Research Paper

MicroRNA-34a mediates ethanol-induced impairment of neural differentiation of neural crest cells by targeting autophagy-related gene 9a

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ABSTRACT

Neural crest cells (NCCs) are multipotent progenitor cells that are sensitive to ethanol and are implicated in Fetal Alcohol Spectrum Disorders (FASD). The objective of this study is to test whether ethanol exposure can inhibit the neural differentiation of NCCs by inhibiting autophagy and whether miR-34a is involved in ethanol-induced inhibition of autophagy in NCCs. We found that ethanol exposure resulted in the inhibition of neural differentiation of NCCs. Exposure to ethanol also significantly decreased autophagy in NCCs, as indicated by a decreased LC3II/I ratio and an elevated expression of p62 protein. Knockdown of p62 restored the expression of the neurogenesis genes, NF and Mash1, in ethanol-exposed NCCs, suggesting that ethanol exposure can inhibit the neural differentiation of NCCs by inhibiting autophagy. We also found that ethanol exposure resulted in a significant increase in miR-34a expression in NCCs. Inhibition of miR-34a restored the expression of *Atg9a*, a direct target of miR-34a and significantly decreased ethanol-induced inhibition of autophagy in NCCs. Down-regulation of miR-34a also prevented ethanol-induced inhibition of neural differentiation of NCCs. These results demonstrate that ethanol-induced inhibition of neural differentiation of NCCs is mediated by the miR-34a through targeting *Atg9a*.

1. Introduction

Fetal alcohol spectrum disorders (FASD) is a term that describes a series of disorders that can be observed in an individual who was prenatally exposed to alcohol (Chen et al., 2004; Herrmann et al., 1980; Spiegel et al., 1979). The neural crest cells (NCCs) are originated from the junction between neural and epidermal ectoderm in neurula-stage of vertebrate embryos, contributing to various differentiated cell types, including peripheral neurons, glia, melanocytes, chondrocytes and connective tissue (Achilleos and Trainor, 2012; Zhang et al., 2014). NCCs are one of the most vulnerable cell populations to ethanol exposure (Chen et al., 2000; Dunty Jr et al., 2001). Studies have demonstrated that ethanol-induced excessive apoptosis in NCCs is one of the major mechanisms underlying the pathogenesis of FASD (Cartwright and Smith, 1995; Chen et al., 2013a; Dunty Jr et al., 2001; Kotch and Sulik, 1992).

NCCs can differentiate to form the facial bones, cartilage and the sensory components of certain cranial nerves. The neurons contained in varied ganglia, including trigeminal ganglia, dorsal root ganglia, and sympathetic ganglia of the peripheral nervous system (PNS), are mostly originated from NCCs (Bhatt et al., 2013; Pavan and Raible, 2012). In addition to the impairment of the central nervous system (CNS) in FASD, studies using zebrafish model had shown that ethanol exposure significantly decreased the cranial ganglia volume, suggesting inhibition of neural differentiation in PNS (Muralidharan et al., 2013). Ethanol exposure also resulted in significant apoptosis in the neural crest and placodal components of the cranial sensory ganglia in mice (Dunty Jr et al., 2001). While there is considerable evidence to support that impairment of neural differentiation also contributes to the pathogenesis of FASD, the ethanol-induced impairment of neural differentiation of NCCs and the underlying mechanisms have remained elusive.

Autophagy is a process that is considered to be an adaptive response when eukaryotic cells undergo various stress stimuli such as nutrition

or energy deprivation, lack of cell growth factors, excessive reactive oxygen species, overloaded malfunctioned sub-cellular organs and misfolded protein aggregation or any other potentially detrimental physical or chemical factors (He and Klionsky, 2009; Kaur and Debnath, 2015; Kundu and Thompson, 2008). Autophagy is a highly regulated process with a series of well-known autophagy-related genes (*Atgs*) involved (He and Klionsky, 2009; Xie and Klionsky, 2007). Emerging evidence showed that there is molecular crosstalk between autophagy and apoptosis (Denton et al., 2015; Marino et al., 2014). While some genes including *beclin1*, *bcl2* family and some genes encoding cellular kinase are common regulators for both autophagy and apoptosis (Oral et al., 2016), in some cases, activation of autophagy prevents cells from apoptosis (Denton et al., 2015). In addition to its protecting role in certain cell context, autophagy plays a crucial role in neurogenesis. It had been shown that developmental defects of the neural tube can be restored by activating autophagy system (Xu et al., 2013). A number of the studies have also demonstrated that activating autophagy promoted neuronal differentiation and embryonic neurogenesis (Li et al., 2016; Lu et al., 2013; Vazquez et al., 2012; Wang et al., 2013), while the inhibition of autophagy or the accumulation of certain autophagy substrates such as p62 protein prevented neural differentiation (Wang et al., 2016; Zhao et al., 2010). Jang et al. also demonstrated that the commitment of human embryonic stem cells to neuroectoderm fate required the activation of autophagy (Jang et al., 2016). Among core autophagy-related proteins, Atg9a is a multi-spanning transmembrane protein that participates in the recruitment of membranes during the initiation of autophagy and is required for the formation of the autophagosome (Imai et al., 2016; Nishimura et al., 2017; Pavel and Rubinsztein, 2017).

MicroRNAs (miRNAs) are small non-coding RNA of 22–24 nucleotides that can regulate gene expression through binding to 3′ untranslated region of the target mRNA (Chim et al., 2010; Kim, 2005). miRNAs are involved in various biological processes, including cell proliferation, differentiation, apoptosis, development, and tumorigenesis (Esquela-Kerscher and Slack, 2006; Kim, 2005). Emerging evidence demonstrated the roles of miR-34 in apoptosis and embryonic stem cells differentiation (Chang et al., 2007; Raver-Shapira et al., 2007; Tarantino et al., 2010; Welch et al., 2007). A recent report had shown that elevation of miR-34a promoted cochlear cell death by impairing autophagy through repressing *Atg9a* (Pang et al., 2017). It has also been shown that the elevation of miR-34a impaired neuronal differentiation via Atg9a-dependent mechanism (Morgado et al., 2015).

