

## Original Article

# Long non-coding RNA ANRIL mitigates neonatal hypoxic-ischemic brain damage via targeting the miR-378b/ATG3 axis

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**Abstract:** Hypoxic-ischemic brain injury (HIBD) is the most common form of brain injury in newborns and is a major burden on society. However, the molecular mechanism of HIBD remains unclear. Long non-coding RNA (lncRNA) has been demonstrated to be a key regulator in brain development and numerous neurological diseases. The present study identified the role and underlying mechanism of lncRNA antisense non-coding RNA in the INK4 locus (ANRIL) in HIBD. The data indicated that ANRIL expression was significantly increased in hypoxia-stressed primary neurons and PC12 cells. Silencing ANRIL aggravated oxygen-glucose deprivation-induced cell injury. Mechanistically, microRNA (miR)-378b was predicted and confirmed as a direct target of ANRIL. A miR-378b inhibitor counteracted the effect of ANRIL on hypoxia-induced cell injury. Furthermore, ANRIL positively regulated autophagy related 3 (ATG3) expression and promoted autophagy through competitively binding to miR-378b. Overall, the present findings suggest that ANRIL exerts its protective effects via binding to miR-378b and upregulating ATG3 expression, suggesting the potential of ANRIL as a protective target for HIBD.

**Keywords:** Hypoxic-ischemic, non-coding RNA, INK4 locus, microRNA-378b, autophagy related 3

## Introduction

Perinatal hypoxic-ischemic brain injury (HIBD) is the most common form of brain injury in newborns, causing global public health problems and placing a significant burden on the society [1, 2]. Perinatal HIBD leads to high mortality and long-term neurological deficits, including mental retardation, seizures, visual impairment, epilepsy, cerebral palsy and learning disabilities [3, 4]. The pathogenesis of HIBD is closely associated with neurotoxicity, overactive immune/inflammation, calcium accumulation and free radical formation [1, 5, 6]. Although various studies have investigated the mechanism of HIBD, no clinically useful method for HIBD is currently available. Therefore, further research is needed to clarify the pathogenesis of HIBD.

Long non-coding RNA (lncRNA) is a non-coding transcript with >200 nucleotides in length. Previous evidence suggests that lncRNA plays

a key role in certain physiological and pathological processes [7-12]. To date, <1% of all human lncRNAs have been characterized [13]. It has been reported that numerous lncRNAs are involved in the protection against HIBD, including lncRNA Opa interacting protein 5-antisense transcript 1 [14], lncRNA small nucleolar RNA host gene 3 [15], lncRNA nuclear paraspeckle assembly transcript 1 (NEAT1) [16], lncRNA metastasis associated lung adenocarcinoma transcript 1 (MALAT1) [17] and lncRNA maternally expressed 3 (MEG3) [18]. lncRNA antisense non-coding RNA in the INK4 locus (ANRIL) is present in numerous types of human cancer, such as non-small cell lung carcinoma [19, 20], melanoma [21, 22], ovarian cancer [23, 24] and breast cancer [25]. Regarding neuronal diseases, knockdown of lncRNA ANRIL can inhibit neuronal apoptosis in rats with cerebral infarction through inhibiting the NF- $\kappa$ B signaling pathway [26]. However, the role and mechanism of ANRIL in HIBD remains unclear.

MicroRNAs (miRNAs/miRs) are a type of small non-coding RNAs that interfere and regulate the expression of protein-coding genes at the post-transcriptional level. miRNAs are involved in cell proliferation, apoptosis and differentiation [27-29]. It has been reported that certain miRNAs, including miR-181a-5p, let-7a-5p, miR-122-5p, miR-497-5p and miR-378b, are potentially regulated by ANRIL expression [30-34]. miR-378b, a member of the miR-378 family located on chromosome 3, is highly conserved across species, from mouse to human. A previous study has shown that miR-378b is expressed in normal human dermal fibroblasts, and circulating miR-378b is a sensitive biomarker for photo-aging. miR-378b has also been reported to be involved in the regulation of cell differentiation processes [35, 36]. However, the role of miR-378b in the pathogenesis of HIBD remains unclear.

The current study mainly explored the role of ANRIL in neonatal primary neurons and PC12 cells with hypoxic-ischemic injury *in vitro*. The results showed that ANRIL silencing had a detrimental effect on neonatal primary neurons and PC12 cells with hypoxia-ischemia injury. ANRIL silencing decreased the viability and increased the death of neuron cells. Autophagy related 3 (ATG3) is a key regulator in the modulation of autophagy during cell death. The results of the present study further showed that ANRIL regulated ATG3 expression level in neurons by sponging miR-378b.

### Materials and methods

#### Cell culture

Primary hippocampal neurons were purchased from Procell Life Science & Technology Co., Ltd. The hippocampal cells were seeded into 6-well dishes ( $1 \times 10^4$  cells/ml) and cultured in DMEM supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, 2 mM glutamine, B27 and 10% FBS. PC12 cells were obtained from The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences and seeded into 6-well dishes ( $1 \times 10^4$  cells/ml) in culture medium (DMEM, 100 U/ml penicillin, 100 mg/ml streptomycin and 10% FBS). The cells were maintained at 37°C in a humidified incubator containing 5% CO<sub>2</sub>.

