagy in ethanol-induced impairment of NCC differentiation. However, it is important to note that the research on the effects of ethanol on NCC differentiation is still limited, and more

Table 1: Effects of ethanol on the induction, migration and differentiation of neural crest cells
<table>
<thead>
<tr>
<th>Experimental models</th>
<th>NCC impairment</th>
<th>Key findings/outcomes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chick NC progenitors</td>
<td>NCC induction</td>
<td>Ethanol decreased the expression of NC inducers, including Id1, bmp4, shh, hoxA1, and fgf8.</td>
<td>(Flentke et al., 2019)</td>
</tr>
<tr>
<td>Xenopus embryos</td>
<td>NCC induction</td>
<td>Ethanol did not affect NCC induction in Xenopus embryos, as revealed by the unaltered expression of neural plate border genes Msx1, Pax3, and Zic1 and neural crest marker gene Slug.</td>
<td>(Shi et al., 2014)</td>
</tr>
<tr>
<td>Chick embryos</td>
<td>NCC induction</td>
<td>Ethanol did not lead to significant changes in the expression levels of key genes involved in NCC induction, such as Snai1, Snai2, Foxd3, Wnt6, and Wnt1 in neural folds.</td>
<td>(Berres et al., 2017)</td>
</tr>
<tr>
<td>Chick cranial NCCs</td>
<td>NCC migration</td>
<td>Ethanol exposure impaired cranial NCC migration by disrupting the actin cytoskeleton.</td>
<td>(Oyedele &amp; Kramer, 2013)</td>
</tr>
<tr>
<td>Chick NCCs</td>
<td>NCC migration</td>
<td>In vitro ethanol exposure resulted in decreased directional movement and shorter travel distances of cephalic and trunk NCCs.</td>
<td>(Rovasio &amp; Battiato, 2002)</td>
</tr>
<tr>
<td>Xenopus cranial NCC culture</td>
<td>NCC migration</td>
<td>NCCs exposed to ethanol exhibited reduced numbers of migrating cells, delayed dispersion, and decreased displacement distances.</td>
<td>(Czarnobaj et al., 2014)</td>
</tr>
<tr>
<td>Chick cranial NCCs/embryos</td>
<td>NCC migration</td>
<td>Ethanol impaired EMT and NCC migration.</td>
<td>(Zhang et al., 2017)</td>
</tr>
<tr>
<td>NCCs/ Zebrafish embryos</td>
<td>NCC migration</td>
<td>Ethanol significantly inhibited EMT and migration of NCCs by increasing the expression of miR-34a and subsequent inhibition of Snail1.</td>
<td>(Fan et al., 2022)</td>
</tr>
<tr>
<td>Chick embryos</td>
<td>NCC migration</td>
<td>Ethanol decreased the chemotactic index, orientation, and repulsive migration of NCCs along the Shh gradient.</td>
<td>(Tolosa et al., 2016)</td>
</tr>
<tr>
<td>Zebrafish embryos</td>
<td>NCC migration</td>
<td>Ethanol exposure resulted in a loss of left-right symmetry and shorter migration distance in NCCs.</td>
<td>(Boric et al., 2013)</td>
</tr>
<tr>
<td>Xenopus embryos</td>
<td>NCC migration</td>
<td>Ethanol increased homocysteine levels and impaired NCC migration.</td>
<td>(Shi et al., 2014)</td>
</tr>
<tr>
<td>Mouse NCCs</td>
<td>NCC differentiation</td>
<td>Ethanol inhibited neural differentiation of NCCs through miR-34a-mediated inhibition of autophagy.</td>
<td>(Fan et al., 2019)</td>
</tr>
<tr>
<td>Zebrafish embryos</td>
<td>NCC differentiation</td>
<td>Ethanol inhibited the expression of a type II collagen gene col2a1a, one of the chondrocyte markers, in ocular and craniofacial NCCs and disrupted eye and jaw development.</td>
<td>(Williams &amp; Bohnsack, 2022)</td>
</tr>
</tbody>
</table>

investigations are required to elucidate the underlying mechanisms by which ethanol impairs NCC differentiation. These studies will contribute to a more comprehensive understanding of the effects of ethanol on NCC differentiation and aid in the development of potential interventions or
preventive measures for FASD. The effects of ethanol on the induction, migration, and differentiation of NCCs are summarized in Table 1.

6. Impact of ethanol exposure on neural crest cell survival

The pathogenesis of FASD is a complex and multifactorial process, and apoptosis is considered a major mechanism underlying the pathogenesis of FASD (Dunty Jr et al., 2001; Kotch & Sulik, 1992; Lu et al., 2023). NCCs are among the most vulnerable cell populations affected by ethanol exposure. Studies have demonstrated that ethanol can induce apoptosis in NCCs (Chen et al., 2000; Chen & Sulik, 1996; Chen et al., 1996a; Kotch & Sulik, 1992; Sun et al., 2014) and that apoptosis in NCCs contributes heavily to ethanol-induced abnormalities (Kotch & Sulik, 1992; Sulik, 2005). For instance, chick embryos exposed to ethanol exhibit a reduction in the frontonasal process, hypoplastic branchial arches, and apoptotic death of cranial NCCs (Ahlgren et al., 2002). Another study reported that ethanol treatment induced apoptosis and reduced cranial NCCs in patterns consistent with observed dysmorphologies of cranial NCC-derived cranial structures (Cartwright & Smith, 1995).

Multiple signaling pathways have been demonstrated to be involved in ethanol-induced apoptosis in NCCs. These include ROS and Nrf2 signaling (Chen et al., 2000; Chen et al., 1996a; Dong et al., 2008; Yan et al., 2010), CaMKII and Wnt/β-catenin signaling (Flentke et al., 2011; Flentke, Garic, et al., 2014), Seven in absentia homolog 1 (Siah1) signaling (Sun et al., 2014; Yuan et al., 2020), Pdgfra/PI3K/mTOR pathways (McCarthy et al., 2013), and p53, p38 MAPK, and B-cell lymphoma 2 (Bcl-2) pathways (Yuan et al., 2018; Yuan et al., 2017). Additionally, miRNAs and other epigenetic mechanisms also contribute to ethanol-induced apoptosis in NCCs (Chen et al., 2015; Yuan et al., 2020). Furthermore, Garic and colleagues used high-throughput
transcriptional profiling to identify gene candidates that could alter susceptibility to ethanol-induced neurotoxicity in neural crest and neuroprogenitors of *Gallus gallus* embryos with differential ethanol sensitivities (W98S and W98D) in the absence of ethanol exposure. They found 363 differentially expressed genes, with the largest cluster being ribosomal proteins, followed by metabolism, oxidative phosphorylation, spliceosome, protein processing, and calcium/β-catenin signaling (Garic et al., 2014).

