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Research Article

Keywords: neural crest cells, placode cells, exosomes, SDF1, miR-126

Posted Date: July 19th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1791954/v1

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Exosome-shuttled miR-126 mediates ethanol-induced disruption of neural crest cell-placode cell interaction by targeting SDF1

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Author Contributions: H.F. and Y.H.L. designed research, performed the experiments, and participated in data analysis and manuscript preparation. H.F., Y.H.L., T.C., H.L., Y.L., S.Z., F.S., J.L. and S.C. participated in data interpretation and discussion. All authors reviewed the manuscript.

Acknowledgments
The study was supported by Young Innovative Talent Project of YongJiang Talent Introduction Programme 2021A-012-G (H.F.), Natural Science Foundation of Ningbo 2021J321 (H.F.), Special Funding for Microfluidic Chip of Biomedicine of Ningbo Institute of Life and Health Industry, University of Chinese Academy of Sciences 2021YJY1006 (H.F.), Natural Science Foundation of Ningbo 2021J328 (Y.L.), Special Funding for Microfluidic Chip of Biomedicine of Ningbo Institute of Life and Health.
Industry, University of Chinese Academy of Sciences 2021YJY1005 (Y.L.), and National Institute of Health Grants R01AA028435, R01AA021434. (S.C.)

**Keywords**: neural crest cells, placode cells, exosomes, SDF1, miR-126
Abstract

During embryonic development, two populations of multipotent stem cells, cranial neural crest cells (NCCs) and epibranchial placode cells (PCs), are anatomically adjacent to each other. The coordinated migration of NCCs and PCs plays a major role in the morphogenesis of craniofacial skeletons and cranial nerves. It’s known that ethanol-induced dysfunction of NCCs and PCs is a key contributor to the defects of craniofacial skeletons and cranial nerves implicated in Fetal Alcohol Spectrum Disorder (FASD). However, how ethanol disrupts the coordinated interaction between NCCs and PCs was not elucidated. To fill in this gap, we established a well-designed cell co-culture system to investigate the reciprocal interaction between human NCCs (hNCCs) and human PCs (hPCs), and also monitored the migration behavior of NCCs and PCs in zebrafish embryos. We found that ethanol exposure resulted in a disruption of coordinated hNCCs-hPCs interaction, as well as in zebrafish embryos. Treating hNCCs-hPCs with exosomes derived from ethanol-exposed hNCCs (Exo_{EtOH}) mimicked ethanol-induced impairment of hNCCs-hPCs interaction. We also observed that a chemoattractant, SDF1, was downregulated in ethanol-treated hPCs and zebrafish embryos. Meanwhile, miR-126 level in Exo_{EtOH} was significantly higher than that in control exosomes (Exo_{Con}). We further validated that Exo_{EtOH}-encapsulated miR-126 from hNCCs can be transferred to hPCs to suppress SDF1 expression in hPCs. Knockdown of SDF1 replicated ethanol-induced abnormalities either in vitro or in zebrafish embryos. On the contrary, overexpression of SDF1 or inhibiting miR-126 strongly rescued ethanol-induced impairment of hNCCs-hPCs interaction and developmental defects.
Fetal Alcohol Spectrum Disorders (FASD) are among the most devastating consequences of maternal ethanol exposure during pregnancy. One of the hallmarks of FASD is the birth defects of craniofacial skeletons and cranial sensory system. The neural crest cells (NCCs) are multipotent progenitor cells that can give rise to a diversity of neural and non-neural cell types, such as melanocytes, neurons, glial, and mesenchymal cells that form craniofacial skeletons and dermis [1-3]. The majority of the cranial mesenchyme are made up by NCCs, which contributes significantly to the craniofacial structures [3]. Cranial placodes are thickened regions of ectoderm originated at the border between the neural plate and neural crest that include the olfactory, lens, otic, trigeminal and epibranchial placodes [4]. The epibranchial placode cells (PCs) can later differentiate into the distal ganglia of facial (VII), glossopharyngeal (IX) and vagus (X) cranial nerves [5-8]. NCCs and PCs are both highly migratory cell populations throughout their development, and they remain in close proximity to each other and interact in a reciprocal manner, which is crucial for the coordinated morphogenesis of head and functional sensory systems [9]. In addition, it has been demonstrated that a PCs-derived paracrine chemokine, SDF1, plays a key role in mediating the interaction between PCs and NCCs [10]. Up to now, most studies just focused on ethanol-induced excessive apoptosis or migration impairment in NCCs [11-14]. How ethanol exposure disrupts the coordinated migration of NCCs and PCs in embryonic development and the detailed mechanism of ethanol-induced concurrence of the dysfunction of NCCs and PCs that leads to craniofacial and cranial nerve defects remains largely unknown.

Exosomes are ~40–150 nm, endosome-derived, small extracellular vesicles secreted by most cells [15]. Exosomes carry a variety of biologically active molecules, including proteins, lipids and miRNAs, and can transfer these molecules from one cell to another to facilitate cell-cell communication [16-18]. For instance, exosomes have been shown to mediate the communication between neurons and oligodendrocytes [19]; During embryogenesis, they have been implicated in important classes of developmental signals
such as WNT, HH and Notch [20]; It has also been reported that maternal exosomes in diabetes that cross the maternal-fetal barrier can result in cardiac defects in embryos [21].

miRNAs are a class of small non-coding RNA molecules that can regulate gene expression and play a pivotal role in numerous biological events [22]. Among exosome cargoes, miRNAs have been considered to be crucial in the therapeutic effects of exosomes [23]. Studies have shown that exosomes can be taken up into neighboring or distant cells and release miRNAs to modulate the function of recipient cells [24-27]. A number of miRNAs have been demonstrated to be involved in the modulation of SDF1-mediated signaling [28, 29]. For instance, miR-342 and miR-137 can act as a tumor suppressor by targeting SDF1 [30, 31], while miR-126 can directly inhibit SDF1 expression and enhance the migration of the CD34+ cells [32]. In particular, exosome-encapsulated miR-126 transferred from endothelial cells to leukemia cells reduced SDF1 expression and cell migration which can be reversed by miR-126 inhibitor [33]. These evidences indicate that exosome-derived miRNAs play an important role in exosome-mediated cell-cell communication.

Here, we aimed to uncover an exosome-mediated mechanism by which ethanol exposure destroys the coordinated hNCCs-hPCs interaction in vitro, and to elucidate whether the restoration of the SDF1-mediated chemotaxis signaling can prevent ethanol-induced teratogenesis in vivo. Using a co-culture system for hNCCs-hPCs, we showed that ethanol exposure results in a significant increase of hNCCs-derived exosome cargo, miR-126, which can be horizontally transferred to hPCs to specifically inhibit SDF1 expression, leading to an impairment of coordinated migration between hNCCs and hPCs. We also demonstrated that a recovery of SDF1 expression through interfering with miR-126/SDF1 axis can reverse ethanol-induced disruption of hNCCs-hPCs interaction and developmental defects. Besides, we established an efficient way for delivering miR-126 inhibitors using the grape-derived exosome-like nanoparticles (GELNs) as a carrier, which might be a translatable strategy for the intervention for FASD.

Materials and Methods

hNCCs and hPLs induction from hESCs
Human Neural crest cells (hNCCs) and human placode cells (hPCs) were both differentiated from human embryonic stem cells (hESCs) purchased from WiCell® (Madison, WI, USA). hESCs were cultured in mTeSR™1 (StemCell Technologies, Inc., Vancouver, Canada) on hESC-qualified Matrigel™ (BD Biosciences, San Jose, CA) coated plates. The differentiation procedures are as follows: For hNCCs induction, the hESCs were adapted to accutase dissociation and single-cell culture before subsequent differentiation [34]. Briefly, hESCs cultures were disaggregated by accutase for 10 minutes. The non-adherent hESCs were washed and plated on matigel-coated dishes. hESCs at 85% confluence were ready to be passaged for hNCCs induction. The hESCs culture medium (mTeSR™1) was then replaced with hNCCs-induction medium (DMEM/F-12 Medium; 14.3 M L-Glutamine+β-mercaptopoethanol; MEM Non-Essential Amino Acid; 10 μg/mL Fgf2; 10 μg/mL Heregulin β-1; 200 μg/mL Long R3-IGF1; 10 mM CHIR 99021; 10 mM SB421542; Penicillin and streptomycin) and change daily. Once reaching proper confluence (75%-85%), the differentiating cells were passaged and maintained in the hNCCs-induction medium. After 10 days, the hNCCs identity was determined by hNCCs-specific marker, HNK1 and p75, through immunocytochemistry.

For hPCs induction, the initial differentiation medium includes knock out serum replacement media (KSR) supplemented with 10 μM TGF-β inhibitor SB431542 (Tocris, Minneapolis, MN, USA) and 250 ng/mL of Noggin (R&D, Minneapolis, MN, USA) [35]. Cells were grown in hPLs-induction medium for 7 days with medium changed daily and Noggin was withdrawn at Day 3 of differentiation. At Day 7, the hPC identity was examined by hPCs marker Six1 using immunocytochemistry.