In this study, we determined whether ethanol exposure can inhibit the neural differentiation of NCCs by down-regulating autophagy and whether miR-34a is involved in ethanol-induced inhibition of autophagy and neural differentiation of NCCs. We found that exposure to ethanol significantly decreased the neural differentiation of NCCs. Ethanol exposure also resulted in the inhibition of autophagy in NCCs. Knockdown of p62 restored the expression of neuronal markers NF and Mash1 in ethanol-exposed NCCs and prevented ethanol-induced inhibition of the neural differentiation of NCCs. We also found that inhibition of miR-34a restored the expression of *Atg9a*, and significantly decreased ethanol-induced inhibition of autophagy and neural differentiation of NCCs. These results demonstrate that ethanol-induced inhibition of neural differentiation of NCCs is mediated by the miR-34a through targeting *Atg9a*, leading to the inhibition of autophagy in NCCs.

2. Materials and methods

2.1. Cell culture and treatment

NCCs (Joma1.3 cells) were cultured as previously described (Chen et al., 2013b). Cells were grown on culture dishes coated with fibronectin and maintained in Dulbecco's modified Eagle's medium (DMEM): Ham's F12 (1:1) at 37 °C in 5% CO2/95% air. For ethanol
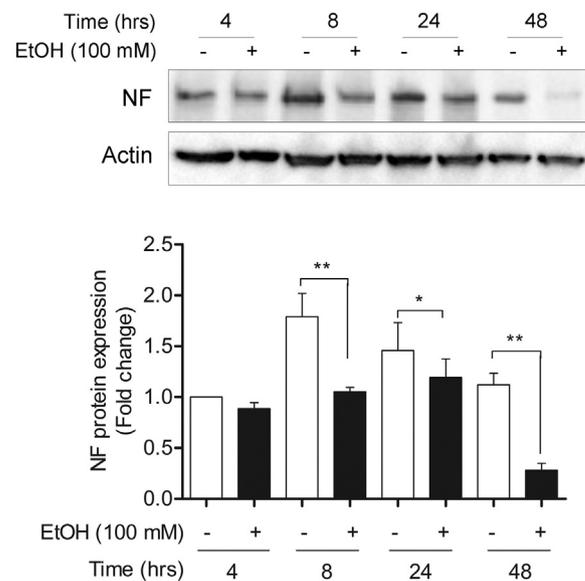

Fig. 1. BMP-2-induced neural differentiation of NCCs was inhibited by ethanol exposure. NCCs pre-treated with BMP-2 (50 ng/mL) for 24 h were treated with or without 100 mM ethanol for 4, 8, 24 or 48 h. After 6 days of differentiation, the protein expression of neurofilament (NF) was determined by Western Blot. Data are expressed as fold change over control and represent the mean ± SEM of three separate experiments. *, $p < .05$, **, $p < .01$ vs. control.

treatment, NCCs were treated with 50 or 100 mM ethanol. The stable ethanol levels were maintained by placing the cell culture dishes or plates in a plastic desiccator containing ethanol in distilled water, as described previously (Yan et al., 2010).

2.2. Induction of neuronal differentiation from NCCs

Neuronal differentiation of NCCs was conducted as described by Maurer et al. (Maurer et al., 2007). Briefly, cells were seeded at approximately 30% confluence onto dishes sequentially coated with Poly-D-Lysin (1 mg/mL, Sigma-Aldrich, Billerica, MA, USA) and fibronectin (1 mg/mL, Bedford, MA, USA). After cells were cultured in NCC medium for 24 h, BMP-2 (50 ng/mL, R&D system, Minneapolis, MN, USA) was added to the culture medium to induce neuronal differentiation. To determine the effects of ethanol exposure on neural differentiation of NCCs, NCCs pre-treated with BMP-2 for 24 h were treated with 100 mM ethanol for 4, 8, 24 or 48 h. After 6 days of differentiation in BMP-2-containing medium, a neuronal marker, Neurofilament (NF), was used to detect the neural differentiation of NCCs.

2.3. qRT-PCR and Western Blotting

Quantitative Real-time PCR was performed as previously described (Yuan et al., 2018). Briefly, the mRNAs were extracted from NCCs by using RNeasy Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. The cDNAs were synthesized by using the QuantiTect® Reverse Transcription Kit (Qiagen, Valencia, CA, USA). Primers used for qRT-PCR analyses were: Neurofilament-MF: forward: 5′-GCAGCCAAACTGAACACAGA-3′; reverse: 5′-CCATCTCCCACTTGGT GTTT-3′. Mash1: forward: 5′-TTGAACTCTATGGCGGGTTC-3′; reverse: 5′-GGGCTTAGGTTCAGACACCA-3′. Atg9a: forward: 5′-TGAGAGCACA GCTTTCCTGG-3′; reverse: 5′-ATCTCGGTGGACGCGTATTC-3′. The qRT-PCR reaction was performed using FastStart SYBR Green Master (Roche Applied, Indianapolis, IN, USA) on a Rotor-Gene 6000 Real-Time PCR system (Corbett Life Science, Sydney, Australia), and the relative gene expression was calculated using ΔΔCT method.