**6.1. Oxidative stress and the Nrf2 signaling pathway in ethanol-induced apoptosis in neural crest cells**

Reactive oxygen species (ROS) are highly reactive chemicals, including superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and the hydroxyl radical (·OH), that are produced during normal cellular activity, such as mitochondrial respiration and xenobiotic metabolism, and participate in cellular signaling (Sies & Jones, 2020). However, various stresses can result in excess production of ROS, which can be scavenged by various antioxidative defense mechanisms, including superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH), and other enzymes. Oxidative stress occurs when ROS production exceeds the ability of the antioxidative system to scavenge them, resulting in damage to cellular membranes, DNA, and proteins (Schieber & Chandel, 2014).

Ethanol exposure can induce apoptosis in NCCs by increasing ROS generation. Chen and Sulik found that exposure of cranial NCCs to ethanol increased the production of superoxide anion radicals and induced cell death. Co-treatment with free radical scavengers, such as SOD, CAT, or α-tocopherol, can improve cell viability (Chen & Sulik, 1996). They have also shown that co-treatment of NCCs with ethanol and iron chelators, such as deferoxamine or phenanthroline,
significantly increased the percentage of viable cells compared with exposure to ethanol alone. Co-treatment with an antioxidant, N-acetylcysteine (NAC), significantly diminished the toxicity of ethanol alone, as well as from the combination of ethanol exposure and iron loading (Chen & Sulik, 2000). These results confirm the role of iron and free radical-mediated damage in ethanol-induced cytotoxicity in NCCs. Additionally, it was found that ethanol exposure induced apoptosis in cranial NCCs, and co-treatment with NAC diminished apoptosis in ethanol-exposed NCCs (Wentzel & Eriksson, 2009). Furthermore, ethanol exposure significantly increased the mRNA expression of NOX regulatory subunits, p22phox, p67phox, NOXA1, and NOXO1, and increased NOX enzyme activity in mouse embryos. Co-treatment with the NOX inhibitor, diphenyleneiodonium (DPI), significantly prevented ethanol-induced increases in NOX enzyme activity, ROS generation, oxidative DNA damage, and diminished apoptosis in ethanol-exposed embryos. These results suggest that NOX is a critical source of ROS in ethanol-exposed embryos and plays an important role in ethanol-induced oxidative stress and apoptosis (Dong et al., 2010). These findings demonstrate that oxidative stress contributes significantly to ethanol-induced apoptosis in NCCs.

Nrf2 is a redox-regulating transcription factor belonging to the basic region leucine zipper group expressed in various tissues (Cuadrado et al., 2019). The Nrf2 signaling is initiated by various oxidative and electrophilic stimuli, such as ROS and heavy metals, as well as by certain disease mechanisms (Hayes & Dinkova-Kostova, 2014). Under normal conditions, Nrf2 is located in the cytoplasm by binding to Kelch-like ECH-associated protein 1 (Keap1), which facilitates the proteolytic degradation of Nrf2 by ubiquitylation (Itoh et al., 1999). Nrf2 activation occurs through nuclear translocation from the cytoplasm, which is mediated through a ubiquitin-26S proteasome pathway regulated by the Keap1/Cul3-independent ubiquitin ligase (E3) (Kobayashi et al., 2004).
Nrf2 plays a vital role in the transcriptional activation of many genes involved in cellular defense and antioxidative mechanisms through the antioxidant response element (ARE) (Cuadrado et al., 2019). The activation of the Nrf2-ARE pathway leads to the induction of phase II detoxification enzymes and antioxidant enzymes such as heme oxygenase-1 (HO-1), NAD(P)H: quinone oxidoreductase 1 (NQO1), superoxide dismutase (SOD), catalase, and glutathione S-transferase (GST) (Dinkova-Kostova et al., 2002).

Dong and coworkers conducted the first study to determine the role of Nrf2 signaling in FASD. They found that maternal ethanol treatment increased Nrf2 protein levels and Nrf2-ARE binding in mouse embryos, resulting in a moderate increase in the mRNA and protein expression of Nrf2 downstream antioxidant genes. Induction of Nrf2 by an Nrf2 inducer, 3H-1,2 dithiole-3-thione (D3T), significantly increased Nrf2 protein levels and Nrf2-ARE binding, strongly induced the mRNA and protein expression of Nrf2 downstream antioxidant genes, and decreased ethanol-induced ROS generation and apoptosis in mouse embryos (Dong et al., 2008). These findings demonstrate that Nrf2 signaling is involved in the induction of the antioxidant response in ethanol-exposed embryos. Further studies from this group have shown that overexpression of Nrf2 significantly increased ARE activity and enhanced protein expression and activities of Nrf2 target antioxidants. Overexpression of Nrf2 also decreased ROS generation and diminished apoptosis in ethanol-exposed NCCs (Chen et al., 2013a). Additionally, induction of Nrf2 by its inducers, tBHQ or sulforaphane (SFN), significantly upregulated Nrf2 and its downstream antioxidants and prevented ethanol-induced oxidative stress and apoptosis in NCCs (Chen et al., 2013b; Yan et al., 2010). These findings demonstrated that Nrf2-mediated antioxidant response plays a vital role in the susceptibility of NCCs to ethanol-induced oxidative stress and apoptosis.
6.2. The involvement of CaMKII and Wnt/β-Catenin signaling pathways in ethanol-induced apoptosis in neural crest cells

Numerous studies have investigated the role of calcium in ethanol-induced apoptosis in NCCs (Flentke, Garic, et al., 2014; Flentke, Klingler, et al., 2014; Smith, Garic, Flentke, et al., 2014). It was reported that ethanol exposure increased intracellular calcium levels in NC progenitors in a G protein signaling/PLC-dependent manner, resulting in the induction of apoptosis (Garic-Stankovic et al., 2006; Garic-Stankovic et al., 2005). A study also showed that calcium transient is followed by the activation of calmodulin-dependent protein kinase II (CaMKII) through the binding of calcium or calmodulin. The activation of CaMKII was rapid after ethanol exposure, and its activity was elevated by 300% compared to the control, which can independently and constantly modulate the cellular activity after the calcium transient elapsed (Garic et al., 2011). It was also found that the transcriptional effector β-catenin is the major target of calcium transient, and its loss of function leads to NCC death (Flentke et al., 2011). Ethanol treatment at clinically relevant concentrations was also found to destabilize the nuclear β-catenin in NC progenitors and reduce downstream signaling gene transcripts, including Slug, FoxD3, and Wnt6, which regulate neural crest induction, expansion, and survival. Additionally, Flentke and colleagues examined how calcium-dependent and -independent Wnt effectors contribute to the neurotoxic effects of ethanol on the NCCs in chick embryos. They found that inhibiting calcium-independent Wnt effectors, such as GSK3β, Protein kinase C (PKC), calpain, and JNK, failed to restore β-catenin activity and cell survival in the presence of ethanol, while inhibition of calcium-dependent CaMKII recovered β-catenin activity and cell survival. This study also reveals that CaMKII can phosphorylate and destabilize β-catenin directly and indirectly through the phosphorylation of LEF-1 and the transforming growth factor β (TGF-β)-activated kinase/Nemo-like kinase (TAK/NLK) kinases, which inhibit
TCF/LEF function. Therefore, ethanol-induced activation of CaMKII contributes to ethanol-induced apoptosis in NCCs through the inactivation of the β-catenin/TCF-LEF complex (Flentke et al., 2011). Furthermore, this group investigated the mechanism of ethanol-induced neurotoxic effects in NCCs in zebrafish embryos. This study found that short-term exposure to ethanol results in calcium-dependent activation of CaMKII, an increase in apoptosis in NCCs, and craniofacial defects in zebrafish embryos, suggesting that this mechanism is evolutionarily conserved (Flentke, Klingler, et al., 2014).