**Analysis of the coordinated migration between NCCs and PCs**

The coordinated migration of hNCCs and hPCs *in vitro* was evaluated by using the CytoSelect Wound Healing Assay Kit, which can be used as a co-culture system for two types of cells (Cell Biolabs, San Diego, CA, USA). At first step, an insert was put into a cell culture plate. hNCCs and hPCs were then seeded into each side of the insert and were cultured to form a monolayer. Next, the insert was removed to generate a 0.9 mm cell-free gap between hNCCs and hPCs. Then the co-cultured hNCCs and hPCs were ready for downstream experiments to evaluate the coordinated migration between hNCCs and hPCs.
Finally, the co-cultured hNCCs-hPCs were fixed and co-stained with antibodies against specific markers of hNCCs (HNK1) and hPCs (Six1), and then the images were captured under a fluorescent microscope (Olympus IMT-2, Tokyo, Japan).

**Isolation, characterization, and labeling of hNCCs-Exo and GELNs**

Exosomes from hNCCs (hNCCs-Exo): Differential ultracentrifugation and Direct Immuno-affinity Capture (DIC) methods are two connecting steps for crude exosome isolation and further immuno-purification of the crude exosomes, respectively [15]. During differential ultracentrifugation process, the collected fresh conditioned media (from hNCCs) were undergone centrifugation at the speed of 300g, 2,000g and 10,000g step by step, and the supernatants from each previous step were kept for the next step until the 10,000g centrifugation step was performed. Finally, the supernatants from 10,000g centrifugation were further ultra-centrifuged at the speed of 120,000g for 70 minutes. The pellet was collected, re-suspended in PBS and then ultra-centrifuged for 1 hour at 120,000g using Beckman Coulter ultracentrifuge with fixed angle rotor (Type 70 Ti Rotor, Beckman, Germany). The final volume of PBS used for re-suspending the pellet was equal to 1/1000 of the initial volume of the hNCCs-conditioned media. All the centrifugation steps were performed at 4 °C. The crude exosomes obtained above were proceeded to be purified by DIC method in the next step. The key idea of DIC is that exosomes can be directly immuno-captured by their specific surface marker, CD63. In this step, exosomes obtained from ultra-centrifugation were further immune-purified by CD63 Exo-Flow Capture Kit (EXOFLOW300A-1, SBI, Palo Alto, CA, USA) according to the manufacturer’s instructions. Exosomes from grapes (GELNs): Grapes skins were removed and homogenized in a high-speed blender for 1 minutes at 4 °C. The collected juice was sequentially centrifuged at 2,000g for 20 minutes and then 10,000g for 1 hour to exclude debris. The supernatants were filtered through a 1-μm membrane filter (Millipore, Bedford, MA, USA). Then the filtered supernatants were pelleted at 120,000g for 1 hour, washed once with PBS and then purified and separated using sucrose gradients centrifugation (8, 30, 45 and 60%, respectively). Band 1 at 8/30% and band 2 at the 30/45% interface was considered to be pure GELNs for collection. For TEM characterization, purified exosomes in PBS were fixed in 2% PFA in PBS for 2 hours at room temperature. The fixed sample
A drop (10 μL) was placed on the surface of formvar carbon-coated copper grids (FCF200-CU, Electron Microscopy Sciences, PA, USA) and allowed to absorb for 5 minutes. Then the grids were blotted by filter paper and placed on a drop of Uranylless solution (Catalog NO. 22409, Electron Microscopy Sciences, PA, USA) for 5 minutes at room temperature. Finally, the grids were blotted and dried for 5 minutes and observed under an electron microscope (Zeiss EM 900, Germany). To quantify hNCCs-Exo or GELNs, a BCA protein assay kit (PIERCE, Rockford, IL, USA) was used to measure protein content. For the cell migration assay, the work concentration of exosomes was 1 μg/10^5 cells, and for the microinjection experiments, each zebrafish embryo was injected with 2 ng of exosomes. To monitor the trafficking and uptake of exosomes, purified hNCCs-Exo or GELNs were labeled with PKH67 (Sigma Aldrich, St. Louis, MO, USA) according to manufacturer’s instructions.

**microRNAs analysis of exosomes**

Direct Immunoaffinity Capture (DIC)-purified exosomes were further proceeded for miRNA extraction using miRNeasy® mini kit (Qiagen, Valencia, CA, USA) followed the manufacturer’s procedures. Extracted total miRNAs were first reverse-transcribed using the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster, CA, USA) in a reaction mixture containing a miR-specific stem-loop reverse transcription primer (hsa-miR-126*: RT-000451, Thermo Fisher, Waltham, MA, USA). Then quantitative PCR amplification was performed using TaqMan® Universal PCR Master Mix kit (Applied Biosystems, Foster, CA, USA) with a sequence-specific Taqman probe (hsa-miR-126*: TM-000451, Thermo Fisher, Waltham, MA, USA) on a Rotor-Gene 6000 Real-Time PCR system (Corbett Life Science, Sydney, Australia). Data were normalized with snoRNA202 as endogenous control, and the relative expression of miR-126 was calculated using the ΔΔCT method.

**Loading of miR-126 inhibitors into exosomes, and the in vitro or in vitro treatments with exosomes**

For in vitro experiments, loading of miR-126 inhibitors (MH10401, mirVana® miRNA inhibitor, Thermo Fisher, Waltham, MA, USA) into exosomes were completed by Exo-FectTM exosome transfection reagent (SBI, Palo Alto, CA, USA) according to the
manufacturer’s protocol. Briefly, hNCCs-Exo were initially re-suspended with sterile PBS.

In a clean 1.5 ml tube, the following reagent containing 10 μL Exo-Fect™ solution, 20 μL miRNA inhibitors (5 μM), 70 μL sterile PBS and 50 μL (200μg/mL) purified exosomes were mixed together to incubate at 37 °C in a shaker for 10 minutes, and then the tube was immediately placed on ice. After stop the reaction by adding 30 μL of the ExoQuick-TC reagent provided in the kit, the transfected exosomes were placed on ice for 30 minutes. Then the samples were centrifuged at 13,000g for 3 minutes. After the supernatants were removed, the pellets composed of transfected exosome were re-suspended in 300 μL PBS and were ready for downstream experiments. For in vitro treatments, 1 µg of hNCCs-Exo (based on protein measurement) were added to 1X10^5 cells and cultured for 24 hours. For in vivo study, zebrafish embryos were microinjected with GELNs or PHK67-labeled GELNs (2 ng/embryo) at 4 hpf.

**Dual luciferase reporter assays**

miR-126 targeting sites in the 3'-untranslational region (3'-UTR) of SDF1 mRNA were predicted by online database tool, Target Scan (http://www.Targetscan.org/), as described previously [36]. The 3'-UTR of SDF1 containing putative miR-126 binding sites were amplified from human genomic DNA using the following primers:

5’-

GTTACTGGCATCTTTACTAGTCAACGCCAGCCCAGTGCATCCCACAGCTACA

GCTT-3’ (forward primer);

5’-

TAGGCTGCGGATGCGGATAAGGGGCAGTAGAACGGTGTCAGTCAGC

GACTA-3’ (reverse primer), and were cloned into pMIR-Luciferase-Report plasmid (Applied Biosystems, Foster, CA, USA) at MluI/SpeI restrictive enzyme site. Renilla luciferase pRL-TK control vector (Promega, Madison, WI, USA) was used as a control.

The pMIR-Luciferase-SDF1 (3’-UTR) constructs (200 ng of plasmid/well of 24-well plates) were co-transfected with 20 ng pRL-TK control vector and 50 nmol of miR-126a mimics or control mimics (Ambion, Austin, TX, USA) into hPCs by Lipofectamine 2000 (Invitrogen, Waltham, MA, USA) according to the manufacturer's procedures. Luciferase activity was measured 48 hours after the transfection using the Dual-luciferase assay kit
(Promega, Madison, WI, USA) with a Lumat LB9507 Ultra Sensitive Tube Luminometer (Berthold Technologies, Bad Wildbad, Germany). The relative activity of luciferase of each sample was normalized to the pRL-TK driven Renilla luciferase activity.

**Zebrafish maintenance and ethanol treatment**

Adult AB zebrafish (Danio rerio) were obtained from the Zebrafish International Resource Center (ZIRC) at the University of Oregon, Eugene, OR and maintained in 14h:10h light: dark cycle at 28°C. Fertilized eggs were collected after natural spawning and used for this study. For ethanol treatment, the zebrafish embryos were exposed to ethanol (1% v/v) during 5-24 hours post fertilization (hpf). After ethanol exposure, the embryos were rinsed three times and transferred to fresh system water. The embryos were collected at 30 hpf, or 2 days post fertilization (dpf) for the whole-mount in situ hybridization (WISH) to view the migration patterns of cranial neural crest and placodes, or the morphology of cranial nerves, respectively. The embryos were also collected at 5 dpf for evaluating morphology of craniofacial cartilages using Alcian Blue staining.