#### Oxygen-glucose deprivation (OGD) model

For the OGD model, primary neurons and PC12 cells were cultured in DMEM without serum and

glucose in an atmosphere of 0.1% O<sub>2</sub>, 94.9% N<sub>2</sub> and 5% CO<sub>2</sub> for 24 h, and then incubated in normal DMEM at 37°C in a humidified incubator containing 5% CO<sub>2</sub>.

#### Cell transfection

To knock down lncRNA ANRIL, three siRNAs against ANRIL (si ANRIL) and si-NC were synthesized by Shanghai GenePharma Co., Ltd. miR-378b mimics, miR-378b inhibitors and NCs were purchased from Shanghai GenePharma Co., Ltd. Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) was used to transfect primary neurons and PC12 cells with the original plasmids at the specified concentrations according to the manufacturer's instructions.

#### MTT assay

The viability of primary neurons and PC12 cells was determined using an MTT kit (Roche Applied Science). Briefly, primary neurons or PC12 cells were seeded at a density of  $5 \times 10^4$  cells/200 µl well onto a 96-well plate. After treatment, the cells were incubated with MTT (0.5 mg/ml) at 37°C for 4 h. MTT stock solution (20 µl at 5 mg/ml) was added to each well, and the culture was continued for 5 h. Subsequently, the medium inside each well was gently removed. Next, 150 µl DMSO was added to each well, and the 96-well plate was placed on a shaker and agitated at low speed for 10 min to completely dissolve the crystals. The absorbance of each well was then measured at an optical density of 490 nm.

#### Annexin V/PI detection by flow cytometry

Analysis of cell apoptosis was performed with Annexin V-FITC Apoptosis Detection kit (eBioscience; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Flow cytometry analysis was performed using a FACScan flow cytometer (Beckman Coulter, Inc.). The data were analyzed with FlowJo software (FlowJo LLC).

#### Dual-luciferase reporter assay

To construct the dual-luciferase reporter vector, a fragment from ANRIL or ATG3 containing the predicted miR-378b binding site was amplified and inserted into the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega Corporation) to construct the reporter vector [ANRIL-wild-type (WT) or ATG3-WT]. A QuikChange II Site-Directed Mutagenesis kit

## Neuroprotection of lncRNA ANRIL

(Agilent Technologies, Inc) was used to mutate the binding site of miR-378b in ANRIL or ATG3 to generate ANRIL-mutant (MUT) or ATG-MUT. All vectors and miRNAs were then co-transfected into PC12 cells, and luciferase activity was determined using a Dual-Luciferase Reporter assay system (Promega Corporation) 48 h after co-transfection.

### *Reverse transcription-quantitative PCR (RT-qPCR)*

Total RNA from primary neurons or PC12 cells was extracted using a RNeasy Plus Mini kit (Qiagen, Inc.) according to the manufacturer's instructions. RNA (1 µg) was reverse transcribed into cDNA using a ReverTra Ace qPCR RT Kit (Toyobo Life Science). RT-qPCR for analysis of the expression of the indicated genes was performed with SYBR Green™ Realtime PCR Master Mix Plus (Toyobo Life Science) in an ABI PRISM 7900 system (Applied Biosystems; Thermo Fisher Scientific, Inc.). GAPDH was used for normalization. The data were calculated using the  $2^{-\Delta\Delta C_q}$  method. The primers used in the present study were as follows: ANRIL-forward (F) 5'-GCCTATTCTGATCAACA-3', ANRIL-reverse (R), 5'-TAGAAAGCAGCTGACTCGG-3', miR-378b-F, 5'-GGCTCGAGTATCATGGCATATCAGCAGAAGC-3', miR-378b-R, 5'-TGAATTCTGCATATACCTGATAAGTCACAGG-3', miR-181a-5p-F, 5'-CGCAACATTCAACGCTGTC-3', miR-181a-5p-R, 5'-GTGCAGGGTCCGAGGT-3', let-7a-5p-F, 5'-CGCTGAGGTAGTAGTTGT-3', let-7a-5p-R, 5'-GTGCAGGGTCCGAGGT-3', miR-122-5p-F, 5'-TATTCGCACTGGATACGACACAAAC-3', miR-122-5p-R, 5'-GCCCGTGGAGTGTGACAATGGT-3', miR-497-5p-F, 5'-AGCGAAGTTTTGAGCCGATCGGGC-3', miR-497-5p-R, 5'-GCCGTGAGTCAGAGGTGGT-3', ATG3-F, 5'-ACACGGTGAAGGGAAGGC-3', ATG3-R, 5'-TGTTGGACTAAGTGATCTCCAG-3', GAPDH-F, 5'-CGTGTTCCTACCCCAA-TGT-3' and GAPDH-R, 5'-TGTCATCATACTTGCC-AGGTTTCT-3'.

### *Western blot analysis*

Cells were scraped off from the culture plates on ice and lysed with RIPA lysis buffer containing protease and phosphatase inhibitors (Thermo Fisher Scientific, Inc.). Protein concentrations were determined using a BCA assay (Thermo Fisher Scientific, Inc.). Protein samples were prepared for SDS-PAGE. Proteins were then transferred to a PVDF membrane (BioRad Laboratories, Inc.) for immunoblotting with antibodies against cleaved caspase 3 (1:1,000; Sigma-

Aldrich; Merck KGaA), Bax (1:1,000; Abcam), Bcl-2 (1:1,000; Cell Signaling Technology, Inc.), ATG3 (1:1,000; Abcam) and GAPDH (1:5,000; Santa Cruz Biotechnology, Inc.).