6.3. The role of seven in absentia homolog 1 signaling pathway in ethanol-induced apoptosis in neural crest cells

Seven in absentia homolog 1 (Siah1) is a member of the E3 ubiquitin ligase family that comprises two zinc finger domains, an N-terminal RING domain, and a substrate-binding domain (Ko et al., 2019; Qi et al., 2013). The Siah is widely expressed in developing embryos and adult tissues and is involved in the ubiquitination and proteasomal degradation of various proteins involved in multiple signaling pathways, such as transcriptional regulators, membrane receptors, and other proteins (Tiedt et al., 2001; Winter et al., 2008). Siah1 also plays a critical role in cell proliferation, migration, axon guidance, and development (Hu & Fearon, 1999; Ko et al., 2019; Lee et al., 2008; Leung et al., 2014). In addition, Siah is involved in the induction of apoptosis and tumor suppression (Nemani et al., 1996).

Sun and coworkers provided the first evidence of Siah1’s role in ethanol-induced apoptosis in NCCs and the pathogenesis of FASD. Their findings showed that ethanol exposure increased the mRNA and protein expression and nuclear translocation and accumulation of Siah1 in NCCs,
ultimately resulting in apoptosis. Knockdown of Siah1 significantly reduced ethanol-induced caspase-3 activation and apoptosis in NCCs, indicating that Siah1 plays a critical role in ethanol-induced apoptosis in NCCs (Sun et al., 2014). Another study by the same group demonstrated that ethanol exposure increased total protein levels of Siah1, phosphorylation of p38 MAPK, phosphorylation of p53 at serine 15, and apoptosis in NCCs. Knockdown of Siah1 or p38 MAPK significantly reduced ethanol-induced accumulations and phosphorylation of p53, and apoptosis in NCCs, indicating that ethanol triggers apoptosis through the Siah1-mediated activation of p38 MAPK/p53 signaling pathway (Yuan et al., 2017). Recently, this group discovered that ethanol-induced downregulation of miR-135a upregulated Siah1, activated the p38 MAPK/p53 pathway, and contributed to ethanol-induced apoptosis in NCCs. Furthermore, overexpression of miR-135a protected against ethanol-induced apoptosis in NCCs and craniofacial defects in a zebrafish model of FASD (Yuan et al., 2020).

6.4. Ethanol induces apoptosis in neural crest cells by disrupting the cell membrane

Ethanol is known to disrupt the physical structure of various membranes, such as the plasma membrane, membranes of cell organelles, and liposomes (Goldstein, 1986; Toth et al., 2014). It can increase membrane fluidity and alter the lipid environment of membranes, resulting in the dysfunction of the membrane-associated proteins (Escriba et al., 2008). Using the technique of fluorescence recovery after photobleaching (FRAP), Chen and coworkers found that ethanol exposure resulted in a dose-dependent increase in membrane lipid lateral mobility, which was negatively correlated with the viability of NCCs. Pre- or co-treatment of NCCs with GM1 ganglioside reduced the ethanol-induced increases in membrane fluidity and decreases in cell viability. These results suggest that a change in membrane fluidity may contribute to ethanol-
induced NCC death, and GM1 ganglioside may protect the cell membrane by stabilizing its biophysical properties and biological functions (Chen et al., 1996b). Another study by the same group (Chen et al., 2000) compared the sensitivity of NCCs to ethanol-induced membrane disruption and cell death between NCCs from two different mouse strains, an inbred mouse strain (C57 BL/6J) and an outbred strain (ICR), that are more or less sensitive to ethanol-induced teratogenicity, respectively. They found that ethanol-induced cell death was significantly higher in C57 NCCs than in ICR NCCs. The membrane GM1 content was lower, and the lateral mobility of the membrane lipids was faster in C57 NCCs compared to ICR NCCs, both in control and ethanol-treated groups. In addition, ethanol exposure resulted in significant increases in the membrane lipid lateral mobility and decreases in the membrane GM1 content in the NCCs from both strains. These results suggest that different strain sensitivities to ethanol-induced teratogenicity may lie, at least in part, in the interstrain differential response of the NCCs and that the vulnerability of the NCCs to ethanol-induced death may be related to their endogenous membrane GM1 content. Additionally, a study examined the role of ceramide, one of the lipids in the cell membrane of eukaryotic cells, in ethanol-induced apoptosis in NCCs. Their findings showed that ethanol exposure increased ceramide levels, which led to apoptosis in NCCs in both \textit{in vitro} and \textit{in vivo} and defective cranial development (Wang & Bieberich, 2010).

6.5. Involvement of Pdgfra/PI3K/mTOR pathway in ethanol-induced apoptosis in neural crest cells

Platelet-derived growth factor receptor alpha (Pdgfra) is a receptor tyrosine kinase that plays a pivotal role in regulating various cellular functions, such as cellular migration, proliferation, and survival, by activating the PI3K (Sápi et al., 2011; Zemskov et al., 2009). PI3K, in turn, regulates
cell migration by activating small GTPases and cellular survival and growth by activating mTOR (Downward, 2004; Klinghoffer et al., 2002; Zhou & Huang, 2010). Pdgfra is expressed in cranial NCCs in mice and zebrafish, and its expression is essential for NCC development (Eberhart et al., 2008; Soriano, 1997; Tallquist & Soriano, 2003). McCarthy and colleagues (McCarthy et al., 2013) investigated the impact of pdgfra on ethanol-induced apoptosis in NCCs and craniofacial defects in zebrafish. Using a neural crest cell labeling fli1:EGFP transgenic line, they found that ethanol exposure slightly increased apoptosis in NCCs of wild-type embryos but caused a significant increase in apoptosis in NCCs and craniofacial defects in pdgfra mutants, suggesting that the interaction of pdgfra and ethanol lead to excessive apoptosis in NCCs, contributing to the ethanol-induced craniofacial defects in zebrafish embryos. Ethanol exposure also resulted in decreased levels of phosphor-eIF4 B, a downstream target of mTOR in mutants compared to the control group. Additionally, the upregulation of PI3K or mTOR signaling can protect against ethanol-induced craniofacial defects in pdgfra mutants. These findings demonstrate that ethanol-induced apoptosis in NCCs contributes to craniofacial defects in zebrafish embryos and that the protective effects of pdgfra against ethanol-induced apoptosis in NCCs and craniofacial defects are mediated by the PI3K/mTOR pathway.