**Microinjection of antisense morpholinos, in vitro-synthesized mRNA, or labeled exosomes**

Morpholinos used for knocking down gene expression were as follows: SDF1: CTACTACGATCAGTTGAGATCCAT [37]. In vitro-synthesis of SDF1 mRNA was performed using mMESSAGE mMACHINE T7 Ultra Kit (Cat. No. AM1345, ambiom, USA) following the manufacturer’s instructions. The primers used for in vitro transcription of SDF1 mRNA were as follows: SDF1: TAATACGACTCACTATAGGGATGCTCTCACAAGTGATCGTAGTA (forward), TTAGACCTGCTGCTGTTGGGCTTT (reverse). Morpholinos or synthesized mRNAs were microinjected into one-cell stage zebrafish embryos (0.3 pmol/egg). PKH-67-labeled exosomes containing miR-126 inhibitors (2 ng/egg) were microinjected into zebrafish embryos at 4 hpf.

**Whole-mount in situ hybridization**

The procedure of the whole-mount in situ hybridization (WISH) were described as in [38]. The PCR primers used for synthesizing digoxigenin-conjugated probes were as follows:
Twist1a: CTCAGTCTCTGAACGAGGCG (forward),
TAATACGACTCACTATAGGGTCTGCTCCCATGCGTAGT (reverse);
Sox3: CTCGGTGCTGACTGGAAACT (forward),
TAATACGACTCACTATAGGGTGCGTGTATGCTGGTGACAT (reverse);
SDF1: TGCCAAATATGCGTCCCAGT (forward),
TAATACGACTCACTATAGGGGAGCGTGAAGCAACAGTGTG (reverse);
Phox2b: GAGGAGCTCGCGCTTAAGAT (forward),
TAATACGACTCACTATAGGGGAGAGTCCGGAATGGAGGTGA (reverse).

The dechorionated zebrafish embryos were fixed in 4% paraformaldehyde at 4 °C overnight and dehydrated in methanol for 2 hours. After rehydration, the embryos were permeated with proteinase K (10μg/mL, Invitrogen, Carlsbad, CA, USA) and then pre-hybridized for 5 hours at 60 °C in a pre-hybridization buffer. The embryos were then incubated with digoxigenin-conjugated mRNA probes in hybridization buffer for 8 hours at 60 °C. Before adding the digoxigenin antibody (Roche, Basel, Switzerland), the embryos were placed in blocking buffer (containing 2mg/mL BSA and 2% sheep serum) for 2 hours. Then the embryos were incubated with digoxigenin antibody overnight at 4 °C following by adding NBT and BCIP buffer to develop the hybridized gene patterns. Finally, the embryos were photographed under a stereoscopic microscope (SZX16, Olympus, USA).

**Alcian Blue staining of the zebrafish cartilages**

The zebrafish embryos were collected at 5 dpf and fixed with 4% paraformaldehyde overnight at 4 °C. The fixed embryos were washed and dehydrated with 50% ethanol at room temperature for 10 min, followed by staining in 0.1% Alcian Blue solution at room temperature overnight and washing in a bleach solution [39]. After the washing, 1 ml solution of 20% glycerol and 0.25% KOH was added and incubated at room temperature for 30 min. The embryos were then photographed under a stereoscopic microscope (SZX16, Olympus, USA).

**Statistical analysis**

Statistical analyses were performed using GraphPad Prism software (GraphPad, San Diego, CA, USA). All data were expressed as means ± S.E.M. of at least three
independent experiments. Comparisons between groups were analyzed by one-way ANOVA. Differences between groups were considered significant at $p < 0.05$

**Results**

*Ethanol exposure disrupted the coordinated migration of NCCs and PCs in vitro and in zebrafish embryos*

To examine whether ethanol can disrupt the coordinated migration of hNCCs and hPCs in vitro, hNCCs and hPCs were cultured and treated with 50 mM ethanol for 24 hours and the migration of hNCCs and hPCs was analyzed in the CytoSelect Wound Healing Assay Kit, as described in the *Materials and Methods* section. We designed a cell co-culture system to evaluate the coordinated migration between hNCCs and hPCs (Fig. 1A). Briefly, hNCCs (green) and hPCs (red) were simultaneously seeded in each semi-section of the same cell dish with an insert (blue) to physically insulate the two cell types. As the cells were attached to the bottom of the dish, the insert was taken away to form a central cells-free cleft on the dish bottom, and in the meantime, the hNCCs would begin to migrate towards hPCs. In control group (Fig. 1B, left panel), we observed that hNCCs (stained with anti-HNK1 antibody, green) migrate towards hPCs (stained with anti-sox3 antibody, red) and hPCs move away from hNCCs, manifesting a coordinated efficient directional migration between hNCCs and hPCs in a “chase-and-run” manner as previously described by Theveneau et.al [10]. However, directional migration of hNCCs and hPCs were lost after they were exposed to ethanol for 24 hours and both hNCCs and hPCs move randomly with a low directionality and poor net displacement (Fig. 1B, right panel), indicating that ethanol can directly disrupt the coordinated migration of hNCCs and hPCs in vitro. We then investigated whether ethanol can affect the coordinated migration in vivo. Using WISH technique, the migration patterns of NCCs and PCs were displayed by the NCC marker *twist1a* and PC marker *sox3* in 30 hpf zebrafish embryos, respectively. Three streams of migratory NCCs had migrated to the ventral site of neural tube in 30 hpf zebrafish embryos in control group (Fig. 1C, upper panel), whereas exposure to 1% (v/v) ethanol at 5-24 hpf resulted in a significant delay of NCCs’ migration that three stream of migratory NCCs was accumulated near to the dorsal site of neural tube (Fig. 1C, lower panel). The migratory path (dorsal-to-ventral axis) of NCCs in ethanol-treated group was
significantly shorter than that in control group (Fig. 1D). Similarly, the migration of PCs was also strongly delayed under ethanol treatment (Fig. 1E and F). These data demonstrated that ethanol exposure can also disrupt the migration of NCCs and PCs in vivo.

**Treatment with exosomes derived from ethanol-exposed hNCCs (hNCCs-Exo\textsubscript{EtOH}) disrupted the coordinated migration of hNCCs and hPCs**

Exosomes can carry various biological molecules, such as proteins or RNAs, for intercellular communication. Regarding their emerging roles in embryonic development and neural system [20, 40, 41], we hypothesized that NCCs-secreted exosomes may mediate the interaction between NCCs and PCs. To determine the role of hNCCs-Exo\textsubscript{EtOH} in ethanol-induced disruption of the coordinated migration of NCCs and PCs, control PCs were cultured with exosomes derived from control hNCCs (hNCCs-Exo\textsubscript{Con}) or hNCCs-Exo\textsubscript{EtOH} (1 μg/10^5 cells) for 24 hours before they were cultured with control hNCCs in our cell co-cultured system as described above. We found that exposure of control hPCs to hNCCs-Exo\textsubscript{EtOH} significantly disrupted the coordinated migration of control hNCCs and hPCs (Fig. 2, right panel), suggesting that hNCC-Exo\textsubscript{EtOH} may mediate ethanol-induced impairment of hNCCs-hPCs interaction. To further confirm this, NCCs were pretreated with or without exosomes secretion inhibitor GW4869 (10 μM) for 24 hours, to block the release of hNCCs-Exo before NCCs were co-cultured with hPCs and then exposed to 50 or 0 mM ethanol for 24 hours (Appendix, Fig. 11). As seen obviously, GW4869 restored coordinated migration of hNCCs and hPCs (Appendix, Fig. 11, the fourth panel) compared with ethanol-treated group (Appendix, Fig. 11, the second panel). Together, these data provide a primary foundation indicating that hNCCs-Exo\textsubscript{EtOH} may contain certain inhibitory molecules that contribute ethanol-induced disruption of coordinated migration of hNCCs and hPCs.

**Ethanol exposure decreased the expression of SDF1 in hPCs and in zebrafish embryos**

To examine the effects of ethanol on the SDF1 expression, hPCs were treated with or without 50 mM ethanol for 24 hours. After that, cells were harvested for protein analysis. We found that SDF1 level was significantly decreased (Fig. 3A). Next, we detected the SDF1 expression in zebrafish embryos. Zebrafish embryos were first treated with or
without 1% (v/v) ethanol at 5–24 hpf, and then were collected at 30 hpf for qRT-PCR analysis and WISH. The mRNA level of zebrafish embryos in ethanol-treated group was much lower than in control group (Fig. 3B). Typically, the SDF1 expression pattern in control group resembles to the pattern of Sox3 expression as they are both appeared in the position where epibranchial placodes located (Fig. 3C, upper right, arrows). However, in ethanol-treated group, the normal distribution of SDF1 was abolished (Fig. 3C, lower right, arrow). These results demonstrated that ethanol exposure can destroy normal expression of SDF1 in vitro and in vivo.