Western Blotting was performed by the standard protocol as previously described (Yuan et al., 2017). NCCs were washed with
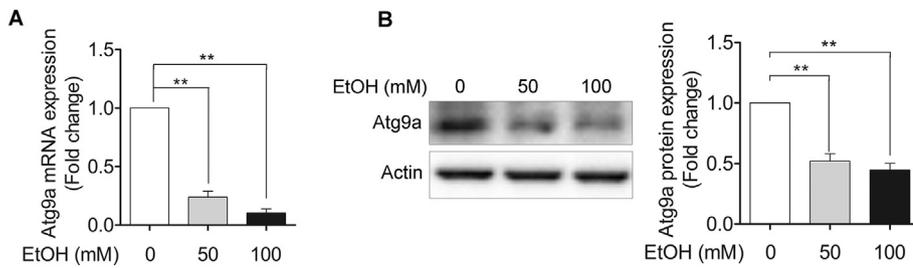
Fig. 2. Ethanol exposure significantly decreased *Atg9a* mRNA and protein expression in NCCs. NCCs were treated with 50 or 100 mM ethanol for 24 h. The mRNA (A) and protein (B) expression of *Atg9a* was determined by qRT-PCR or Western Blot, respectively. Data are expressed as fold change over control and represent the mean ± SEM of three separate experiments. *, $p < .05$, **, $p < .01$ vs. control.

phosphate-buffered saline (PBS) and then lysed in a buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate and protease inhibitors (Cocktail, Roche, Indianapolis, IN, USA). The whole cell lysates were centrifuged at $12,000 \times g$ for 10 min at 4 °C, and the proteins in supernatants were separated by 12% SDS-PAGE and then transferred onto PVDF membranes (Millipore, USA). Primary antibodies used for this study were as follows: Mouse monoclonal anti-Neurofilament antibody (CST, Beverly, MA, USA), mouse monoclonal anti-Mash1 antibody (Invitrogen, Waltham, MA, USA), rabbit monoclonal anti-Atg9a antibody (CST, Beverly, MA, USA), rabbit polyclonal anti-LC3B antibody (CST, Beverly, MA, USA), rabbit polyclonal anti-p62 antibody (Abcam, Cambridge, MA, USA), and mouse monoclonal anti-β-actin antibody (Santacruz, Santa Cruz, CA, USA). After incubation with secondary antibodies, the proteins were visualized using a SuperSignal West Femto kit (Thermo Fisher, Waltham, MA, USA) under Gel Doc™ XR Imaging System (Bio-RAD, Hercules, CA, USA). The densitometry of the blots was analyzed using Adobe Photoshop CS software (Adobe Systems, San Jose, CA, USA).

2.4. Immunofluorescence

NCCs from control and treated groups were washed with PBS and then fixed with 4% PFA followed by adding 0.25% TritonX-100 for 10 min. After blocking in 4% fetal bovine serum for 1 h, the cells were incubated with rabbit polyclonal anti-p62 (1: 300, Abcam, Cambridge, MA, USA), rabbit polyclonal anti-LC3B (1:300, CST, Beverly, MA, USA), or mouse monoclonal anti-Neurofilament antibody (CST, Beverly, MA, USA) for 2 h at room temperature, followed by incubation with FITC-conjugated goat anti-rabbit secondary antibody (1: 300, Invitrogen, Waltham, MA, USA) or Alexa Fluor 594-conjuagated donkey anti-mouse antibody (1:500, Thermo Fisher, Waltham, MA, USA) for 1 h at room temperature. Cells were then counterstained with DAPI and photographed under an inverted fluorescence microscope (Olympus IMT-2, Tokyo, Japan).

2.5. Analysis of microRNA expression

The expression of miRNA was analyzed as described previously (Chen et al., 2015). Briefly, total RNA was isolated from NCCs using the *mir*Vana™ miRNA Isolation Kit (Thermo Fisher, Waltham, MA, USA), according to the manufacturer's instructions. Total RNA was reverse-transcribed by 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 (has-miR-34a: RT-000426, Thermo Fisher, Waltham, MA, USA). Quantitative PCR amplification was performed using TaqMan® Universal PCR Master Mix kit (Applied Biosystems, Foster, CA, USA) with a sequence-specific Taqman probe (has-miR-34a: TM-000426, 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-34a was calculated using the ΔΔCT method.

2.6. Cell transfection

For transient transfection of NCCs, control and p62 siRNA (Dharmacon, Lafayette, CO, USA), miR-34a mimic (Ambion, Foster, CA, USA), or miR-34a inhibitor (Ambion, Foster, CA, USA) were transfected into NCCs, respectively, by using Lipofectamine 2000 (Invitrogen, Waltham, MA, USA), according to the manufacturer's instructions. The cells were harvested 24 h after transfection for additional treatment and analysis.

2.7. Dual luciferase reporter assays

miR-34a target sites in the 3′-UTR regions of Atg9a mRNA were predicted by using the online database, Target Scan (http://www.Targetscan.org/), as described previously (Chen et al., 2015). The 3′-UTR of Atg9a containing putative miR34a binding sites were amplified from mouse genomic DNA using the following primers: 5′-GACTACTAGTCAGCCCAGTCCCAGTACTGCCATCTTTGCATCCACCCAAGCTTGCTA-3′; 5′-TAGCAAGCTTGGGTGGATGCAAAGATGGCAGTACTGGGACTGGGCTGACTAGTAGTC-3′ and cloned into pMIR-Luciferase-Report plasmid (Applied Biosystems, Foster, CA, USA). Renilla luciferase pRL-TK control vector was used as a control. The constructs (200 ng of plasmid/well of 24-well plates) were co-transfected with 20 ng Renilla luciferase pRL-TK control vector (Promega, Madison, WI, USA) and 50 nmol of miR-34a mimics or control mimics (Ambion, Austin, TX, USA) into NCCs by Lipofectamine 2000 (Invitrogen, Waltham, MA, USA) according to the manufacturer's instruction. Luciferase activity was measured 48 h after the transfection by 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.

2.8. Statistical analysis

Statistical analyses were performed using GraphPad Prism software (GraphPad, San Diego, CA, USA). All data were expressed as means ± SEM of at least three independent experiments. Comparisons between groups were analyzed by one-way ANOVA. Differences between groups were considered significant at $p < .05$.