### *ANRIL siRNA-mediated silencing*

Three specific ANRIL siRNAs were designed to target ANRIL, while an universal negative control siRNA was used as a negative control. Lipofectamine® 3000 was purchased from Thermo Fisher Scientific, Inc. and was used as a transfection reagent according to the manufacturer's instructions. The siRNAs used in the present study were as follows: siRNA 1, 5'-GA-ACCAGGACUGGAACCUA-3'; siRNA 2, 5'-GAAUG-UCAGUUUUGAACUA-3'; and siRNA 3, 5'-GGUCA-UCUCAUUGCUCUAU-3'. All the siRNAs contained 3'dTdT overlaps.

### *RNA immunoprecipitation (RIP)*

RIP experiments were performed using a Magna RIP RNA-Binding Protein Immunoprecipitation kit (Millipore) according to the manufacturer's instructions. RIP experiments were conducted on PC12 cells treated with miR-378b inhibitors or negative control, which were transfected into the cells using Lipofectamine® 3000. Cells were harvested after 6 h of incubation, and IP was performed using an anti-argonaute-2 (Ago2) monoclonal antibody and Protein G beads (New England BioLabs, Inc.). After IP, the beads were washed and subjected to western blotting with anti-Ago2 antibodies, as previously described [37].

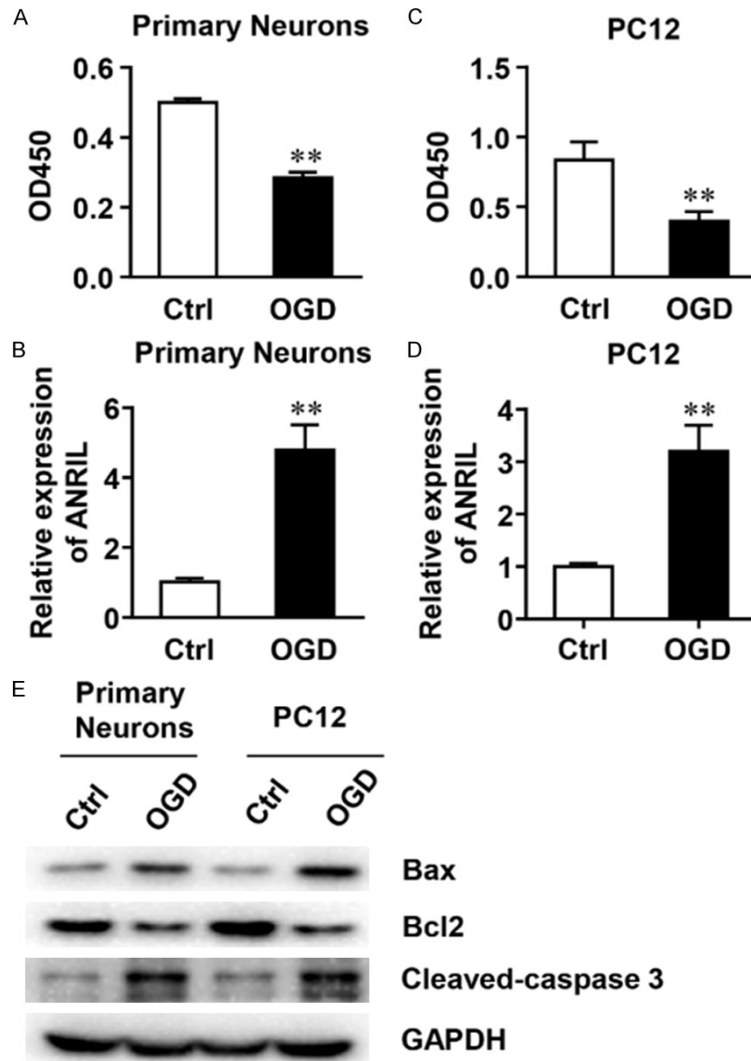
### *Statistical analysis*

Data are expressed as the mean ± SEM. Comparisons between multiple groups were performed with one-way or two-way ANOVA followed by a Bonferroni post hoc test, or by two-tailed Student's t-test using GraphPad Prism version 6.1 software (GraphPad Software, Inc.).  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

### *ANRIL is markedly induced in hypoxia-injured neonatal neurons*

To examine whether ANRIL expression could be affected by hypoxic/ischemic stress, primary neurons were exposed to OGD stress, and cell viability after OGD stimulation was significantly decreased (**Figure 1A**). ANRIL expression was significantly upregulated under OGD condition



**Figure 1.** ANRIL was markedly induced in hypoxia-injured neonatal neuronal cells. (A) Cell viability of primary neurons treated with OGD for 24 h was detected using MTT assay. (B) Relative expression of lncRNA ANRIL in primary neurons treated with OGD for 24 h. (C) Viability of PC12 cells treated with OGD for 24 h, as detected using MTT assay. (D) Relative expression of lncRNA ANRIL in PC12 cells treated with OGD for 24 h. E. Western blot analysis of Bax, Bcl-2, cleaved caspase 3 and GAPDH protein expression in primary neurons and PC12 cells under normoxia and OGD conditions. \* $P < 0.05$ , \*\* $P < 0.01$  vs. the control group. Data represent the mean  $\pm$  SEM of three independent experiments. Comparisons between two groups were performed using a two-tailed Student's t-test. ANRIL, antisense non-coding RNA in the INK4 locus; OGD, oxygen-glucose deprivation; lncRNA, long non-coding RNA.