**Overexpression of SDF1 diminished ethanol-induced disruption of the coordinated migration of NCCs and PCs in zebrafish embryos**

To investigate the role of SDF1 in mediating ethanol-induced disruption of the coordinated migration of NCCs and PCs, SDF1 was overexpressed in zebrafish embryos by the microinjection of in vitro-synthesized SDF1 mRNA. Overexpression of SDF1 significantly restored the normal migration pattern of NCCs (Fig. 4A, row 3 of left panel, arrows) and PCs (Fig. 4A, row 3 of right panel, arrows), as detected by their marker Twist1a and Sox3, respectively. SDF1 overexpression also strongly increased the migratory distance from dorsal site to ventral site (DV axis) of neural tube of NCCs and PCs (Fig. 4B and C). Together with Figure 3, these data demonstrated that SDF1, a chemoattractant cytokine, is essential for the coordinated migration of NCCs and PCs and that reduction of SDF1 in PCs may contribute to ethanol-induced disruption of the coordinated migration of NCCs and PCs.

**Ethanol exposure resulted in a significant increase in the expression of miR-126 in hNCC-Exo**

It’s been reported that miR-126 wrapped in exosomes can be shuttled among different types of cells [33, 42]. Particularly, miR-126 is the most relevant miRNAs in regulating migration of various tumor cells through its potential target SDF1 [33, 43]. To determine whether ethanol exposure can alter miR-126 expression, hNCCs-Exo (Fig. 5A) derived from control or ethanol-treated hNCCs were isolated for qRT-PCR analysis. We found that the level of miR-126 in hNCC-Exo_{EtOH} was significantly
higher than that in hNCC-Exo^{Con} (Fig. 5B), suggesting that ethanol exposure of hNCCs can facilitates more miR-126 assembled into hNCCs-derived exosomes.

_Treatment with hNCCs-Exo^{EtOH} dramatically increased the levels of miR-126 and decreased the expression of SDF1 in hPCs_

To further determine whether elevated miR-126 in hNCCs-Exo^{EtOH} can be horizontally transferred into control hPCs to suppress the SDF1 expression, hPCs were cultured with hNCCs-Exo^{EtOH} (1µg/10^5 cells) for 24 hrs. We observed that hPC can efficiently uptake hNCCs-Exo^{EtOH} (Fig. 6A, labelled with green fluorescent PHK-67), and that the culture of hPCs with hNCCs-Exo^{EtOH} significantly increased the levels of miR-126 (Fig. 6B) and reduced the mRNA and protein expression of SDF1 in hPCs (Fig. 6D and E).

Using dual luciferase reporter assay, we further validated that SDF1 can be directly targeted by miR-126 in hPCs. Taken together, these data indicated that ethanol-induced reduction of SDF1 in hPCs might be caused by the transfer of overloaded miR-126 of Exo^{EtOH} from hNCCs to hPCs.

_Inhibition of miR-126 significantly diminished the disruption of the coordinated migration of hNCCs and hPCs induced by hNCC-Exo^{EtOH}_

To further explore the role of exosome-derived miR-126 in mediating ethanol-induced impairment of the coordinated migration of NCCs and PCs, we examined if inhibition of miR-126 can diminish such an effect induced by hNCCs-Exo^{EtOH}. We found that treatment with miR-126 inhibitor-loaded hNCCs-Exo^{EtOH} (Fig. 7, the fifth panel) significantly diminished the disruption of the coordinated migration of hNCCs and hPCs induced by hNCC-Exo^{EtOH} (Fig. 7, the third panel). These data provided a solid evidence that ethanol-induced elevation of miR-126 in Exo^{EtOH} highly contributes to ethanol-induced disruption of coordinated migration between hNCCs and hPCs.

_Knockdown of SDF1 recapitulated ethanol-induced disruption of coordinated migration of hNCCs and hPCs, and mimicked developmental defects induced by ethanol in zebrafish embryos_

The above data have demonstrated that the Exo^{EtOH}-miR-126-SDF1 axis is involved in mediating ethanol-induced disruption of NCCs-PCs interaction _in vitro_ and _in vivo_. To further confirm the role of SDF1 in ethanol-induced teratogenesis, we silenced SDF1
expression in hPCs or in zebrafish embryos. Before seeded into the cell dishes of our co-culture system, hPCs was transfected with SDF1 siRNAs for 24 hrs, and then both hNCCs and hPCs were seeded into the same cell dish to proceed the migration assay. We found that either knockdown of SDF1 (Fig. 8A, middle column) or exposure to ethanol (Fig. 8A, third column) can lead to a severe disruption of coordinated migration between hNCCs and hPCs. Further in zebrafish, after microinjecting SDF1 morpholinos into one-cell stage embryos, we assessed the morphological changes of craniofacial skeletons and cranial nerves using WISH and Alcian Blue staining, respectively. Knockdown of SDF1 (Fig. 8B, middle column) or exposure to 1% (v/v) ethanol (Fig. 8B, right column) at 5-24 hpf resulted in significant defects in craniofacial skeletons, as indicated by severely reduced anterior arches, a loss of many brachial arches. Moreover, knockdown of SDF1 or ethanol exposure also induced cranial nerve defects in zebrafish embryos. As indicated by ganglia-specific marker Phox2b (Fig. 8C, left column, arrows), the ganglia of the cranial nerves VII, IX and X are absent, or diffused, or the ganglia volume was significantly decreased in treated embryos (Fig. 8C, second and third column). Together, these data demonstrated that downregulating SDF1 can mimic the effect of ethanol-induced impairment of hNCCs-hPCs interaction, as well as craniofacial and cranial anomalies of zebrafish embryos.

**Overexpression of SDF1 diminished ethanol-induced craniofacial and cranial nerve defects in zebrafish**

To determine whether exogenous supplement of SDF1 can rescue ethanol-induced malformations of craniofacial and cranial nerves, we microinjected *in vitro*-synthesized SDF1 mRNA into one-cell stage zebrafish eggs. While ethanol resulted in malformations or loss of hypobranchials, ceratobranchials, or basibranchials (Fig. 9, second row), overexpression of SDF1 significantly diminished these phenotypic abnormalities (Fig. 9, second row).
third row). These data, in combination with Figure 8, demonstrated that lack of SDF1 expression plays a pivotal role in ethanol-induced teratogenesis.

*Uptake of miR-126 inhibitor-loaded GELNs significantly rescued SDF1 expression, and diminished ethanol-caused craniofacial anomalies in zebrafish embryos.*

It’s been reported that edible plant-derived exosome-like nanoparticles (EPDENs) can be used for therapeutic or delivery purposes [44-46]. To figure out whether delivery of miR-126 inhibitors by grape-derived exosome-like nanoparticles (GELNs) can prevent ethanol-induced reduction of SDF1, and malformations of craniofacial skeletons in zebrafish embryos, GELNs-loaded miR-126 inhibitor (GELNs-miR-126 inhibitor) was used for delivering to zebrafish embryos. Using PKH67 as a green fluorescence tag for GELNs, miR-126 inhibitor-loaded GELNs were microinjected into one-cell stage zebrafish eggs. We found that the assembly of GELNs-miR-126 inhibitor (AGmiR126I) can be efficiently taken up by zebrafish embryos and distributed to the position where NCCs and PCs are located (Fig. 10A, *third row, arrows*). Interestingly, we observed that in AGmiR126I-injected group, the level of SDF1 (Fig. 10B) and the migration pattern of SDF1 were strikingly restored (Fig. 10C, *third row, arrows*) compared with ethanol-treated group. Furthermore, the ethanol-induced severe defects of craniofacial skeletons of zebrafish embryos were significantly diminished by exogenous supplement with AGmiR126I (Fig. 10D, *third column*). Collectively, these results demonstrated that GELNs can be used as a carrier for miR-126 inhibitor to efficiently attenuate ethanol-caused downregulation of SDF1 and subsequently to prevent ethanol-induced teratogenesis.