3. Results

3.1. Ethanol exposure significantly inhibited neural differentiation of NCCs

To determine whether ethanol can inhibit neural differentiation of NCCs, NCCs pre-treated with BMP-2 for 24 h were treated with 100 mM ethanol for 4, 8, 24 or 48 h. This ethanol concentration was chosen because previous studies have indicated that a peak maternal blood ethanol concentration of 400–500 mg/100 mL (approximately 85–105 mM) is required to induce major malformation with the characteristics of fetal alcohol syndrome in mouse embryos (Dunty Jr et al., 2001; Kotch and Sulik, 1992; Sulik et al., 1981). This relatively high concentration is not beyond that which can be observed in chronic
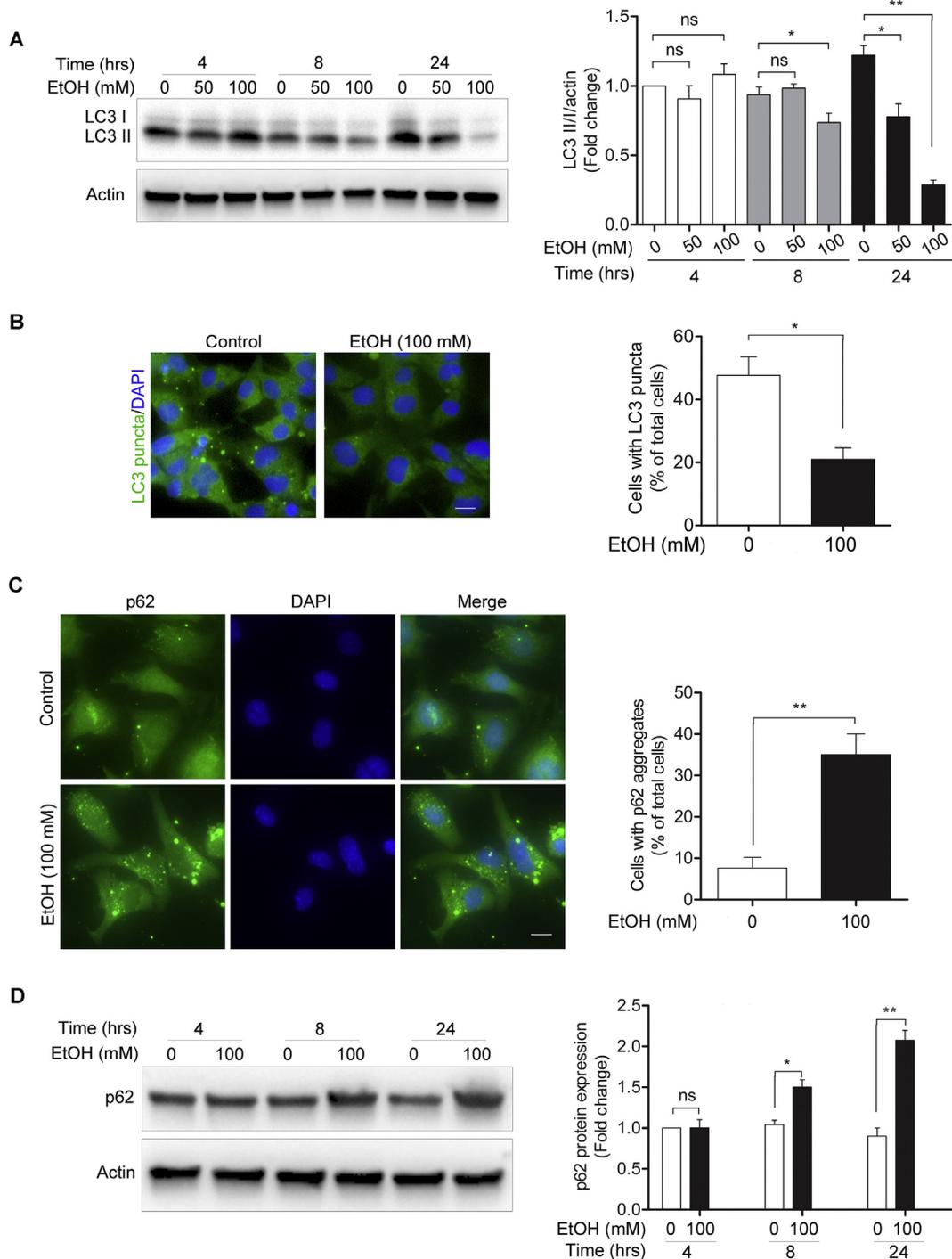
Fig. 3. Ethanol exposure resulted in the inhibition of autophagy and elevated expression of p62 in NCCs. (A) NCCs were treated with 50 or 100 mM ethanol and the expression of LC3 I and LC3 II was determined 4, 8 and 24 h following the ethanol exposure by Western Blot. (B) The formation of autophagosomes was detected by immunofluorescences staining with anti-LC3 antibody (for LC3 puncta). Bar, 10 μm. (C) NCCs were treated with 100 mM ethanol for 24 h and the expression of p62 was determined by immunofluorescence staining with the p62 antibody (left panel) and DAPI (middle panel), respectively. Bar, 10 μm. (D) NCCs were treated with 100 mM ethanol for 4, 8 and 24 h and the expression of p62 was determined by Western Blot. Data are expressed as fold change over control and represent the mean ± SEM of three independent experiments. ns, no statistical difference, *, $p < .05$, **, $p < .01$ vs. control.

alcoholics (Adachi et al., 1991). After 6 days of differentiation in BMP-2-containing medium, the expression of neurofilament (NF), a neuronal marker, was determined by Western blot. As shown in Fig. 1, while exposure of NCCs to ethanol for 4 h did not result in a change in NF expression, ethanol treatment for 8, 24 or 48 h resulted in a significant decrease in NF expression, indicating that ethanol treatment can inhibit neural differentiation of NCCs.