(Figure 1B). PC12 cells were also exposed to OGD, which reduced the viability of PC12 cells (Figure 1C) and significantly upregulated the expression of ANRIL (Figure 1D).

Western blot analysis of the expression levels of apoptosis markers revealed that OGD stimulation decreased Bcl-2 levels, while increased

those of Bax and cleaved caspase 3 (Figure 1E), which indicated the successful induction of the *in vitro* OGD model. These results suggest that hypoxic/ischemic stimulation increased ANRIL expression in neonatal neurons *in vitro*.

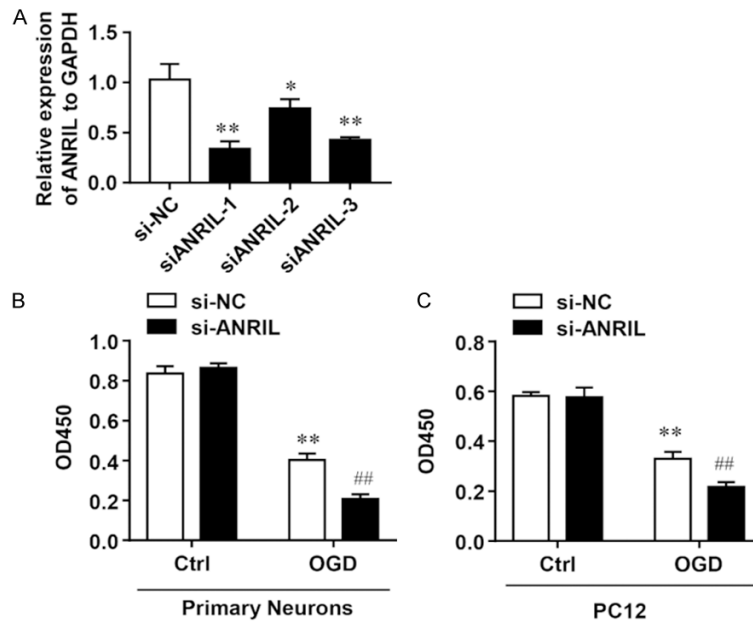
#### Knockdown of ANRIL exacerbates hypoxia-induced neuronal cell injury

Next, we investigated the effect of the ANRIL knockdown on neuronal function *in vitro*. Three different ANRIL siRNAs were compared, and it was found that transfection with si-ANRIL significantly reduced ANRIL levels in primary neurons (Figure 2A). Therefore, si-ANRIL-1 was selected for further experiments.

MTT assays demonstrated that, in response to hypoxic stress, knockdown of ANRIL by si-ANRIL-1 significantly exacerbated the OGD-induced decrease in viability of primary neurons and PC12 cells (Figure 2B and 2C). Therefore, the present data suggest that ANRIL can protect primary neurons from hypoxic/ischemic stress *in vitro*.

#### ANRIL sponges miR-378b in neonatal neuronal cells

We then explored the mechanism of ANRIL-mediated neuronal protection. lncRNAs can act as 'sponges' to regulate the expression of miRNAs. According to previous studies, there are several targeted miRNAs that are potentially regulated by ANRIL expression, including miR-181a-5p, let-7a-5p, miR-122-5p, miR-497-5p and miR-378b [30-34]. Cells were transfected with mimics of these miRNAs, and miR-378b had the most significant effect on the expression of ANRIL (Figure 3A).



**Figure 2.** Knockdown of ANRIL exacerbates hypoxia-induced neuronal cell injury. (A) Reverse transcription-quantitative PCR analysis of ANRIL expression in primary neurons transfected with three different ANRIL siRNAs. (B and C) MTT assays of OGD-induced (B) primary neurons and (C) PC12 cells transfected with ANRIL siRNA. \* $P < 0.05$ , \*\* $P < 0.01$  vs. the si-NC + control group; ## $P < 0.01$  vs. the si-NC + OGD group. Data represent the mean  $\pm$  SEM of three independent experiments. Comparisons between multiple groups were performed using one-way ANOVA. ANRIL, antisense non-coding RNA in the INK4 locus; siRNA, short interfering RNA; OGD, oxygen-glucose deprivation; NC, negative control.

The binding of ANRIL and human or rat miR-378b was predicted using miRanda software (Figure 3B). To further evaluate the potential association between miR-378b and ANRIL, miR-378b inhibitor or mimics were transfected into cells to reduce or increase, respectively, the expression of miR-378b. Inhibition of miR-378b expression resulted in significant upregulation of ANRIL, while overexpression of miR-378b significantly reduced the expression of ANRIL (Figure 3C and 3D). These results indicated that ANRIL could act as a sponge by specifically binding to miR-378b in neurons.

#### *Inhibition of miR-378b eliminates the injury-promoting effect of ANRIL depletion on hypoxic neonatal neurons*

To confirm whether ANRIL improves the damage caused by hypoxia via sponging miR-378b, a miR-378b inhibitor or the corresponding scramble control was co-transfected into PC12 cells after silencing ANRIL with siRNA. The effect of miR-378b on cell viability and apopto-

sis induced by OGD was studied in PC12 cells. As shown in Figure 4A, inhibition of miR-378b with miR-378b inhibitors restored the decrease in cell viability and the increase in apoptosis induced by ANRIL knockdown (Figure 4A). In addition, suppression of miR-378b by the miR-378b inhibitor reduced the notable increase in cell apoptosis induced by ANRIL knockdown (Figure 4B). Taken together, these results indicate that ANRIL exerts its effect on OGD injury by sponging miR-378b.