**Discussion**

While craniofacial anomaly is considered as a key diagnostic feature of FASD, the defects of the cranial nerves might be associated with functional deficits that have been reported in up to 95% of children with FAS, such as voice dysfunction, and impaired tongue, mouth and larynx movements [47-50]. Over the past decades, by using different animal models for FASD, investigators recapitulated craniofacial defects or the dysfunctions of sensory system that were remarkably similar to those of affected humans [51-56]. Mechanism study for these malformations and malfunctions have revealed that exposure to ethanol results in excessive apoptosis in NCCs and PCs [11-14, 57-63] and
that ethanol-induced apoptosis in NCCs contributes heavily to the subsequent craniofacial
defects [13, 54, 64]. In addition, ethanol-induced cell death in NCCs and PCs resulted in
anomalies of cranial nerves, including the fusion of the trigeminal (V) and the facial
vestibulocochlear (VII–VIII) ganglia complex, a loss of the dorsal root of the IX nerve and
partial fusion between the roots of the IX and X cranial nerves, and the disorganization of
the dorsal roots of the X nerve [65]. Moreover, ethanol exposure has been demonstrated to
strongly suppress NCC migration, resulting in short travel distance and less directional
movements of NCCs [14, 66]. Studies have also shown that fewer NCCs emigrated from
the forebrain, midbrain and hindbrain in ethanol-exposed embryos [12, 67]. Real-time
mapping of the abnormal NCC migration in ethanol-exposed zebrafish embryos
demonstrated that the migration of NCCs loses left-right symmetry and that NCCs travel a
shorter distance [68]. Based on above evidences, three points can be generated: 1) dysfunctions of NCCs and PCs contribute to the craniofacial abnormalities and cranial
nerves, respectively; 2) NCCs migration was intensively studied whereas PCs migration
was seldom paid attention, although these two tightly-neighbored cell populations are
simultaneously affected by ethanol exposure; 3) how ethanol impact the NCCs-PCs
interaction remains poorly understood. In our study, we acquired solid evidences
demonstrating that ethanol exposure can directly disrupt NCCs-PCs interaction and
directional migration of NCCs and PCs in vitro or in vivo.

Reported by Ammann decades ago, FASD and DiGeorge Syndrome share high
similarities of clinical and laboratory features in human [69]. Through acute maternal
alcohol administration, Sulik et al reproduced the typical FASD craniofacial phenotype
that is similar to that noted in DiGeorge anomaly [55]. Further genetic studies revealed that
disruption of a chemokine SDF1, and its receptor CXCR4-mediated signaling plays a key
role in the pathogenesis of DiGeorge Syndrome [70]. Specifically, downregulation of
SDF1/CXCR4 signaling resulted in misrouting of pharyngeal NCCs migration and
remarkable morphological defects in the craniofacial skeletons and cranial sensory ganglia
[71, 72]. Repression of SDF1/CXCR4 signaling has also been shown to impair NCCs
migration [73-75] or to cause major defects in many organisms [74-78]. In particular, an
outstanding study by Theveneau and colleagues have demonstrated that SDF1-positive PCs
can attract CXCR4-positive NCCs to move towards PCs, which result in a physical contact between these two cell populations and the subsequent contact-inhibition of locomotion (CIL), leading to the moving away of PCs from the NCCs, a process termed “chase and run” [10]. This coordinated and efficient directional migration of cranial NCCs and epibranchial PCs towards lateral and ventral regions of embryo is pivotal for craniofacial development. In addition, it have been shown that alcohol consumption can reduce blood SDF1 levels in patients [79]. Based on these existed findings, we come up with the hypothesis that dysregulation of SDF1/CXCR4 signaling induced by ethanol is highly correlated with the defects of craniofacial structures and cranial sensory ganglia observed in FASD. As expected, we found that ethanol exposure can decrease the SDF1 expression in hPCs and in zebrafish embryos. Furthermore, the silence of SDF1 expression either in vitro or in vivo mimicked ethanol-induced interruption of hNCC-hPC interaction or FASD-related morphological defects in zebrafish, respectively. Conversely, restoration of SDF1 expression by delivering exogenous SDF1 mRNA into zebrafish embryos protected against ethanol-induced craniofacial anomalies.

PCs-secreted SDF1 is considered to be a motor to initiate the PCs-guided NCCs migration. As demonstrated in our experiments, NCCs and PCs synergistically migrate in a chemotaxis-mediated mechanism, whereas ethanol exposure can disrupt such a process. However, through what exact mechanism that ethanol impairs the NCCs-PCs interaction is unclear. During NCCs-PCs crosstalk, PCs can emit a chemoattractant signaling molecule, SDF1, to NCCs, so it is of particular interest to quest whether NCCs can also send feedback signal to PCs through the similar way as the PCs utilize or through other unknown mechanisms. With versatile biological activities, exosomes have recently emerged as an important mode of intercellular communication especially in brain development and nervous system [40, 80-83], which carry a defined but mixed cargo of bioactive molecules to modulate the molecular configuration and behavior of target cells. For instance, during neurons communication, cells can deploy a membrane protein, EphB2 receptor into exosomes to activate ephrinB signaling resulting in growth cone collapse [84, 85]. During embryogenesis of zebrafish, neurons can secrete miR-132-containing exosomes to regulate endothelial integrity of brain [41]. Regarding these existed
knowledge, we came up with the idea that it is of high possibility that NCCs-derived exosomes may transmit negative or positive signal to PCs to regulate NCCs-PCs interaction. To address this issue, we have demonstrated that ethanol-treated hNCCs-derived exosomes (Exo$^{\text{EtOH}}$) can strongly suppress the coordinated migration of hNCCs and hPCs in our co-culture system, indicating that certain inhibitory signaling might be conducted through Exo$^{\text{EtOH}}$. Then, we proceeded to pretreat hNCCs with exosome inhibitor, GW4869, to block exosome releasing. We observed that ethanol-induced disruption on hNCCs-hPCs interaction was dramatically blocked, indicating that hNCCs-derived exosomes play a key role in mediating ethanol’s detrimental effect.

As mentioned above, exosomes take advantage of their enwrapped contents to conduct biological effects. Most important contents of exosomes are miRNAs. Among numerous miRNAs, miR-126 was highly related with SDF1/CXCR4 signaling in modulating cell migration and cancer metastasis [86-89]. Also, studies have shown that SDF1 is directly repressed by miR-126 in breast cancer cells and endothelial cells [43, 90]. More interestingly, exosomal shuttling of miR-126 in endothelial cells can modulates adhesive and migratory abilities of leukemia cells through targeting SDF1 [33]. Taken together, these studies provided us a clue that miR-126 might act as a negative regulator encapsulated in NCCs-secreted exosomes during coordinated migration of NCCs and PCs.

To test this hypothesis, we examined the miR-126 level in hNCC-derived exosomes and found that miR-126 level in Exo$^{\text{EtOH}}$ was significantly higher than that in control exosomes (Exo$^{\text{Con}}$). Furthermore, consistent with the other studies, we confirmed that miR-126 can specifically inhibit SDF1 expression in hPCs. Then we loaded ethanol-treated hNCCs-derived exosomes with miR-126 inhibitors and demonstrated that inhibiting exosome-shuttled miR-126 can significantly diminish ethanol-induced impairment of hNCCs-hPCs interaction. Also in zebrafish experiments, we observed that fruit-derived exosomal delivery of miR-126 inhibitors into zebrafish embryos can strongly rescue ethanol-induced repression of SDF1 and the subsequent craniofacial defects.

Exosome therapy is recently emerging as a promising treatment for many diseases. The advantages of exosome therapy include that they are natural carriers of proteins and RNAs with a favorable size and are well-tolerated in vivo [91-93]. However, the difficulties
in large scale production of exosomes from mammalian cells limit their clinical application. A promising solution for this problem is to produce exosome-like nanoparticles from edible plants. Recently, edible plant-derived exosome-like nanoparticles (EPDENs) that share similar properties as mammalian exosomes have been reported in grapes, grapefruit, ginger, and carrots [44-46, 94]. Several studies have demonstrated that EPDENs can be used for therapeutic or delivery purposes. For instance, it has been demonstrated that systemic deliver siRNA by using ginger exosome-like nanoparticles as a siRNA delivery vesicle could suppress tumor growth [95]. In our experiments, we used grape-derived exosome-like nanoparticles (GELNs) as a carrier for miR-126 inhibitors, showing that GELNs-encapsulated miR-126 inhibitors can be easily absorbed by zebrafish embryos and exert a strongly protective effect against ethanol-induced craniofacial anomalies.

In summary, we uncovered a novel mechanism by which exosomal delivery of miR-126 act as a negative regulator for SDF1-mediated chemotaxis process involved in ethanol-induced disruption of the coordinated NCCs-PCs interaction and developmental defects. Also, our in vivo model utilizing GELNs as an efficient delivery method for miR-126 inhibitors might pave a new way for effective intervention of FASD.
References


Statements & Declarations

Funding

The study was supported by Young Innovative Talent Project of YongJiang Talent Introduction Programme 2021A-012-G (H.F.), Natural Science Foundation of Ningbo 2021J321 (H.F.), Special Funding for Microfluidic Chip of Biomedicine of Ningbo Institute of Life and Health Industry, University of Chinese Academy of Sciences 2021YJY1006 (H.F.), Natural Science Foundation of Ningbo 2021J328 (Y.L.), Special Funding for Microfluidic Chip of Biomedicine of Ningbo Institute of Life and Health Industry, University of Chinese Academy of Sciences 2021YJY1005 (Y.L.), and National Institute of Health Grants R01AA028435, R01AA021434. (S.C.)

Competing Interests

The authors declare no conflict of interest.

Author Contributions

H.F. and Y.H.L. designed research, performed the experiments, and participated in data analysis and manuscript preparation. H.F., Y.H.L., T.C., H.L., Y.L., S.Z., F.S., J.L. and S.C. participated in data interpretation and discussion. All authors reviewed the manuscript.