3.2. Ethanol exposure significantly decreased the mRNA and protein expression of Atg9a in NCCs

To examine whether ethanol treatment can inhibit autophagy in NCCs, we first determined whether ethanol exposure can down-regulate Atg9a, a multi-spanning membrane protein essential for autophagy. Using real-time PCR analysis, we found that exposure to 50 or 100 mM ethanol for 24 h resulted in a significant decrease in mRNA expression
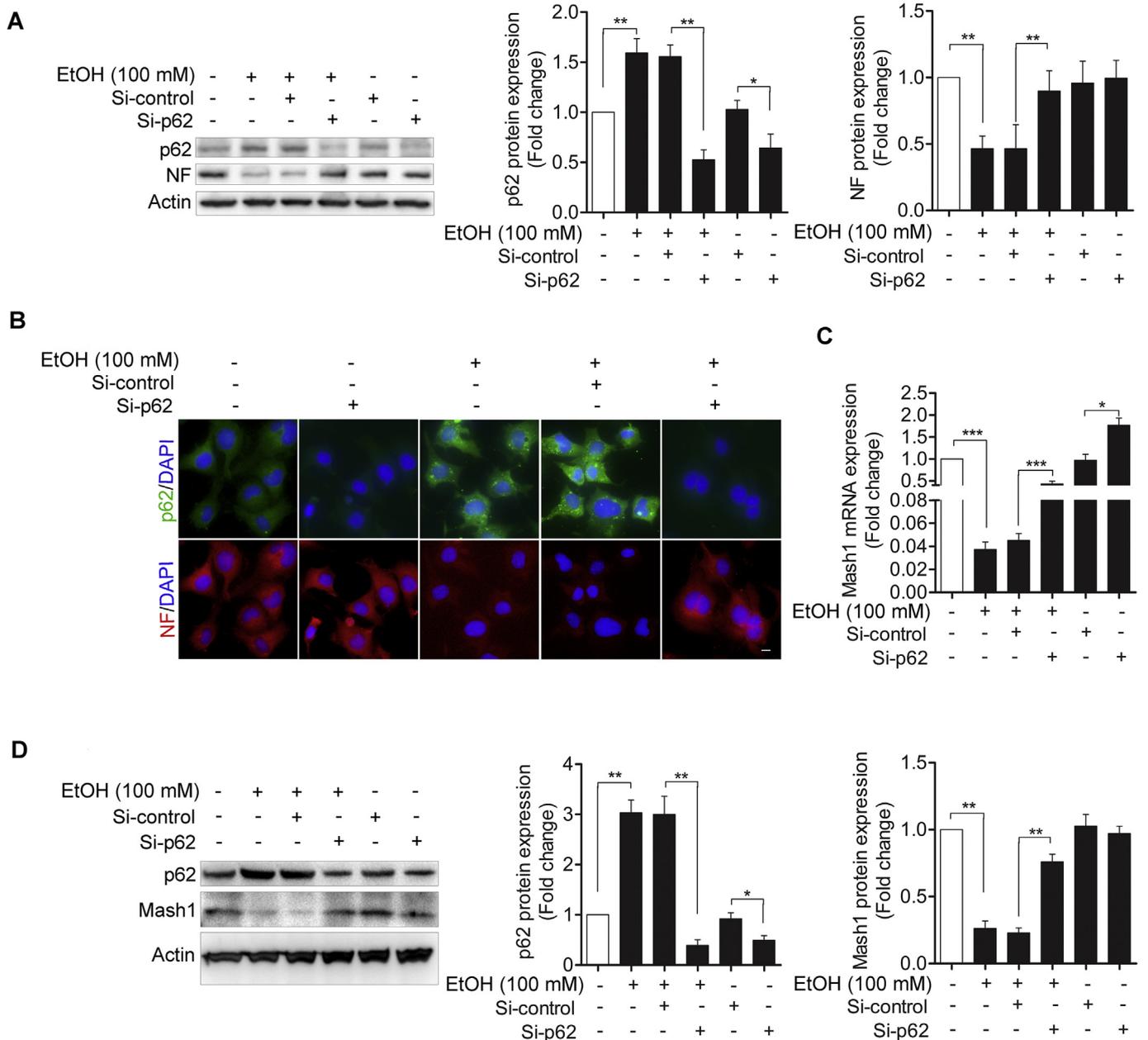
Fig. 4. Knockdown of p62 diminished ethanol-induced inhibition neural differentiation of NCC. NCCs transfected with control siRNA or p62-siRNA were treated with or without 100 mM ethanol for 24 h. The protein expression of NF and p62 were analyzed by Western Blot (A) and immunofluorescence staining (B). Bar, 10 μm. The mRNA (C) and protein (D) expression of Mash1 was determined by RT-PCR and Western Blot, respectively. Data are expressed as fold change over control and represent the mean ± SEM of three separate experiments. *, $p < .05$, **, $p < .01$ vs. control.

of *Atg9a* (Fig. 2A). Ethanol exposure also significantly decreased the protein expression of Atg9a (Fig. 2B). These results demonstrate that ethanol exposure can down-regulate the expression of the gene crucial for the induction of autophagy.

3.3. Ethanol exposure resulted in the inhibition of autophagy and elevated expression of p62 and in NCCs

To determine whether ethanol treatment can inhibit autophagy, the ratio of LC3II/I was analyzed in ethanol-exposed NCCs. LC3 is a soluble protein that is distributed ubiquitously in cells and tissues. During autophagy, LC3I, a cytosolic form of LC3 is converted to LC3-phosphatidylethanolamine conjugate, LC3II. LC3II is then recruited to autophagosomal membranes. The LC3II level is correlated with the number of autophagosomes, and an increased LC3II/I ratio is considered as a

hallmark of autophagy (Fader and Colombo, 2009; Xie and Klionsky, 2007). We found that exposure to 100 mM ethanol for 8 or 24 h resulted in a significant decrease in LC3II/I ratio (Fig. 3A). We also found that treatment with 100 mM ethanol resulted in a significant inhibition of autophagosome formation (Fig. 3B), indicating that ethanol exposure can inhibit autophagy in NCCs. In addition, immunofluorescence and Western Blot analysis showed that exposure to 100 mM ethanol significantly increased the protein expression and aggregation of p62, a selective autophagy substrate, in NCCs (Fig. 3C, D), further confirming that ethanol exposure can inhibit autophagy in NCCs.