6.6. Epigenetic mechanisms underlying the ethanol-induced apoptosis in neural crest cells.

Epigenetic mechanisms, such as DNA methylation, histone modifications, and microRNAs play a crucial role in gene regulation (Gibney & Nolan, 2010). Epigenetic modifications are known to regulate cellular processes such as differentiation, proliferation, and apoptosis (Srinageshwar et al., 2016). In particular, epigenetic mechanisms are crucial for the development of the nervous system and the formation and differentiation of NCCs (Weiner, 2018). Dysregulation of epigenetic
mechanisms can lead to various diseases, including cancer and developmental and neurological disorders. In particular, a number of studies have shown that epigenetic mechanisms play a critical role in ethanol-induced apoptosis in NCCs. These findings highlight the importance of epigenetic mechanisms in the pathogenesis of FASD.

6.6.1. DNA methylation

DNA methylation is an important epigenetic modification by which methyl groups are added to the cytosine base of DNA, which can lead to the repression of gene expression. The catalysis of this process is facilitated by DNA methyltransferases (DNMTs), which include DNMT1, DNMT3a, and DNMT3b (Mattei et al., 2022; Moore et al., 2013). DNMT1 maintains the existing DNA methylation pattern, while DNMT3a and DNMT3b establish de novo DNA methylation (Cheng & Blumenthal, 2022; Jones & Liang, 2009). Changes in the activity of DNMTs can alter the DNA methylation pattern of gene promoters, leading to hypermethylation or hypomethylation, which can inhibit or activate gene expression, respectively (Hervouet et al., 2013; Mattei et al., 2022). Recent research has shown that hypermethylation induced by increased DNMT activity is involved in ethanol-induced apoptosis in human neural crest cells (hNCCs). In particular, a recent study (Li et al., 2021) investigated the role of DNMT and hypermethylation of the promoters regions of genes encoding the inhibitor of apoptosis proteins (IAPs), including neuronal apoptosis inhibitory protein (NAIP) and X-linked inhibitor of apoptosis protein (XIAP), in ethanol-induced apoptosis in hNCCs derived from human embryonic stem cells. They found that exposure of hNCCs to ethanol significantly increased the expression of DNMT3a and the activity of DNMTs. Ethanol exposure also resulted in hypermethylation at the promoter regions of two IAP proteins, NAIP and XIAP, in hNCCs, reduced mRNA expression of NAIP and XIAP, and induced apoptosis.
These findings suggest that ethanol-induced apoptosis in hNCCs is mediated by increasing DNMT activity, leading to hypermethylation at the promoter regions of the genes encoding the IAP proteins and reduced expression of these anti-apoptotic genes.

### 6.6.2. Histone modifications

Histone modifications are a crucial epigenetic mechanism that regulates gene expression by modifying chromatin structure and function. Two common types of histone modifications are histone acetylation and histone methylation (Lawrence et al., 2016). Histone acetylation involves the addition of an acetyl group to lysine residues on histone proteins, which generally results in transcriptional activation by loosening the chromatin structure and facilitating access of transcription factors to DNA. The enzymes responsible for histone acetylation include histone acetyltransferases (HAT) and histone deacetylases (HDAC) (Lawrence et al., 2016; Marks et al., 2000). Recent research has demonstrated that HDAC and histone acetylation are involved in ethanol-induced apoptosis in NCCs. Specifically, it was found that ethanol exposure significantly increased HDAC activities and decreased the expression of acetyl-histone H3 in NCCs, which reduced the binding of acetyl-histone H3 to the Bcl-2 promoter, leading to reduced Bcl-2 expression and apoptosis in NCCs (Yuan et al., 2018). These findings indicate that ethanol can induce apoptosis in NCCs by altering histone acetylation and reducing the expression of anti-apoptotic genes.

Histone methylation is also critical in modulating the chromatin accessibility of transcription factors to target genes and gene transcription. In general, transcriptional activation marks increase the permissibility of gene transcription, whereas transcriptional silencing marks repress gene transcription (Lawrence et al., 2016). For instance, the triple methyl modification on the fourth
lysine of histone 3 (H3K4me3) facilitates gene transcription. In contrast, the triple methyl modification on the twenty-seventh lysine of histone 3 (H3K27me3) typically represses gene transcription (Barski et al., 2007). Studies have shown that H3K4me3 modification is associated with neurodevelopmental disorders (Vallianatos & Iwase, 2015; Wynder et al., 2010). Histone methylation also plays a critical role in ethanol-induced apoptosis in NCCs. A recent study reported that exposure to ethanol significantly decreased the levels of H3K4me3 at the promoters of Snail1 and reduced the expression of Snail1 in NCCs. This reduction in Snail1 expression resulted in increased expression of Snail1’s target gene, E-cadherin, leading to the inhibition of EMT and apoptosis in NCCs (Li et al., 2019).

6.6.3. microRNAs

MicroRNAs are small noncoding RNA molecules that are crucial in modulating gene expression. They are known to be involved in regulating a wide range of cellular and physiological processes, including apoptosis (D. Baek et al., 2008; Bartel, 2009). For instance, decreased expression of miR-125b has been associated with excessive apoptosis in rat embryos exposed to retinoic acid (Zhao et al., 2008), and decreased expression of miR-125b has also been reported in zebrafish embryos treated with gamma-irradiation or camptothecin, resulting in increased apoptosis (Le et al., 2009). Downregulation of miR-125b has also been associated with ethanol-induced apoptosis in NCCs. It was reported that ethanol exposure significantly decreased miR-125b expression in NCCs and mouse embryos, leading to increased PUMA and Bak1 protein expression, caspase-3 activation, and apoptosis. Up-regulation of miR-125b resulted in a significant decrease in PUMA and Bak1 expression, reduced ethanol-induced caspase activation, and diminished ethanol-induced embryotoxicity in cultured mouse embryos (Chen et al., 2015). As mentioned above, a recent study
revealed that ethanol exposure significantly decreased miR-135a expression in NCCs and zebrafish embryos. Downregulation of miR-135a by ethanol significantly increased the expression of Siah1 and activated the p38 MAPK/p53 pathway, leading to increased apoptosis in NCCs in

Figure 3. Schematic diagram depicting the mechanisms of ethanol-induced apoptosis in NCCs. Upward and downward vertical red arrows indicate upregulation and repression, respectively, in ethanol-exposed NCCs.