Data Availability Statements

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.
Figures Legends

**Fig. 1** Exposure to ethanol significantly disrupted the coordinated migration *in vitro* and *in vivo* (A) A cartoon depicts hNCCs-hPCs co-culture system to evaluate the interaction between hNCCs and hPCs. An insert was put into a cell culture plate, and then hNCCs and hPCs were seeded into each side of the insert and cultured to form a monolayer. The insert was then removed to generate a 0.9mm cells-free gap between hNCCs and hPCs. hNCCs and hPCs were cultured in the medium with or without ethanol and allowed to migrate for 24 hours. (B) hNCCs and hPCs were treated with or without ethanol (50mM) for 24 hours and then fixed for immunofluorescence that co-stained with antibodies for hNCCs-specific marker HNK1 (green) and hPCs-specific marker Six1(red). Bright-field photos of the two cell groups were captured at the 0 time point when the monolayer of hNCCs and hPCs was just formed (*first row*). The second to fourth row showed the photos of bright field and fluorescence field of control group or ethanol-treated group, in which hNCCs and hPCs had migrated for 24 hours. (C) Zebrafish embryos were treated with 1% ethanol at 5-24 hours post fertilization (hpf), and then the embryos were collected for whole mount *in situ* hybridization (WISH) at 30 hpf. Twist1a was used as a specific probe for detecting the NCCs in zebrafish embryos (*arrows*). Scale bar, 200μm. As quantified in (D), the approximately migratory distance of NCCs was measured as the migrated length towards Ventral position from the Dorsal site of neural tube (*labeled as “migration along the DV axis”*). Data represent the mean±S.E.M. of three independent experiments. **, *p*<0.01 vs. control. (E) After the same treatment as in (C), Sox3 was used as a specific probe for detecting the PCs in zebrafish embryos (*arrows*). Scale bar, 200μm. (F) Quantification of approximately migratory distance of PCs were also expressed as the migrated length towards Ventral position from the Dorsal site of neural tube (*labeled as “migration along the DV axis”*). Data represent the mean ± S.E.M. of three independent experiments. **, *p*<0.01 vs. control.

**Fig. 2** Treatment with hNCCs-Exo\textsuperscript{EtOH} significantly disrupted the coordinated migration between hNCCs and hPCs At first step, hNCCs were treated with or without ethanol (50mM) for 24 hours, and then the hNCCs-cultured medium was collected for isolating hNCCs-derived exosomes. Secondly, hNCCs and hPCs were seeded into the cell
co-culture system as described in Fig. 1A, and treated with control hNCCs-derived exosomes (ExoCon, $1\mu g/10^5$ cells) or ethanol-treated hNCCs-derived exosomes (ExoEtOH, $1\mu g/10^5$ cells) for 24 hours. Finally, the cells were subject to immunofluorescence that co-stained with antibodies for with hNCCs-specific marker HNK1 (green), and hPCs-specific marker Six1 (red). Bright-field photos of ExoCon-treated group or ExoEtOH-treated group were captured at the 0 time point when the monolayer of the two group cells was just formed (first row). The second to fourth row displayed the photos of bright field and fluorescence field of ExoCon-treated group or ExoEtOH-treated group, in which hNCCs and hPCs had migrated for 24 hours.

Fig. 3 Exposure to ethanol significantly decreased SDF1 level in hPCs and zebrafish embryos (A) hPCs were treated with or without ethanol (50mM) for 24 hours and then the cells were lysed for immunoblot analysis using anti-SDF1 or anti-actin antibodies. (B) Zebrafish embryos were treated with or without ethanol (1% v/v) at 5-24 hpf. At 30 hpf, embryos were collected for detecting SDF1 expression by qRT-PCR analysis. (C) Zebrafish embryos were treated with or without ethanol (1% v/v) at 5-24 hpf and then the embryos were fixed for WISH at 30 hpf, as indicated in control or ethanol-treated group, the patterns of SDF1 expression (arrows) were detected using SDF1 probe. Scale bar, 200μm. Data are expressed as fold change over control and represent the mean ± S.E.M. of three independent experiments. * $p < 0.05$, ** $p < 0.01$ vs. control.

Fig. 4 Overexpression of SDF1 diminished ethanol-induced disruption of the coordinated migration between NCCs and PCs in zebrafish embryos (A) Zebrafish embryos were divided into three groups. In addition to control group, embryos in the second group were treated with ethanol (1% v/v) at 5-24 hpf; in the third group, embryos were first microinjected with in vitro-synthesized SDF1 mRNA (0.6pmol/embryo) at one-cell stage and then treated with ethanol (1% v/v) at 5-24 hpf. At 30 hpf, the embryos were collected for WISH to visualize the migration pattern of NCCs and PCs probed with Twist1a and Sox3, respectively. Scale bar, 200μm. (B) and (C) As the same statistic method in Fig. 1D and E, the approximately migratory distance of NCCs or PCs was expressed as their migrated length towards Ventral position from the Dorsal site of neural tube (labeled...
as “migration along the DV axis”), respectively. Data are expressed as the percentage of control and represent the mean ± S.E.M. *p < 0.05, **p < 0.01 vs. control.

**Fig. 5 Ethanol exposure resulted in a robust increase of miR-126 level in hNCCs-Exo<sub>EtOH</sub>** (A) Representative of TEM image of hNCCs-Exo. Magnification, 80,000 X. Scale bar, 100 nm. (B) hNCCs were treated with or without ethanol (50mM) for 24 hours. Then the cultured media for hNCCs were collected for exosomes isolation. After purification, exosomes of hNCCs-Exo<sub>Con</sub> and hNCCs-Exo<sub>EtOH</sub> were lysed for detecting miR-126 expression by qRT-PCR analysis. Data are expressed as fold change over control and represent the mean±S.E.M. of three independent experiments. **p < 0.01 vs. control.

**Fig. 6 Treatment with hNCCs-Exo<sub>EtOH</sub> significantly increased miR-126 level and decreased SDF1 expression in control hPCs** (A) Representative immunofluorescence image showed the uptake of PHK67-labeled hNCCs-Exo (green dots) by hPCs, and the nuclei were stained with DAPI (blue). Scale bar, 10 μm. (B) hPCs were set as blank control, or treated with Exo<sub>Con</sub> (1μg/10<sup>5</sup> cells), or with Exo<sub>EtOH</sub> (1μg/10<sup>5</sup> cells) for 24 hours, and then the cells were harvested for detecting miR-126 expression by qRT-PCR analysis. (C) As shown in the schematic figure, there’s a presumable binding site at 3’-untranslational region (3’-UTR) of SDF1 for miR-126. 3’-UTR of SDF1-assembled pMIR-luciferase plasmid (pMIR-luciferase-SDF1(3’-UTR)) and Renila luciferase plasmid (pRL-TK-luciferase) were co-transfected with or without control mimic or miR-126 mimic into hPCs for 48 hours. Then the cells were subject to dual luciferase reporter assays. (D) hPCs were treated with blank control, Exo<sub>Con</sub> (1μg/10<sup>5</sup> cells), or Exo<sub>EtOH</sub> (1μg/10<sup>5</sup> cells) for 24 hours, and then the mRNA expression of SDF1 in hPCs were determined by qRT-PCR analysis. (E) Treated as the same condition within (E), hPCs were lysed for detecting the protein level of SDF1 by immunoblot. Data are expressed as fold change over control and represent the mean ± S.E.M. of three separate experiments. **p < 0.01 vs. control.

**Fig. 7 Inhibition of miR-126 in hNCCs-secreted Exo<sub>EtOH</sub> significantly diminished Exo<sub>EtOH</sub>-induced disruption of coordinated migration between hNCCs and hPCs** hNCCs were treated with or without ethanol (50 mM) for 24 hours and the hNCCs-cultured media were collected for isolating Exo<sub>Con</sub> or Exo<sub>EtOH</sub>. Exo<sub>EtOH</sub> were loaded with control inhibitor or miR-126 inhibitor. Immediately after hNCCs and hPCs were seeded in the
hNCCs-hPCs co-culture system, they were treated with blank control, Exo^{Con} (1µg/10^5 cells), Exo^{EtOH} (1µg/10^5 cells), Exo^{EtOH} (1µg/10^5 cells) plus control inhibitor (0.1 µM), or Exo^{EtOH} (1µg/10^5 cells) plus miR-126 inhibitor (0.1 µM). After 24 hours’ migration, the cells were co-stained with antibodies for hNCCs marker HNK1 (green), and hPCs marker Six1 (red). Bright-field photos were captured at the 0 time point when the monolayer of the five group cells was just formed (first row). The second to fourth row showed the photos of bright field and fluorescence field for these five groups, in which hNCCs and hPCs had migrated for 24 hours.