3.4. Knockdown of p62 diminished ethanol-induced inhibition of neural differentiation of NCCs

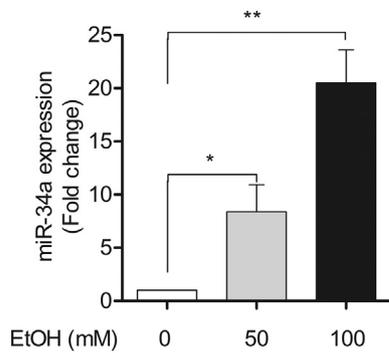To determine whether ethanol-induced inhibition of neural

Fig. 5. Ethanol exposure significantly increased the expression of miR-34a in NCCs. NCCs were treated with 50 or 100 mM ethanol for 24 h. The expression of miR-34a was determined by qRT-PCR. Data are expressed as fold change over control and represent the mean ± SEM of three separate experiments. *, $p < .05$, **, $p < .01$, vs. control.
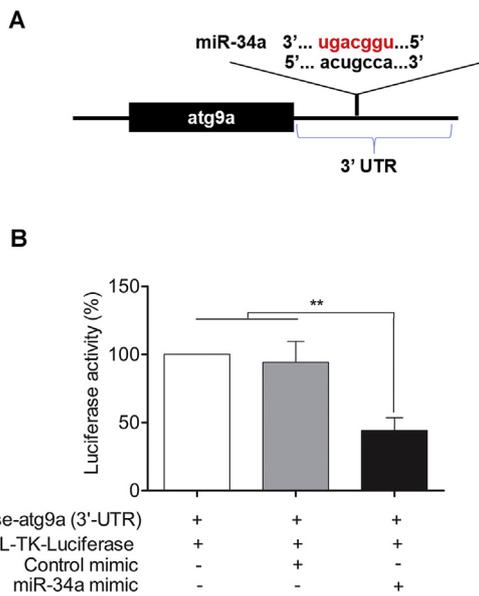

Fig. 6. *Atg9a* is a direct target of miR-34a in NCCs. (A) The predicted binding sites of miR-34a in the 3′-UTR of *Atg9a* mRNA. (B) Luciferase reporter assays validated the binding of miR-34a to the 3′-UTR of *Atg9a* in NCCs. Data are expressed as a percentage of control and represent the mean ± SEM of three separate experiments. **, $p < .01$, vs. control.

differentiation of NCCs was mediated by the elevation of p62 resulting from the inhibition of autophagy, NCCs transfected with p62 siRNA were treated with 100 mM ethanol for 24 h. We found that knockdown of p62 by siRNA significantly diminished ethanol-induced down-regulation of NF (Fig. 4A), which was confirmed by the results from immunofluorescence staining that clearly demonstrated that the cells expressed lower levels of p62 protein have a higher levels of NF (Fig. 4B). Moreover, a neurogenesis marker gene *Mash1* was also robustly restored by knockdown of p62 (Fig. 4C and D), indicating that neural differentiation of NCCs can be restored by reducing the autophagy substrate p62.

3.5. Ethanol exposure significantly increased the expression of miR-34a in NCCs

To investigate whether miR-34a is involved in ethanol-induced inhibition of autophagy and subsequent suppression of neural differentiation of NCCs, miR-34a expression was examined in ethanol-exposed NCCs. As shown in Fig. 5, exposure to 50 or 100 mM ethanol
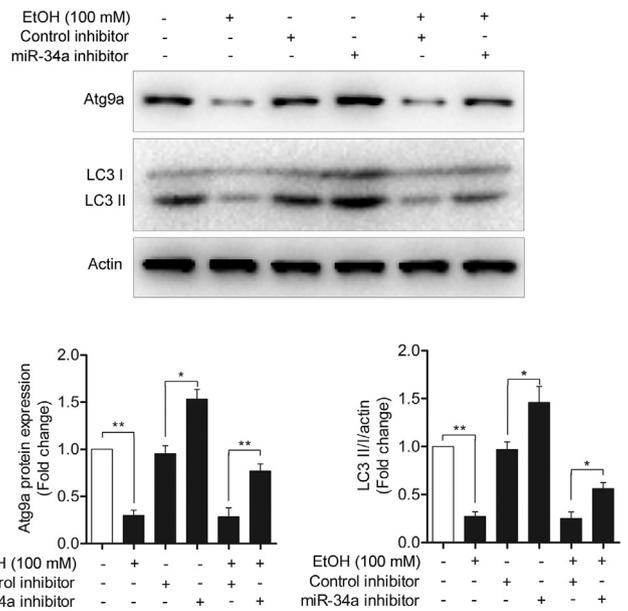

Fig. 7. Inhibition of miR-34a restored the expression of Atg9a and significantly reversed ethanol-induced autophagy inhibition in NCCs. NCCs transfected with control inhibitor or miR-34a inhibitor were treated with 100 mM ethanol for 24 h. The protein expression of Atg9a and LC3 II/I was determined by Western Blot. Data are expressed as fold change over control and represent the mean ± SEM of three separate experiments. *, $p < .05$, **, $p < .01$ vs. control.

significantly increased miR-34a expression in NCCs, indicating that ethanol exposure at the concentrations that can inhibit autophagy and neural differentiation of NCCs can significantly increase the expression of miR-34a.