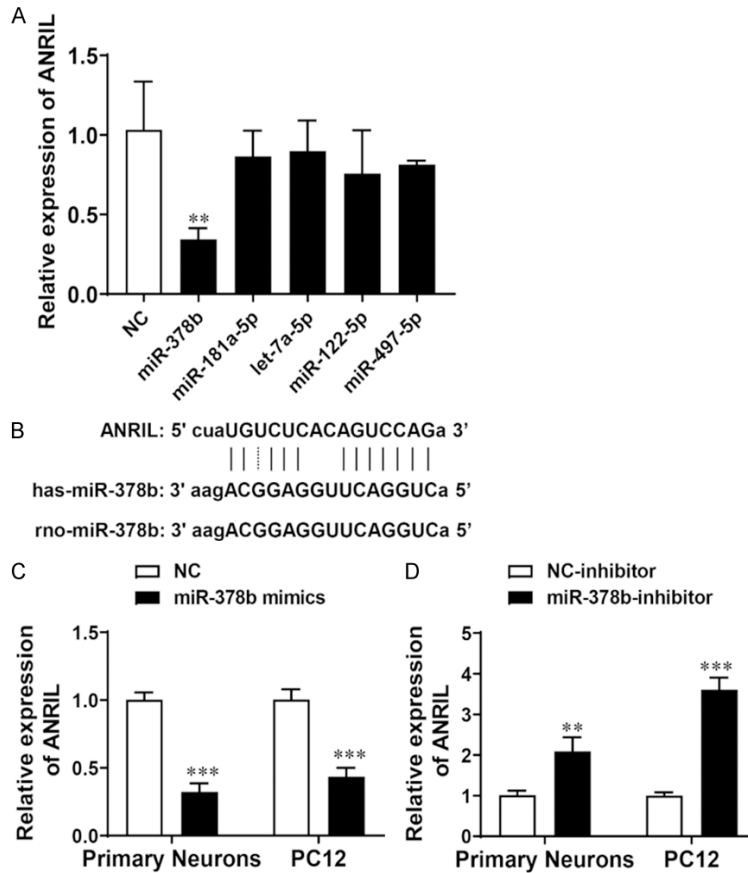
#### *ANRIL 3'-untranslated region (UTR) is a direct target of miR-378 and binds to miR-378b-RNA-induced silencing complex (RISC)*

A dual-luciferase reporter assay was used to confirm whether miR-378b can bind to lncRNA ANRIL. A putative binding site for miR-378b was identified in the 3'-UTR of lncRNA

ANRIL (Figure 5A). Compared with the findings in the NC-mimic group, miR-378b-mimic reduced the luciferase activity of a reporter gene containing a WT lncRNA binding site. By contrast, miR-378b mimics had little influence on the reporter that contained MUT lncRNA binding sites (Figure 5B).

To confirm this result, a dual-luciferase reporter assay was used with miR-378-inhibitor/NC-inhibitor instead of miR-378-mimic/NC-mimic to verify whether miR-378 could target lncRNA ANRIL. Notably, miR-378-inhibitor increased the luciferase activity of reporters harboring an ANRIL-WT binding site compared with that of the NC-inhibitor group, while miR-378-inhibitor had little effect on the reporter containing ANRIL-MUT binding site (Figure 5C). Collectively, these results reveal that the 3'-UTR of ANRIL is a direct target of miR-378.

Numerous reports have shown that miRNAs exist as miRNA ribonucleoprotein complexes



**Figure 3.** ANRIL sponges miR-378b in neonatal neuronal cells. (A) RT-qPCR analysis of ANRIL expression in cells transfected with miRNA mimics, which were predicted to bind to ANRIL with high scores. ANRIL level was normalized to GAPDH expression. (B) Sequence complementarity of ANRIL and miR-378b. The short vertical lines indicate complementary nucleotides. Expression of ANRIL after transfection with (C) miR-378b-mimic or (D) miR-378b-inhibitor was analyzed using RT-qPCR. Data represent the mean  $\pm$  SEM of three independent experiments. \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . Comparisons between multiple groups were performed using one-way ANOVA. ANRIL, antisense non-coding RNA in the INK4 locus; miR, microRNA; RT-qPCR, reverse transcription-quantitative PCR.

that contain Ago2, a key component of RISC [38, 39]. To investigate whether ANRIL binds to miR-378b, RIP experiments were performed on PC12 cells transfected with miR-378b inhibitors or NC. Ago2 protein was efficiently immunoprecipitated by the anti-Ago2 antibody (**Figure 5D**). RIP analysis showed that, although ANRIL was detected in the Ago2-immunoprecipitated fraction of the control group, its level was greatly reduced in the purified Ago2 complex of cells transfected with miR-378b inhibitor (**Figure 5E**), indicating that ANRIL likely binds to miR378b-RISC.