**Fig. 8** The knockdown of SDF1 mimicked the effect of ethanol-induced disruption of coordinated migration between hNCCs and hPCs, as well as the effects of ethanol-induced anomalies of craniofacial skeletons and cranial nerves in zebrafish embryos (A) For the first and the third column, hNCCs-hPCs co-culture system were treated with or without ethanol (50mM) for 24 hours. For the middle column, before hPCs and hNCs were seeded into the co-culture system, SDF1 expression in hPCs was knocked down by SDF1 siRNA. Then, after 24 hours’ migration of hNCCs and hPCs in the co-culture system, these two cell types were co-stained with antibodies for hNCCs marker HNK1 (green), and hPCs marker Six1 (red). Bright-field photos were captured at the 0 time point when the monolayer of the cells in control, SDF1-knockdown, and ethanol-exposed groups was just formed (first row). The second to fourth row showed the photos of bright field and fluorescence field for these three groups, in which hNCCs and hPCs had migrated for 24 hours. (B) Zebrafish embryos were microinjected with SDF1 morpholinos at one-cell stage or exposed to ethanol (1% v/v) at 5-24 hpf, and then the craniofacial cartilages were stained with Alcian blue at 5 dpf (days post fertilization). bb: basibranchials; hb: hypobranchials; cb: ceratobranchial. Scale bar, 200µm. (C) After performing the same treatment within (B), the patterns of cranial nerves at 2 dpf embryos were determined by WISH using cranial ganglia-specific probe Phox2b. VII: facial nerves; IX: glossopharyngeal nerves; X: vagal nerves. Scale bar, 200µm.

**Fig. 9** Overexpression of SDF1 diminished ethanol-induced defects of craniofacial skeletons and cranial nerves in zebrafish embryos (A) The first row of control group showed a normal phenotype of craniofacial cartilages (black arrows). In the second row,
zebrafish embryos were treated with ethanol (1% v/v) at 5-24 hpf. For the third row, the embryos were microinjected with in vitro-synthesized SDF1 mRNA (0.6 pmol/embryo) at one-cell stage and then treated with ethanol (1% v/v) at 5-24 hpf. Zebrafish embryos in these three groups were stained with Alcian blue at 5 dpf for visualizing the morphology of craniofacial cartilages. bb: basibranchials; hb: hypobranchials; cb: ceratobranchial. Scale bar, 200 μm. (B) After the same treatment within (A), zebrafish embryos were subject to WISH at 2 dpf. The patterns of cranial nerves were indicated by ganglia-specific probe Phox2b (white arrows in first and third row). VII: facial nerves; IX: glossopharyngeal nerves; X: vagal nerves. Scale bar, 200 μm.

Fig. 10 Uptake of miR-126 inhibitor-loaded GELNs diminished ethanol-induced repression of SDF1 expression, and rescued the defects of craniofacial skeletons induced by ethanol in zebrafish embryos (A) For the first and second rows, zebrafish embryos were treated without or with ethanol (1% v/v) at 5-24 hpf. In the third row, the embryos were microinjected with miR-126 inhibitor-loaded GELNs that labeled with PHK67 (PHK67-GELNs-miR-126 inhibitor) at one-cell stage and then were treated with ethanol (1% v/v) at 5-24 hpf. The embryos were dechorionized at 30 hpf and photographed under a fluorescence microscope. The PHK67-GELNs-miR-126 inhibitors were distributed throughout the craniofacial position of the embryos as indicated by the white arrows. Scale bar, 200 μm. (B) For the first and second groups, zebrafish embryos were treated without or with ethanol (1% v/v) at 5-24 hpf. For the group, the embryos were microinjected with miR-126 inhibitor-loaded GELNs (GELNs-miR-126 inhibitor) at one-cell stage and then were treated with ethanol (1% v/v) at 5-24 hpf. The embryos of these three groups were collected at 30 hpf and subject to qRT-PCR analysis for detecting SDF1 expression. (C) and (D) As treated with the same methods in (B), zebrafish embryos were subject to WISH for visualizing the expression pattern of SDF1 (indicated by black arrows in the three rows) detected by SDF1 probes at 30 hpf, or were stained by Alcian blue to view the morphology of craniofacial cartilages at 5 dpf, respectively. bb: basibranchials; hb: hypobranchials; cb: ceratobranchial. Scale bars, 200 μm.
Fig. 11 GW4869, an exosome inhibitor, mitigates ethanol-induced impairment of hNCCs-hPCs interaction Four groups of hNCCs and hPCs cultured in the hNCCs-hPCs co-culture system were set as control, ethanol (50 mM), control plus GW4869, or ethanol (50 mM) plus GW4869 (10µM). After the cells migrated for 24 hours, hNCCs and hPCs were co-stained with antibodies for hNCCs-specific marker HNK1 (green) and hPCs-specific marker Six1 (red). Bright-field photos were captured at the 0 time point when the monolayer of the four group cells was just formed (first row). The second to fourth row showed the photos of bright field and fluorescence field for these four groups, in which hNCCs and hPCs had migrated for 24 hours.
Exposure to ethanol significantly disrupted the coordinated migration \textit{in vitro} and \textit{in vivo} (A) A cartoon depicts hNCCs-hPCs co-culture system to evaluate the interaction between hNCCs and hPCs. An insert was put into a cell culture plate, and then hNCCs and hPCs were seeded into each side of the insert and cultured to form a monolayer. The insert was then removed to generate a 0.9mm cells-free gap between hNCCs and hPCs. hNCCs and hPCs were cultured in the medium with or without ethanol and allowed to migrate for 24 hours. (B) hNCCs and hPCs were treated with or without ethanol (50mM) for 24 hours and then fixed for immunofluorescence that co-stained with antibodies for hNCCs-specific marker HNK1 (green) and hPCs-specific marker Six1(red). Bright-field photos of the two cell groups were captured at the 0 time point when the monolayer of hNCCs and hPCs was just formed (first row). The second to fourth row showed the photos of bright field and fluorescence field of control group or ethanol-treated group, in which hNCCs and hPCs had migrated for 24 hours. (C) Zebrafish embryos were treated with 1% ethanol at 5-24 hours post fertilization (hpf), and then the embryos were collected for whole mount \textit{in situ} hybridization (WISH) at 30 hpf. Twist1a was used as a specific probe for detecting the NCCs in zebrafish embryos (arrows). Scale bar, 200μm. As quantified in (D), the approximately migratory distance of NCCs was measured as the migrated length towards Ventral position from the Dorsal site of neural tube (labeled as “migration along the DV axis”). Data represent the mean ± S.E.M. of three independent experiments. **, \( p < 0.01 \) vs. control. (E) After the same treatment as in (C), Sox3 was used as a specific probe for detecting the PCs in zebrafish embryos (arrows). Scale bar, 200μm. (F) Quantification of approximately migratory distance of PCs were also expressed as the migrated length towards Ventral position from the Dorsal site of neural tube (labeled as “migration along the DV axis”). Data represent the mean ± S.E.M. of three independent experiments. **, \( p < 0.01 \) vs. control.
Treatment with hNCCs-Exo$^{\text{EtOH}}$ significantly disrupted the coordinated migration between hNCCs and hPCs. At first step, hNCCs were treated with or without ethanol (50mM) for 24 hours, and then the hNCCs-cultured medium was collected for isolating hNCCs-derived exosomes. Secondly, hNCCs and hPCs were seeded into the cell co-culture system as described in Fig. 1A, and treated with control hNCCs-derived exosomes (Exo$^{\text{Con}}$, 1μg/105 cells) or ethanol-treated hNCCs-derived exosomes (Exo$^{\text{EtOH}}$, 1μg/105 cells).
for 24 hours. Finally, the cells were subject to immunofluorescence that co-stained with antibodies for with hNCCs-specific marker HNK1 (green), and hPCs-specific marker Six1 (red). Bright-field photos of Exo\textsuperscript{Con}-treated group or Exo\textsuperscript{EtOH}-treated group were captured at the 0 time point when the monolayer of the two group cells was just formed (first row). The second to fourth row displayed the photos of bright field and fluorescence field of Exo\textsuperscript{Con}-treated group or Exo\textsuperscript{EtOH}-treated group, in which hNCCs and hPCs had migrated for 24 hours.

**Figure 3**

Exposure to ethanol significantly decreased SDF1 level in hPCs and zebrafish embryos (A) hPCs were treated with or without ethanol (50mM) for 24 hours and then the cells were lysed for immunoblot analysis using anti-SDF1 or anti-actin antibodies. (B) Zebrafish embryos were treated with or without ethanol (1% v/v) at 5-24 hpf. At 30 hpf, embryos were collected for detecting SDF1 expression by qRT-PCR analysis. (C) Zebrafish embryos were treated with or without ethanol (1% v/v) at 5-24 hpf and then the embryos were fixed for WISH at 30 hpf, as indicated in control or ethanol-treated group, the patterns of SDF1 expression (arrows) were detected using SDF1 probe. Scale bar, 200 μm. Data are expressed as
fold change over control and represent the mean ± S.E.M. of three independent experiments. * $p < 0.05$, ** $p < 0.01$ vs. control.