3.6. Atg9a is a direct target of miR-34a in NCCs

Bioinformatic prediction revealed that *Atg9a* is a direct target of miR-34a. To validate that *Atg9a* is a direct target of miR-34a in NCCs, the 3′-UTR of *Atg9a* mRNA that contains a miR-34a binding site was cloned into the pMIR-Report vector to create a luciferase reporter system. Co-transfection of the 3′-UTR of *Atg9a* mRNA and miR-34a mimic into NCCs resulted in a significant reduction in luciferase activity as compared to the cells co-transfected with *Atg9a* 3′-UTR and a control miRNA (Fig. 6A). In contrast, co-transfection of NCCs with miR-34a mimic and pMIR-Report control did not alter the luciferase activity, validating that *Atg9a* is a direct target of miR-34a in NCCs.

3.7. Inhibition of miR-34a restored the expression of Atg9a and significantly decreased ethanol-induced inhibition of autophagy in NCCs

We next determined whether down-regulation of miR-34a can restore the expression of Atg9a and decrease ethanol-induced inhibition of autophagy in NCCs exposed to ethanol. NCCs transfected with miR-34a inhibitor were treated with 100 mM ethanol for 24 h. We found that down-regulation of miR-34a by miR-34a inhibitor significantly increased the expression of Atg9a in ethanol-treated NCCs. Inhibition of miR-34a expression also diminished ethanol-induced inhibition of autophagy in NCCs, as indicated by an increased LC3II/I ratio (Fig. 7). These results indicate that ethanol-induced up-regulation of miR-34a contributes to the inhibition of autophagy in ethanol-exposed NCCs by targeting *Atg9a*.
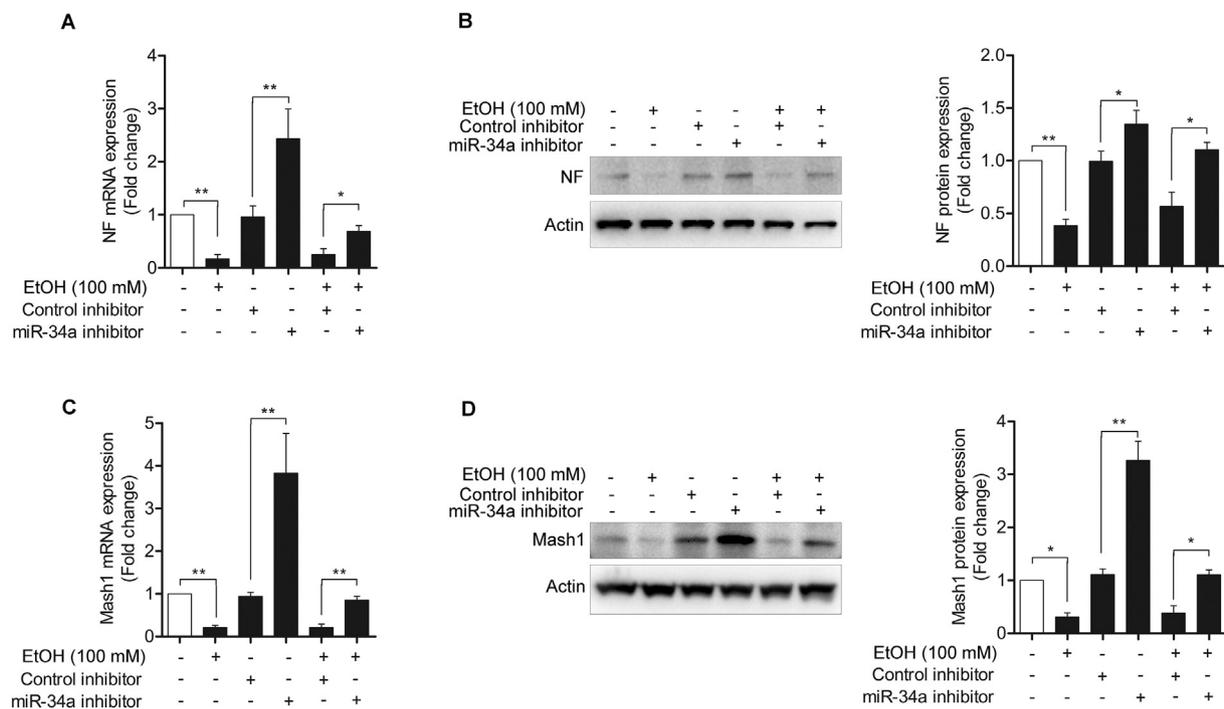
Fig. 8. Down-regulation of miR-34a prevented ethanol-induced inhibition of neural differentiation of NCCs. NCCs transfected with control inhibitor or miR-34a inhibitor were treated with 100 mM ethanol for 24 h. The mRNA expression of *NF* (A) and *Mash1* (C) were analyzed by qRT-PCR. The protein expression of NF and Mash1 were determined by Western Blot (B) and (D). Data are expressed as fold change over control and represent the mean ± SEM of three separate experiments. *, $p < .05$, **, $p < .01$ vs. control.

3.8. Down-regulation of miR-34a prevented ethanol-induced inhibition of neural differentiation of NCCs

To determine the role of miR-34a in modulating ethanol-induced inhibition of neural differentiation of NCCs, NCCs transfected with miR-34a inhibitor were treated with 100 mM ethanol for 24 h. We found that down-regulation of miR-34a significantly increased mRNA expression of the neurogenesis genes, *NF* and *Mash1* (Fig. 8A, C). Treatment with miR-34a inhibitor also resulted in a significant increase in the protein expression of NF and Mash1 in ethanol-exposed NCCs (Fig. 8B, D). These data demonstrate that down-regulation of miR-34a can diminish ethanol-induced inhibition of neural differentiation of NCCs.

4. Discussion

The NCCs are multipotent progenitors that give rise to a diverse cell lineage, including craniofacial cartilage and bone, melanocytes, peripheral and enteric neurons, and glia. While growing evidence demonstrates that ethanol-induced excessive apoptosis in NCCs contributes significantly to craniofacial abnormalities observed in the individuals with FASD, the role of the ethanol-induced impairment of neural differentiation of NCCs in the pathogenesis of FASD has remained elusive. In this study, we have shown that neural differentiation of NCCs was significantly inhibited by ethanol exposure, as indicated by a dramatic decrease in the expression of NF, a neuronal marker. These findings clearly demonstrate that ethanol exposure can impair the neural differentiation of NCCs.