### ANRIL mitigates neonatal HIBD via sponging miR-378b and upregulating ATG3

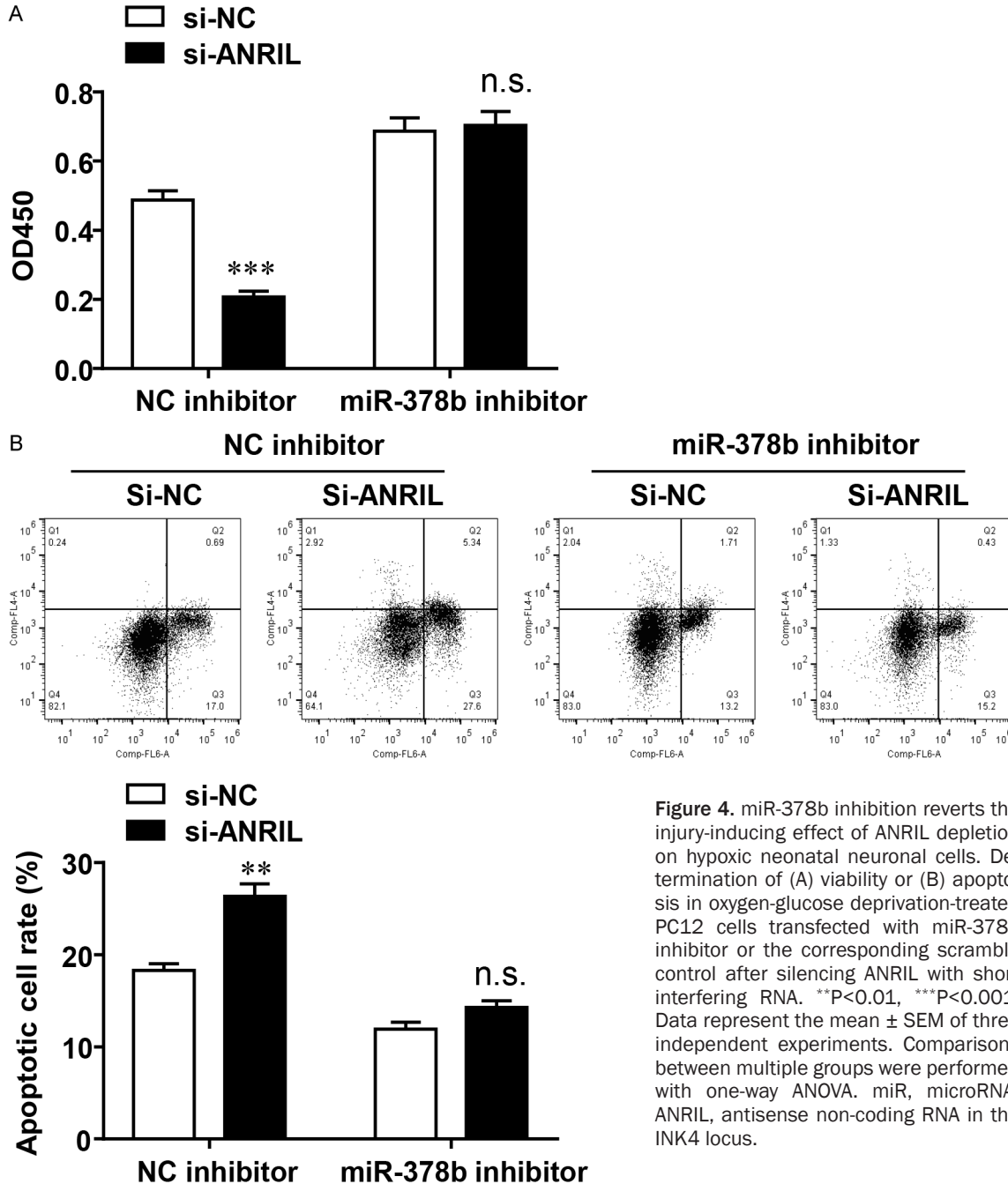
The present study used the TargetScan database to predict potential target genes of miR-378b. One of the candidates, ATG3 (**Figure 6A**), is a ubiquitin-like binding enzyme that forms part of the ubiquitin-like system involved in autophagy. ATG3 is known to play an important role in the regulation of autophagy during cell death.

In the present OGD-injury model of neonatal neuronal cells, the mRNA and protein expression levels of ATG3 were significantly upregulated (**Figure 6B and 6C**). Transfection with miR-378b mimic also reduced the luciferase activity of reporter constructs containing the ATG3 target sequence (**Figure 6D**). ANRIL transfection with miR-378b mimic reduced this decrease in luciferase activity (**Figure 6D**). miR-378b mimic transfection inhibited ATG3 expression at both the mRNA and protein levels, while ANRIL abolished the ATG3 inhibition induced by miR-378b (**Figure 6E and 6F**). miR-378b mimic transfection also reduced the luciferase activity of

reporter constructs containing the ATG3 target sequence, while ANRIL abolished the effect of miR-378b on ATG3 expression (**Figure 6E and 6F**). Knockdown of ANRIL by siRNA transfection also inhibited ATG3 expression in PC12 cells (**Figure 6E and 6F**). Thus, it was concluded that ANRIL/miR-378b/ATG3 crosstalk is involved in regulating neuronal survival in hypoxic/ischemic damage.