Table 2: Effects of ethanol on neural crest cell survival

<table>
<thead>
<tr>
<th>Experimental models</th>
<th>Mechanisms /Signaling pathways</th>
<th>Key findings/outcomes</th>
<th>Reference</th>
</tr>
</thead>
</table>


<table>
<thead>
<tr>
<th>Animal Cell Line</th>
<th>Pathway/Target</th>
<th>Summary</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse NCCs</td>
<td>ROS/oxidative stress</td>
<td>Exposure of NCCs to ethanol increased the production of superoxide anion radicals and induced cell death, which can be diminished by antioxidants.</td>
<td>(Chen &amp; Sulik, 1996, 2000; Wentzel &amp; Eriksson, 2009)</td>
</tr>
<tr>
<td>Mouse NCCs</td>
<td>Nrf2 signaling</td>
<td>Nrf2-mediated antioxidant response plays a vital role in the susceptibility of NCCs to ethanol-induced oxidative stress and apoptosis.</td>
<td>(Chen et al., 2013a, 2013b; Yan et al., 2010)</td>
</tr>
<tr>
<td>Chick NCCs/ Chick embryos</td>
<td>Calcium/ CaMKII/ β-catenin</td>
<td>Ethanol exposure induced apoptosis in NCCs through an intracellular calcium transient that can activate CaMKII, which in turn destabilized transcriptionally-active β-catenin.</td>
<td>(Flentke et al., 2011; Flentke, Garic, et al., 2014; Flentke, Klingler, et al., 2014; Garic-Stankovic et al., 2006; Garic-Stankovic et al., 2005; Garic et al., 2011)</td>
</tr>
<tr>
<td>Mouse NCCs</td>
<td>Membrane</td>
<td>Alteration of membrane fluidity contributed to ethanol-induced NCC death. GM1 ganglioside may protect the cell membrane by stabilizing its biophysical properties and biological functions.</td>
<td>(Chen et al., 2000; Chen et al., 1996a)</td>
</tr>
<tr>
<td>Mouse NCCs/Zebrafish embryos</td>
<td>Siah1/p38/p53 pathways</td>
<td>Ethanol triggered apoptosis in NCCs through the Siah1-mediated activation of the p38 MAPK/p53 signaling pathway.</td>
<td>(Sun et al., 2014; Yuan et al., 2017; Yuan et al., 2020)</td>
</tr>
<tr>
<td>Zebrafish embryos</td>
<td>Pdgfra/PI3K/mTOR</td>
<td>The interaction of pdgfra and ethanol led to excessive apoptosis in NCCs through PI3K/mTOR pathway.</td>
<td>(McCarthy et al., 2013)</td>
</tr>
<tr>
<td>Human NCCs</td>
<td>DNA methylation/ NAIP &amp; XIAP</td>
<td>Ethanol resulted in hypermethylation at the promoter regions of NAIP and XIAP in hNCCs, reduced mRNA expression of NAIP and XIAP, and induced apoptosis.</td>
<td>(Li et al., 2021)</td>
</tr>
<tr>
<td>Mouse NCCs</td>
<td>Histone acetylation/ Bcl-2</td>
<td>Ethanol increased HDAC activities and decreased the binding of acetyl-histone H3 to the Bcl-2 promoter, leading to reduced Bcl-2 expression and apoptosis in NCCs.</td>
<td>(Yuan et al., 2018)</td>
</tr>
<tr>
<td>Mouse NCCs</td>
<td>Histone methylation/ Snail1</td>
<td>Ethanol decreased the levels of H3K4me3 at the promoters of Snail1 and reduced the expression of Snail1 in NCCs, leading to the inhibition of EMT and increased apoptosis in NCCs.</td>
<td>(Li et al., 2019)</td>
</tr>
<tr>
<td>Mouse NCCs/Cultured mouse embryos</td>
<td>microRNA/ miR-125b</td>
<td>Ethanol exposure significantly decreased miR-125b expression in NCCs and mouse embryos, leading to increased PUMA and Bak1 protein expression and apoptosis.</td>
<td>(Chen et al., 2015)</td>
</tr>
<tr>
<td>Mouse NCCs/Zebrafish embryos</td>
<td>microRNA/ miR-135a</td>
<td>Ethanol-induced downregulation of miR-135a contributes to ethanol-induced apoptosis in NCCs by upregulating Siah1 and activating the p38 MAPK/p53 pathway.</td>
<td>(Yuan et al., 2020)</td>
</tr>
</tbody>
</table>

vitro and apoptosis, growth retardation, and craniofacial defects in zebrafish embryos (Yuan et al., 2020). These findings suggest that ethanol-induced downregulation of miR-135a contributes to
ethanol-induced apoptosis in NCCs by upregulating Siah1 and activating the p38 MAPK/p53 pathway.

Overall, the findings presented in this section provide invaluable insights into the diverse signaling pathways and mechanisms involved in ethanol-induced apoptosis in NCCs. These include oxidative stress and the Nrf2 signaling pathway, CaMKII and Wnt/β-Catenin signaling pathways, Siah1 signaling pathway, disruption of the cell membrane, the Pdgfra/PI3K/mTOR pathway, and epigenetic mechanisms. These findings shed light on the intricate interplay of these pathways and mechanisms involved in the detrimental effects of ethanol on NCC survival and development and provide essential foundational knowledge that can inform future research and interventions in the field of FASD. Figure 3 and Table 2 provide a concise overview of the impact of ethanol on NCC survival and the molecular mechanisms that mediate ethanol-induced apoptosis in NCCs.

7. Novel molecular targets and therapeutic strategies for preventing ethanol-induced impairments in neural crest cell development and FASD

FASD is a complex and multifactorial condition with no known cure. While the prevention of FASD is as simple as avoiding alcohol consumption during pregnancy, it remains a significant issue since approximately half of the pregnancies are unplanned. In humans, NCC development occurs as early as three weeks after conception, and the impairments of embryonic development associated with prenatal ethanol exposure can occur within the first three to eight weeks of pregnancy, often before a woman realizes she is pregnant. Consequently, FASD persists as a major public health problem, highlighting the need to develop effective strategies to prevent FASD therapeutically.
Animal studies are essential in understanding the underlying mechanisms of FASD and identifying potential treatments. The insights gained from the studies using various models have revealed several targets and strategies for attenuating the deficits associated with FASD, such as neurotrophic factors, including insulin-like growth factor-I, BDNF and NGF (McGough et al., 2009; Mitchell et al., 1999), antioxidants like superoxide dismutase/catalase mimetic EUK134 (Chen et al., 2004), N-acetylcysteine (NAC) (Parnell et al., 2010), vitamins E, C, and beta-carotene (Cohen-Kerem & Koren, 2003), Nrf2 inducer (Dong et al., 2008; Dong et al., 2011; Yan et al., 2010), agents that can antagonize ethanol’s effects on L1 cell adhesion, such as 1-octanol (Chen et al., 2001) and peptides NAP and SAL (Chen et al., 2005; Parnell et al., 2007; Wilkemeyer et al., 2003), and nutritional factors including zinc, folic acid, and choline supplementation (Summers et al., 2009; Thomas et al., 2009; Thomas et al., 2010; Wang et al., 2009).