Increasing evidence has shown that autophagy plays a pivotal role in stem cells differentiation. Studies have shown that the depletion of Eva1a, an autophagy-related gene, impaired the generation of newborn neurons both in vivo and in vitro (Li et al., 2016). It has also been reported that an increase in the autophagy-activating genes, including *atg6, atg7,* and *atg8,* was observed in mouse embryonic olfactory bulb (OB) during the period of neuronal differentiation (Vazquez et al., 2012). Activation of autophagy is also required for apoptotic cells

clearance and normal neural differentiation during early otic development and the inhibition of autophagy impaired neurogenesis (Aburto et al., 2012). Furthermore, ablation of *FIP200*, another gene that is essential for autophagy induction, resulted in a progressive loss of neural stem cells and impairment in neuronal differentiation (Wang et al., 2013). Trehalose, an autophagy inducer, can prevent neural tube defects by promoting autophagy-mediated neurogenesis (Xu et al., 2013). In our present study, we found that exposure to ethanol resulted in a significant decrease in mRNA and protein expression of Atg9a, a multi-spanning membrane protein essential for autophagy. Ethanol exposure also resulted in a significant decrease in LC3II/I ratio, indicating that ethanol can inhibit autophagy in NCCs. We also found that ethanol treatment significantly decreased the protein expression and aggregation of p62, a selective autophagy substrate, in NCCs, confirming that ethanol exposure can inhibit autophagy in NCCs.

p62 is a multi-functional scaffold protein that can bind to autophagy regulator LC3 through the LC3-interacting region and be degraded by autophagy (Moscat and Diaz-Meco, 2009). It was reported that the accumulation of p62 impaired neural stem cells differentiation by increasing ROS generation (Wang et al., 2016). In contrast, autophagy-mediated degradation of p62 can promote the differentiation of human embryonic stem cells to neuroectoderm (Jang et al., 2016). In this study, we found that knockdown of p62 by siRNA restored the expression of NF and Mash1 in ethanol-exposed NCCs, indicating that neural differentiation of NCCs can be restored by reducing the autophagy substrate p62. These results demonstrated that abnormal p62 aggregate accumulation caused by the ethanol-induced autophagy inhibition plays a pivotal role in ethanol-induced inhibition of neural differentiation of NCCs.

The key process of autophagy induction or activation is the formation of the intact double-membrane structure which can engulf malfunctioned proteins or organelles and subsequently degrade them through fusing with the lysosome (Imai et al., 2016; Nishimura et al., 2017; Pavel and Rubinsztein, 2017). Among key autophagy-related proteins, Atg9a plays a pivotal role in the assembling of autophagosome

and subsequent autophagy activation (Imai et al., 2016). Recent studies have shown that defective trafficking of Atg9a impaired autophagy and caused neuroaxonal dystrophy (De Pace et al., 2018; Mattera et al., 2017). It has also been reported that Atg9a protein was increased and autophagy was activated during neural differentiation (Morgado et al., 2015). Our present study has shown that ethanol treatment decreased Atg9a protein, inhibited autophagy, and subsequently impaired neural differentiation of NCCs, suggesting that down-regulation of Atg9a contributes to ethanol-induced inhibition of autophagy and neural differentiation of NCCs.

MicroRNAs are small noncoding RNAs that regulate gene expression and play important roles in the regulation of various cellular processes, including apoptosis, proliferation, differentiation, and autophagy. Studies have shown that miR-30a can decrease autophagic activity by targeting autophagy-related gene 6 (*BECN1*) and reducing the expression of *BECN1* and that the down-regulation of *BECN1* by miR-30a mimic diminished the activation of autophagy (Zhu et al., 2009). miR-34a was also found to inhibit autophagy by directly binding to the 3′-UTR of *Atg9a* and inhibiting the expression of *Atg9a* (Yang et al., 2013). In addition, miR-34a has been shown to regulate Ang II-induced cardiomyocyte hypertrophy by decreasing Atg9a expression and autophagic activity (Huang et al., 2014). More importantly, miR-34a has been demonstrated to regulate the neural stem cell differentiation by inhibiting the expression of *Atg9a* (Morgado et al., 2015). Consistent with these studies, we found that miR-34a expression was significantly increased in ethanol-exposed NCCs. Inhibition of miR-34a by miR-34a inhibitor restored the expression of Atg9a, significantly diminished ethanol-induced inhibition of autophagy and prevented ethanol-induced inhibition of neural differentiation of NCCs. Collectively, these data demonstrated that miR-34a contributes to ethanol-induced inhibition of neural differentiation of NCCs by directly targeting *Atg9a*, reducing the expression of Atg9a, and subsequently inhibiting autophagic activity in ethanol-exposed NCCs.

In summary, our studies demonstrated for the first time that ethanol exposure resulted in the inhibition of neural differentiation of mouse NCCs. We have also shown that abnormal p62 aggregate accumulation caused by the ethanol-induced autophagy inhibition plays a pivotal role in ethanol-induced inhibition of neural differentiation of NCCs. In addition, ethanol exposure resulted in a significant increase in miR-34a expression in NCCs. Inhibition of miR-34a restored the expression of Atg9a and significantly decreased ethanol-induced inhibition of autophagy in NCCs. Down-regulation of miR-34a also prevented ethanol-induced inhibition of neural differentiation of NCCs. Together, these results demonstrate that ethanol-induced inhibition of neural differentiation of NCCs is mediated by the miR-34a through inhibiting autophagy. The findings from this study suggest that inhibition of miR-34a by microRNA inhibitor may represent an attractive novel therapeutic target for the intervention and prevention of FASD.

Declaration of Competing Interest

None.

Acknowledgments

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