### Discussion

It has been reported that lncRNA ANRIL influences multiple physiological and pathological

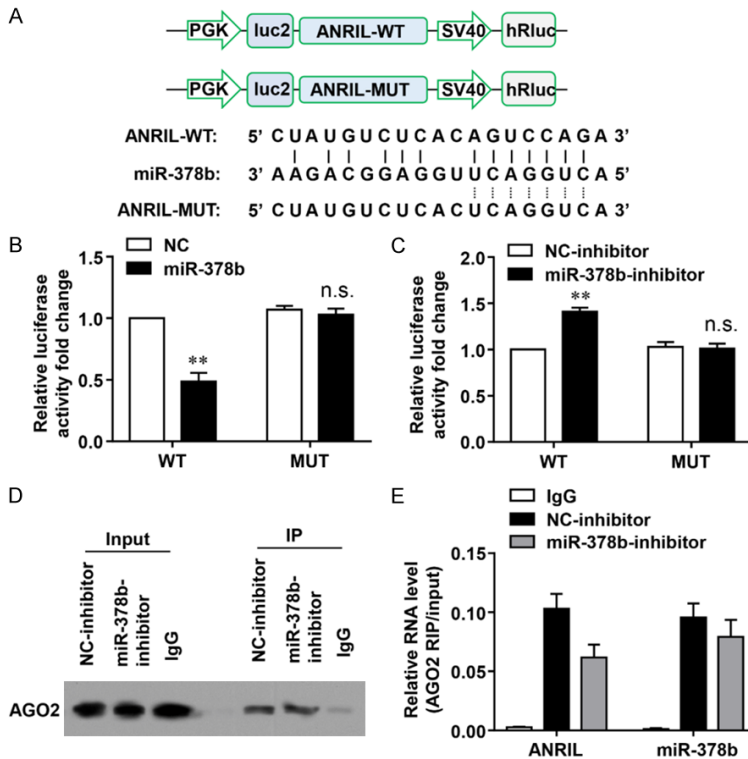


**Figure 4.** miR-378b inhibition reverts the injury-inducing effect of ANRIL depletion on hypoxic neonatal neuronal cells. Determination of (A) viability or (B) apoptosis in oxygen-glucose deprivation-treated PC12 cells transfected with miR-378b inhibitor or the corresponding scramble control after silencing ANRIL with short interfering RNA. \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . Data represent the mean  $\pm$  SEM of three independent experiments. Comparisons between multiple groups were performed with one-way ANOVA. miR, microRNA; ANRIL, antisense non-coding RNA in the INK4 locus.

processes, including those within cardiovascular diseases and tumors [20, 23-25, 40, 41]. However, to the best of our knowledge, the role and mechanism of ANRIL in HIBD remain unclear. In the present study, the effect and mechanism of lncRNA ANRIL on OGD-induced neuronal cell damage were investigated. Inhibiting ANRIL expression reduced the viability and promoted the apoptosis of OGD-treated neuronal cells. Additional results indicated that ANRIL acted as a sponge for miR-378b, and its

inhibitory effect aggravated the OGD-induced damage to primary neuronal cells by upregulating miR-378b. Furthermore, it was found that ATG3 was a target of miR-378b, while ATG3 levels were negatively correlated with miR-378b levels and positively correlated with ANRIL levels.

lncRNAs modulate cell survival, viability and death. Numerous studies have indicated that lncRNAs are involved in a variety of human dis-



**Figure 5.** ANRIL's 3'-untranslated region is a direct target for miR-378, and ANRIL binds to miR-378b-RNA-induced silencing complex. (A) Prediction of the binding site between ANRIL and miR-378b. (B) Dual-luciferase reporter assay showing the luciferase activity of ANRIL-WT/MUT in cells transfected with control (miR-NC) or miR-378b. (C) Dual-luciferase reporter assay showing the luciferase activity of ANRIL-WT/MUT in cells transfected with control (miR-NC-inhibitor) or miR-378-inhibitor. (D) Ago2 protein immunoprecipitated by anti-Ago2 antibody or IgG was detected using western blotting. (E) Quantity of ANRIL and miR-378b bound to Ago2 or IgG was determined using reverse transcription-quantitative PCR in the presence of miR-378b inhibitor or NC. \*\* $P < 0.01$ . Data represent the mean  $\pm$  SEM of three independent experiments. Comparisons between multiple groups were performed with one-way ANOVA. ANRIL, antisense non-coding RNA in the INK4 locus; miR, microRNA; WT, wild-type; MUT, mutant; NC, negative control; Ago2, argonaute-2.

eases, including cardiovascular diseases, cancer and neurodegenerative diseases. Recently, it has been confirmed that lncRNAs play a basic role in the development of the brain and central nervous system, neuronal function and maintenance, and progression of the majority of neurodegenerative diseases. For example, lncRNA NEAT1 binds to miR-339-5p to increase homeobox A1 and reduce ischemic brain damage in newborn mice [16]. lncRNA MALAT1 inhibits miR-429 by regulating the apoptosis of hippocampal neurons in HIBD [17]. lncRNA MEG3 sponges miR-129-5p and weakens the cerebral protection of dexmedetomidine against HIBD in neonatal mice [18]. However, the role of