The NCCs are particularly vulnerable to the toxic effects of ethanol, and their impairment has been shown to contribute significantly to the pathogenesis of FASD. Therefore, understanding the mechanisms underlying ethanol-induced impairments in NCC development is crucial for developing effective strategies for preventing FASD. Recent advances in understanding the molecular mechanisms underlying ethanol-induced impairments in NCC development have identified several potential molecular targets and therapeutic strategies for preventing ethanol-induced impairments in NCC development and FASD. These approaches hold promise for mitigating the impact of prenatal ethanol exposure on NCCs and reducing the incidence of FASD.

7.1. Sulforaphane

Sulforaphane (SFN) is an isothiocyanate present in cruciferous vegetables and is particularly high in broccoli and broccoli sprouts (Fahey et al., 1997). In broccoli and broccoli sprouts, SFN
exists as the glucosinolate precursor glucoraphanin. Upon consumption, plant myrosinases or microbial hydrolases present in gut bacteria convert glucoraphanin to SFN (Ho et al., 2009). SFN has gained increasing attention as a potential therapeutic agent for various diseases due to its anti-inflammatory, antioxidant, and anticancer properties (Chung et al., 2000; Fahey et al., 1997; Zhang et al., 1994). In addition, SFN’s cytoprotective properties have been demonstrated in several in vivo models associated with a variety of disorders, including focal cerebral ischemia, intracerebral hemorrhage, brain inflammation, nephrotoxicity, diabetes, hepatotoxicity and cardiac ischemia and reperfusion (S. H. Baek et al., 2008; Innamorato et al., 2008; Piao et al., 2010; Song et al., 2009; Yoon et al., 2008; Zhao et al., 2006). Moreover, studies have reported that SFN can inhibit NF-kappaB activation (Song et al., 2009), activate the Nrf2 pathway (Kim et al., 2008), increase proteasome activities (Kwak et al., 2007), and regulate gene expression through epigenetic mechanisms (Ho et al., 2009). These findings suggest that SFN has promising therapeutic potential for various health conditions. Recent studies have demonstrated that SFN can prevent ethanol-induced apoptosis in NCCs by activating the Nrf2 pathway or epigenetically modulating gene expression.

7.1.1. Sulforaphane prevents ethanol-induced impairments in neural crest cell development by acting as an Nrf2 inducer

Various studies have reported that SFN-rich broccoli sprouts and other dietary sources of SFN can activate Nrf2 signaling, leading to the induction of phase 2 detoxifying genes and antioxidant enzymes, which can prevent cancer and other diseases (Dinkova-Kostova et al., 2002). SFN has been shown to react with thiol groups of Keap1, resulting in the formation of thionyl adducts. The modification of Keap1 results in the release of Nrf2 from its sequestration and activation of Nrf2
signaling (Hong et al., 2005). Several studies have demonstrated the cytoprotective effects of SFN through Nrf2 activation. For example, SFN rescued rat lens epithelial cells from UVB-induced toxicity by activating Nrf2/ARE interaction (Kubo et al., 2017). SFN-induced Nrf2 activation also showed a protective effect against elevated ROS and apoptosis in granulosa-lutein cells of patients with polycystic ovary syndrome (Taheri et al., 2021). Additionally, SFN has been shown to have alleviative effects against hydrogen peroxide-induced oxidative stress in bovine granulosa cells (Sohel et al., 2018) and goat mammary epithelial cells (Shao et al., 2023).

Nrf2 signaling has been shown to play a critical role in ethanol-induced apoptosis in NCCs and FASD. Dong and coworkers reported that the induction of Nrf2 by an Nrf2 inducer, 3H-1,2 dithiole-3-thione (D3T), resulted in increased Nrf2 protein levels and Nrf2-ARE binding, which strongly induced the mRNA and protein expression of Nrf2 downstream antioxidant genes, decreased ethanol-induced ROS generation, and prevented apoptosis in mouse embryos (Dong et al., 2008). Moreover, in addition to a study that has shown that tBHQ significantly increased Nrf2 protein expression and the expression of its downstream antioxidants, prevented oxidative stress, and reduced apoptosis in NCCs (Yan et al., 2010), SFN has also been demonstrated to protect against ethanol-induced apoptosis in NCCs. It was reported that SFN treatment significantly increased Nrf2 protein expression and ARE promoter activity and increased the mRNA and protein expression of Nrf2 target genes, SOD1 and catalase, and their enzyme activities in ethanol-exposed NCCs. Treatment with SFN also significantly decreased ethanol-induced oxidative stress and apoptosis in NCCs (Chen et al., 2013b). These findings suggest that the induction of Nrf2-mediated antioxidant response by SFN is a promising, safe, and effective approach for the prevention of FASD.
7.1.2. Sulforaphane attenuates ethanol-induced impairments in neural crest cell development by acting as an epigenetic modulator

DNA methyltransferase (DNMT) and histone deacetylases (HDACs) are the essential enzymes involved in DNA methylation and histone deacetylation, respectively (Gibney & Nolan, 2010; Lawrence et al., 2016; Mattei et al., 2022). They play a crucial role in regulating gene expression, the cell cycle, cell proliferation, differentiation, and apoptosis (Srinageshwar et al., 2016). In addition to acting as an Nrf2 inducer, recent studies have shown that SFN can inhibit the activity of HDACs and DNMTs and modulate epigenetic regulation of gene expression in various cell types (Meeran et al., 2012; Su et al., 2018).