Figure 4

Overexpression of SDF1 diminished ethanol-induced disruption of the coordinated migration between NCCs and PCs in zebrafish embryos (A) Zebrafish embryos were divided into three groups. In addition to control group, embryos in the second group were treated with ethanol (1% v/v) at 5-24 hpf; in the third group, embryos were first microinjected with *in vitro*-synthesized SDF1 mRNA (0.6pmol/embryo) at one-cell stage and then treated with ethanol (1% v/v) at 5-24 hpf. At 30 hpf, the embryos were collected for

![Diagram showing the effect of SDF1 overexpression on neural crest cell and placode migration](image_url)
WISH to visualize the migration pattern of NCCs and PCs probed with Twist1a and Sox3, respectively. Scale bar, 200μm. (B) and (C) As the same statistic method in Fig. 1 D and E, the approximately migratory distance of NCCs or PCs was expressed as their migrated length towards Ventral position from the Dorsal site of neural tube (labeled as “migration along the DV axis”), respectively. Data are expressed as the percentage of control and represent the mean ± S.E.M. * p < 0.05, ** p < 0.01 vs. control.

Figure 5

Ethanol exposure resulted in a robust increase of miR-126 level in hNCCs-Exo^{EtOH} (A) Representative of TEM image of hNCCs-Exo. Magnification, 80,000 X. Scale bar, 100 nm. (B) hNCCs were treated with or without ethanol (50mM) for 24 hours. Then the cultured media for hNCCs were collected for exosomes isolation. After purification, exosomes of hNCCs-Exo^{Con} and hNCCs-Exo^{EtOH} were lysed for detecting miR-126 expression by qRT-PCR analysis. Data are expressed as fold change over control and represent the mean±S.E.M. of three independent experiments. ** p < 0.01 vs. control.
Treatment with hNCCs-Exo$^{\text{EtOH}}$ significantly increased miR-126 level and decreased SDF1 expression in control hPCs (A) Representative immunofluorescence image showed the uptake of PHK67-labeled hNCCs-Exo (green dots) by hPCs, and the nuclei were stained with DAPI (blue). Scale bar, 10 μm. (B) hPCs were set as blank control, or treated with Exo$^{\text{Con}}$ (1 μg/105 cells), or with Exo$^{\text{EtOH}}$ (1 μg/105 cells) for 24 hours, and then the cells were harvested for detecting miR-126 expression by qRT-PCR analysis. (C) As shown in the schematic figure, there’s a presumable binding site at 3'-untranslational region (3'-UTR) of SDF1 for miR-126. 3'-UTR of SDF1-assembled pMIR-luciferase plasmid (pMIR-luciferase-SDF1(3'-UTR)) and Renila luciferase plasmid (pRL-TK-luciferase) were co-transfected with or without control mimic or miR-126 mimic into hPCs for 48 hours. Then the cells were subject to dual luciferase reporter assays. (D) hPCs were treated with blank control, Exo$^{\text{Con}}$ (1 μg/105 cells), or Exo$^{\text{EtOH}}$ (1 μg/105 cells) for 24 hours, and then the mRNA expression of SDF1 in hPCs were determined by qRT-PCR analysis. (E) Treated as the same condition within (E), hPCs were lysed for detecting the protein level of SDF1 by immunoblot. Data
are expressed as fold change over control and represent the mean ± S.E.M. of three separate experiments. **p < 0.01 vs. control.

**Figure 7**

Inhibition of miR-126 in hNCCs-secreted Exo^{EtOH} significantly diminished Exo^{EtOH}-induced disruption of coordinated migration between hNCCs and hPCs hNCCs were treated with or without ethanol (50 mM) for 24 hours and the hNCCs-cultured media were collected for isolating Exo^{Con} or Exo^{EtOH}. Exo^{EtOH} were loaded with control inhibitor or miR-126 inhibitor. Immediately after hNCCs and hPCs were seeded in the hNCCs-hPCs co-culture system, they were treated with blank control, Exo^{Con} (1µg/105 cells), Exo^{EtOH} (1µg/105 cells), Exo^{EtOH} (1µg/105 cells) plus control inhibitor (0.1 µM), or Exo^{EtOH} (1µg/105 cells) plus miR-126 inhibitor (0.1 µM). After 24 hours’ migration, the cells were co-stained with antibodies for hNCCs marker HNK1 (green), and hPCs marker Six1(red). Bright-field photos were captured at the 0 time point when the monolayer of the five group cells was just formed (*first row*). The second to fourth row showed the photos of bright field and fluorescence field for these five groups, in which hNCCs and hPCs had migrated for 24 hours.
The knockdown of SDF1 mimicked the effect of ethanol-induced disruption of coordinated migration between hNCCs and hPCs, as well as the effects of ethanol-induced anomalies of craniofacial skeletons and cranial nerves in zebrafish embryos (4) For the first and the third column, hNCCs-hPCs co-culture system were treated with or without ethanol ($50mM$) for 24 hours. For the middle column, before hPCs and hNCs were seeded into the co-culture system, SDF1 expression in hPCs was knocked down by SDF1
siRNA. Then, after 24 hours’ migration of hNCCs and hPCs in the co-culture system, these two cell types were co-stained with antibodies for hNCCs marker HNK1 (green), and hPCs marker Six1 (red). Bright-field photos were captured at the 0 time point when the monolayer of the cells in control, SDF1-knockdown, and ethanol-exposed groups was just formed (first row). The second to fourth row showed the photos of bright field and fluorescence field for these three groups, in which hNCCs and hPCs had migrated for 24 hours. (B) Zebrafish embryos were microinjected with SDF1 morpholinos at one-cell stage or exposed to ethanol (1% v/v) at 5-24 hpf, and then the craniofacial cartilages were stained with Alcian blue at 5 dpf (days post fertilization). bb: basibranchials; hb: hypobranchials; cb: ceratobranchial. Scale bar, 200μm. (C) After performing the same treatment within (B), the patterns of cranial nerves at 2 dpf embryos were determined by WISH using cranial ganglia-specific probe Phox2b. VII: facial nerves; IX: glossopharyngeal nerves; X: vagal nerves. Scale bar, 200μm.
Overexpression of SDF1 diminished ethanol-induced defects of craniofacial skeletons and cranial nerves in zebrafish embryos (A) The first row of control group showed a normal phenotype of craniofacial cartilages (black arrows). In the second row, zebrafish embryos were treated with ethanol (1% v/v) at 5-24 hpf. For the third row, the embryos were microinjected with *in vitro*-synthesized SDF1 mRNA (0.6 pmol/embryo) at one-cell stage and then treated with ethanol (1% v/v) at 5-24 hpf. Zebrafish embryos in
these three groups were stained with Alcian blue at 5 dpf for visualizing the morphology of craniofacial cartilages. bb: basibranchials; hb: hypobranchials; cb: ceratobranchial. Scale bar, 200μm. (B) After the same treatment within (A), zebrafish embryos were subject to WISH at 2 dpf. The patterns of cranial nerves were indicated by ganglia-specific probe Phox2b (white arrows in first and third row). VII: facial nerves; IX: glossopharyngeal nerves; X: vagal nerves. Scale bar, 200μm.

**Figure 10**

Uptake of miR-126 inhibitor-loaded GELNs diminished ethanol-induced repression of SDF1 expression, and rescued the defects of craniofacial skeletons induced by ethanol in zebrafish embryos (A) For the first and second rows, zebrafish embryos were treated without or with ethanol (1% v/v) at 5-24 hpf. In the third row, the embryos were microinjected with miR-126 inhibitor-loaded GELNs that labeled with PHK67 (PHK67-GELNs-miR-126 inhibitor) at one-cell stage and then were treated with ethanol (1% v/v) at 5-24 hpf. The embryos were dechorionized at 30 hpf and photographed under a fluorescence microscope. The
PHK67-GELNs-miR-126 inhibitors were distributed throughout the craniofacial position of the embryos as indicated by the white arrows. Scale bar, 200μm. (B) For the first and second groups, zebrafish embryos were treated without or with ethanol (1% v/v) at 5-24 hpf. For the group, the embryos were microinjected with miR-126 inhibitor-loaded GELNs (GELNs-miR-126 inhibitor) at one-cell stage and then were treated with ethanol (1% v/v) at 5-24 hpf. The embryos of these three groups were collected at 30 hpf and subject to qRT-PCR analysis for detecting SDF1 expression. (C) and (D) As treated with the same methods in (B), zebrafish embryos were subject to WISH for visualizing the expression pattern of SDF1 (indicated by black arrows in the three rows) detected by SDF1 probes at 30 hpf, or were stained by Alcian blue to view the morphology of craniofacial cartilages at 5 dpf, respectively. bb: basibranchials; hb: hypobranchials; cb: ceratobranchial. Scale bars, 200μm.

**Supplementary Files**

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- Fig11.png