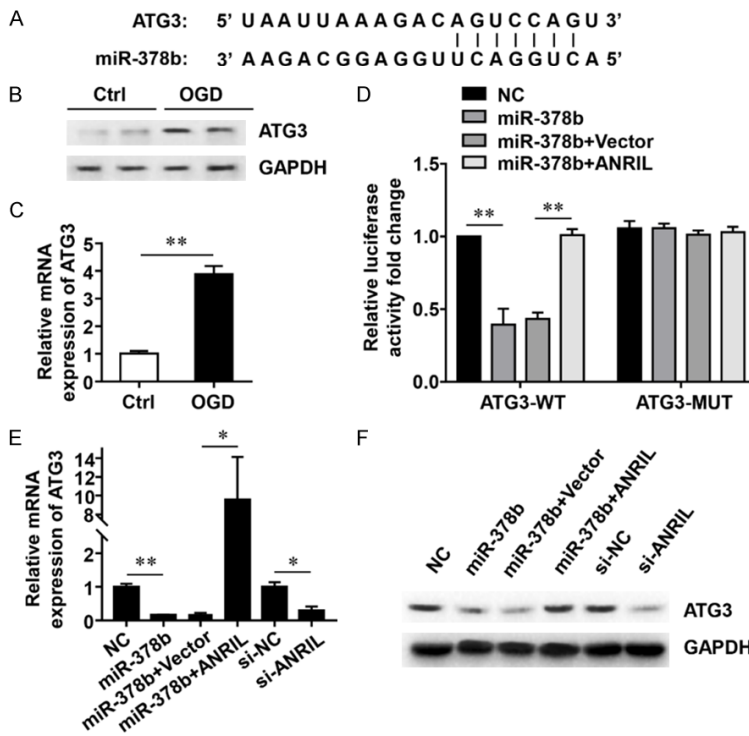
lncRNAs in HIBD remains unclear. The present study first evaluated the role and mechanism of lncRNA ANRIL in the OGD-induced hypoxic/ischemic injury of primary neurons and PC12 cells.

The lncRNA-miRNA-mRNA network has been studied in multiple diseases, including brain damage. lncRNA small nucleolar RNA host gene 3 acted as a sponge for miR-196 in neuronal cells by regulating X-linked inhibitor of apoptosis protein and caspase activity and apoptosis inhibitor 1, and protected HIBD *in vivo* and *in vitro* [15]. lncRNA MEG3 acts as a sponge for miR-21, thus regulating the PI3K/AKT and NF- $\kappa$ B signaling pathways and protects the survival of neuronal cells [42]. The lncRNA Gm-4419/miR-4661/TNF- $\alpha$  network contributes to inhibiting inflammatory damage and trauma-induced astrocyte apoptosis during traumatic brain injury [43]. In the current study, suppression of ANRIL was found to promote OGD-induced injury of primary neurons and PC12 cells by decreasing cell viability and promoting apoptosis. The present study identified that ANRIL acted as

a sponge of miR-378b, and its suppression aggravated OGD-induced injury in primary neurons and PC12 cells through upregulating miR-378b.

Notably, the present study found that ATG3 was a target of miR-378b, and the level of ATG3 was negatively correlated with the miR-378b level, while being positively correlated with the ANRIL level. It is well known that ATG3 is an important regulator of autophagy during cell damage [44, 45]. Although autophagy and apoptosis can be determined separately [46, 47], autophagy has been shown to promote caspase-independent cell death and regulate caspase-mediated ap-





**Figure 6.** ANRIL mitigates neonatal hypoxic-ischemic brain injury by sponging miR-378b and upregulating ATG3. (A) Prediction of the binding site between miR-378b and ATG3. (B and C) Western blot analysis of ATG3 expression of PC12 cells treated with normal conditions or oxygen-glucose deprivation. (D) Dual-luciferase reporter assay showing the luciferase activity of NC miRNA, miR-378b mimic, miR-378b mimic + vector of lncRNA and miR-378b mimic + ANRIL in cells transfected with ATG3-WT or ATG3-MUT. (E) Quantitative PCR analysis of ATG3 mRNA expression in PC12 cells transfected with NC miRNA, miR-378b mimic, miR-378b mimic + vector of lncRNA, miR-378b mimic + ANRIL, si-NC and si-ANRIL. (F) Western blot analysis of ATG3 protein expression in PC12 cells transfected with NC miRNA, miR-378b mimic, miR-378b mimic + vector of lncRNA, miR-378b mimic + ANRIL, si-NC and si-ANRIL. \*P<0.05, \*\*P<0.01. Data represent the mean ± SEM of three independent experiments. Comparisons between multiple groups were performed with one-way ANOVA. ANRIL, antisense non-coding RNA in the INK4 locus; miR or miRNA, microRNA; ATG3, autophagy related 3; WT, wild-type; MUT, mutant; NC, negative control; lncRNA, long non-coding RNA; siRNA, short interfering RNA.

optosis in certain cases [48, 49]. In addition, autophagy and apoptosis share common regulators, such as calcium overload, the presence of reactive oxygen species and endoplasmic reticulum stress [50]. ERK activity has been reported to promote intrinsic or extrinsic apoptotic pathways by triggering the release of mitochondrial cytochrome c [51]. Therefore, lncRNA ANRIL may regulate the apoptosis of neurons via miR-378b and upregulation of ATG3, which may constitute the underlying mechanism of ANRIL in the regulation of neuronal apoptosis.

In conclusion, the present study demonstrated an increase in lncRNA ANRIL in OGD-induced hypoxia/ischemic injury of primary neurons and PC12 cells, and confirmed the interaction between lncRNA ANRIL and miR-378b. ANRIL knock-down increased the apoptosis of primary neurons and PC12 cells via the miR-378b/ATG3 pathway. These findings provide novel insights into therapeutic strategies that may be beneficial for HIBD.

#### Acknowledgements

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#### Disclosure of conflict of interest

None.

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