SFN has been found to modulate gene expression by inhibiting the activity of DNMTs (Meeran et al., 2012). SFN inhibited LPS-induced expression of DNMT3a and conferred resistance to LPS-induced apoptosis in porcine monocyte-derived dendritic cells (Qu et al., 2015). Recently, a study has demonstrated that treatment with SFN diminished the upregulation of DNMT3a and significantly reduced the activity of DNMTs in ethanol-exposed hNCCs. SFN treatment also prevented ethanol-induced hypermethylation at the promoter regions of the genes encoding the inhibitor of apoptosis proteins (IAP), NAIP and XIAP, and diminished the ethanol-induced repression of NAIP and XIAP, and apoptosis in hNCCs (Li et al., 2021). These results suggest that SFN can diminish ethanol-induced apoptosis in hNCCs by reducing ethanol-induced hypermethylation at the promoter regions of the anti-apoptotic genes through modulating DNMT3a expression and DNMT activity.
An increasing number of studies have highlighted the role of dietary factors in promoting health and preventing diseases. In this context, several dietary HDAC inhibitors have been identified, including EGCG, curcumin, quercetin, butyrate, and SFN (Myzak & Dashwood, 2006). SFN has been shown to inhibit HDAC activity and increase histone hyperacetylation in human colon cancer cells (Myzak & Dashwood, 2006). Supplementation of broccoli sprouts by humans has also been shown to increase the acetylation of histones H3 and H4 in blood cells (Myzak et al., 2007). In ethanol-exposed NCCs, SFN treatment has been reported to decrease the activities of HDACs and significantly increase the expression of acetyl-histone H3 in ethanol-exposed NCCs. SFN also reversed the ethanol-induced reduction in acetyl histone H3 binding to the Bcl-2 promoter, restored the expression of Bcl-2, and diminished apoptosis in ethanol-exposed NCCs (Yuan et al., 2018). These results demonstrate that SFN can epigenetically restore the expression of Bcl-2 and attenuate ethanol-induced apoptosis by increasing histone acetylation at the Bcl-2 promoter. SFN has also been found to diminish the reduction of H3K4me3 at the promoter regions of the Snail1 gene, restore the expression of Snail1 and down-regulate Snail1 target gene E-cadherin, restore EMT, and reduce apoptosis in ethanol-exposed NCCs (Li et al., 2019).

These findings suggest that SFN is a promising candidate for attenuating ethanol-induced impairment in NCCs and FASD. Consumption of broccoli sprout extracts has been found to significantly increase isothiocyanate levels in human plasma (Ye et al., 2002). It has also been demonstrated that SFN can cross the placenta during maternal dietary supplementation of broccoli sprout extracts in mice (Shorey et al., 2013). In addition, the consumption of broccoli sprout extracts in human subjects significantly inhibited HDAC activity, accompanied by the induction of acetylation of histones H3 and H4 (Myzak et al., 2007). A clinical phase I study of safety,
tolerance, and pharmacokinetics of repeated oral doses of broccoli sprout extracts has demonstrated no evidence of any systematic, clinically significant adverse events (Shapiro et al., 2006). The potency of SFN in activating Nrf2 signaling, epigenetically regulating anti-apoptotic gene expression, and preventing ethanol-induced apoptosis in NCCs, along with the fact that SFN is a bioactive compound derived from broccoli and other cruciferous vegetables, offer optimism for the development of clinically applicable strategies for the treatment or prevention of FASD.

7.2. Folate

Folate plays a crucial role in one-carbon metabolism, which is essential for various biological processes, including DNA synthesis, amino acid metabolism, and methylation reactions. In the body, dietary folate is converted to 5-methyltetrahydrofolate (5-MTHF), a methyl group donor that donates a methyl group to homocysteine to regenerate methionine. Methionine is further converted to S-adenosylmethionine (SAM), a critical methyl group donor in the body (Menezo et al., 2022). Deficiencies in folate can lead to elevated homocysteine levels and impaired methylation reactions, leading to adverse health effects, including developmental defects, such as neural tube defects and neurocristopathies (Jimenez & Strobl-Mazzulla, 2022; Li et al., 2011). A study has reported that folate deficiency can lead to orofacial abnormalities, particularly clefts of the lip and palate (Jimenez et al., 2018). Moreover, SAM has been shown to play an essential role in the specification of NC progenitors (Jimenez & Strobl-Mazzulla, 2022). It is also crucial in regulating the migration of NCCs, as evidenced by a study demonstrating that knockdown of the reduced folate carrier (RFC) inhibited the ventral migration of NCCs, leading to their failure to segregate into distinct streams in Xenopus embryos (Li et al., 2011). Notably, alcohol consumption is known to decrease folate uptake and increase homocysteine accumulation, further impairing NCC development. It
has been shown that co-injection of reduced folate carrier (XRFC) mRNA rescued NCC migration defects induced by RFC downregulation, suggesting that folate plays a critical role in regulating the directional movement of NCCs (Li et al., 2011). Additionally, it was reported that microinjection of 5-methyltetrahydrofolate (5-MTHF) reduced ethanol-induced increases in homocysteine and alleviated the effects of ethanol on NCC migration in Xenopus embryos (Shi et al., 2014). These findings suggest that supplementation of folic acid may prevent ethanol-induced impairment of NCC development and FASD.

7.3. MicroRNA inhibitors and mimics

MicroRNA (miRNA) therapy is promising for treating various diseases, including cancer, cardiovascular disorders, and metabolic diseases (Rupaimoole & Slack, 2017). miRNAs are small non-coding RNAs that regulate gene expression by binding to mRNA targets and inhibiting their translation or promoting their degradation. Dysregulated expression of miRNAs is associated with many diseases. In miRNA therapy, synthetic miRNA mimics or inhibitors are delivered to target cells or tissues to modulate the expression of specific miRNAs and restore normal cellular functions (Rupaimoole & Slack, 2017). Studies have demonstrated the potential of miRNA therapy in preclinical models of various diseases, including cancer, diabetes, and cardiovascular disorders (Li & Rana, 2014). Various studies have shown that dysregulated expression of miRNAs is associated with ethanol-induced impairment in the migration, differentiation, and survival of NCCs (Chen et al., 2015; Fan et al., 2022; Fan et al., 2019; Yuan et al., 2020), making them attractive therapeutic targets for preventing ethanol-induced impairments in NCC development and FASD.

7.3.1. microRNA mimics
MicroRNA mimics are a promising class of therapeutic agents that can mimic the functions of endogenous miRNAs, thereby regulating the expression of specific genes and pathways. Numerous studies have highlighted the potential of miRNA mimics as therapeutic agents for various diseases, including cancer, cardiovascular diseases, and neurological disorders. For instance, a study has demonstrated that miR-124 mimics treatment decreased retinal inflammation and photoreceptor cell death and improved retinal function in degenerating retinas (Chu-Tan et al., 2018). Similarly, it was reported that injection of miR-19a/19b mimics increased cardiomyocyte proliferation and enhanced cardiac regeneration in response to myocardial infarction injury (Gao et al., 2019). In the context of FASD, Chen and colleagues (Chen et al., 2015) demonstrated that miR-125b mimics significantly reduced ethanol-induced increase in PUMA and Bak1 protein expression, caspase-3 activation, and apoptosis in NCCs. Microinjection of miR-125b mimics also significantly decreased the protein expression of PUMA and Bak1, reduced ethanol-induced caspase-3 activation, and diminished ethanol-induced growth retardation in cultured mouse embryos. This is the first demonstration that the microinjection of miRNA mimics can prevent ethanol-induced embryotoxicity. Additionally, a recent study from the same group found that miR-135a mimics significantly reduced the upregulation of Siah1 and the activation of the p38 MAPK/p53 pathway and decreased apoptosis in ethanol-exposed NCCs and zebrafish embryos. miR-135a mimics also protected against ethanol-induced craniofacial defects in a zebrafish model of FASD by reducing Siah1 expression and inhibiting the activation of the p38 MAPK/p53 pathway (Yuan et al., 2020). These findings suggest that the delivery of miRNA mimics may hold promise for mitigating the detrimental effects of fetal alcohol exposure on NCCs and preventing FASD.

7.3.2. microRNA inhibitors
In addition to miRNA mimics, miRNA inhibitors have been explored for therapeutic purposes. These inhibitors can be used to decrease the expression of specific miRNAs that are upregulated in certain diseases or conditions. For example, one study found that inhibition of miR-188-5p by its inhibitors diminished hepatic fibrosis by reducing the activation and proliferation of hepatic stellate cells (Riaz et al., 2021). Another study showed that inhibition of miR-665-3p increased autophagy and reduced inflammation in fusarium solani-induced keratitis (Guo et al., 2021). Notably, miRNA inhibitors have also been shown to reduce the effects of ethanol on NCC development. Fan and coworkers (Fan et al., 2019) found that miR-34a inhibitors restored the expression of its direct target, Atg9a, decreased the inhibition of autophagy in ethanol-exposed NCCs, and prevented ethanol-induced inhibition of neural differentiation of NCCs. Furthermore, a study from the same group also demonstrated that miR-34a inhibitors prevented the repression of Snail1 and diminished the upregulation of Snail1 target gene E-cadherin1 in ethanol-exposed NCCs, restoring EMT and preventing ethanol-induced inhibition of NCC migration \textit{in vitro} and in zebrafish embryos \textit{in vivo} (Fan et al., 2022). These studies demonstrate the potential of miRNA inhibitors as a targeted therapy for the prevention of FASD.

Although the findings discussed in this section are promising, several challenges need to be addressed before miRNA-based therapeutics can be used to prevent FASD. One challenge is the delivery of miRNA therapeutics to target embryonic tissues. Additionally, minimizing off-target effects is crucial to avoid unintended consequences of miRNA therapy. Despite these challenges, the rapid development of miRNA-based therapeutics has shown great promise for the treatment and prevention of FASD. Further studies are needed to evaluate the efficacy and safety of miRNA-based therapeutic strategies for preventing ethanol-induced birth defects. Nonetheless, with the increasing understanding of miRNA biology and the availability of advanced delivery methods,
the potential for miRNA-based therapies to prevent FASD is a promising avenue of research.

Figure 4 and Table 3 provide a comprehensive overview of the molecular mechanisms of various compounds and molecular targets that have been identified to have the potential to prevent ethanol-induced impairments in NCC development and FASD.

**Figure 4.** Schematic representation of the mechanisms of action of therapeutic agents in preventing ethanol-induced impairment in NCCs. Upward and downward vertical red arrows denote upregulation and repression induced by ethanol exposure, respectively, in NCCs. Upward and downward vertical green arrows indicate upregulation and repression induced by the therapeutic agents, respectively, in NCCs.

**Table 3:** Potential therapeutic agents and gene targets in attenuating ethanol-induced impairments of neural crest cell development

<table>
<thead>
<tr>
<th>Therapeutic agents</th>
<th>Experimental models</th>
<th>Mechanisms/ gene targets</th>
<th>Key findings/outcomes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfuraphane (SFN)</td>
<td>Mouse NCCs</td>
<td>Nrf2 signaling</td>
<td>SFN prevented apoptosis in ethanol-exposed NCCs by increasing Nrf2 expression and ARE activity, increasing the expression of Nrf2 target antioxidant genes, and decreasing oxidative stress.</td>
<td>(Chen et al., 2013b)</td>
</tr>
</tbody>
</table>
8. Future perspectives

This review article presents compelling evidence that ethanol exposure during embryonic development leads to significant impairment of NCC induction, migration, differentiation, and survival. The impairments in NCC development caused by ethanol exposure lead to a broad range
of developmental abnormalities, contributing to the pathogenesis of FASD. This review also examines the molecular mechanisms underlying ethanol-induced NCC dysfunction, including oxidative stress, apoptosis, altered gene expression, epigenetic modifications, and other signaling pathways. Furthermore, the review highlights potential therapeutic strategies for preventing or mitigating the detrimental effects of ethanol on NCCs and reducing the risk of FASD. Despite the progress made in understanding the impact of ethanol on NCCs and FASD pathogenesis, many challenges remain. One major challenge in this area of research is the intricate interplay between genetic and environmental factors that contribute to the impairment of NCC development and FASD pathogenesis. A comprehensive understanding of the genetic determinants of NCC development is crucial to unravel the mechanistic complexity of NCC development and its implications in FASD. Another challenge is the need to better understand the epigenetic changes caused by ethanol exposure and their potential intergenerational effects. As our understanding of epigenetic modifications and their role in NCC development advances, new strategies for preventing ethanol-induced impairments in NCCs may emerge. Cutting-edge genetic and epigenetic techniques, such as single-cell RNA sequencing, CRISPR-Cas9, and other novel techniques, provide exciting opportunities to investigate the interplay between ethanol exposure and gene expression in NCCs. Furthermore, the development of innovative in vitro and in vivo models that better reflect the human condition, such as non-human primates, human NCCs derived from human embryonic stem cells, and human organoids, may provide valuable insights into the mechanisms underlying ethanol-induced impairment of NCCs and FASD pathogenesis. Research efforts should also focus on developing effective preventive and therapeutic strategies for reducing the impact of ethanol on NCCs and mitigating the risk of FASD. Such strategies could include antioxidant supplements, epigenetic modulators, and nutrient interventions aimed at mitigating the
harmful effects of ethanol on NCCs. Continued research into the molecular mechanisms underlying ethanol-induced alterations in NCC development may lead to identifying additional therapeutic targets and interventions. Overall, this review underscores the critical need for further research on the underlying mechanisms of ethanol-induced impairment in NCC development, with the ultimate goal of developing preventative and therapeutic strategies to reduce the impact of FASD on individuals, families, and communities.

Declaration of Competing Interest

The authors declare no competing financial interests.

Authors’ Contributions
S-YC and MK conceptualized and wrote this manuscript.

Acknowledgments

This work was supported by the National Institutes of Health Grants AA028435, and AA024337 (S.-Y.C.) from the National Institute on Alcohol Abuse and Alcoholism.

References:


https://doi.org/10.1007/s00441-018-2953-4


Graphical abstract

Pregnancy

Neural crest cells

Induction

Migration

Differentiation

Survival

Fetal Alcohol Spectrum Disorders (FASD)

Therapeutic agents
(Sulforaphane, tBHQ, folate, miR-34a inhibitors, miR-125b mimics, miR-135a mimics)
Credit Author Statement

Drs. Shao-yu Chen and Maharajan Kannan conceptualized and wrote this manuscript.
Declaration of Competing Interest

The authors declare no competing financial interests.