● RESEARCH ARTICLE

Altered microRNA expression profiles in a rat model of spina bifida

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Graphical Abstract
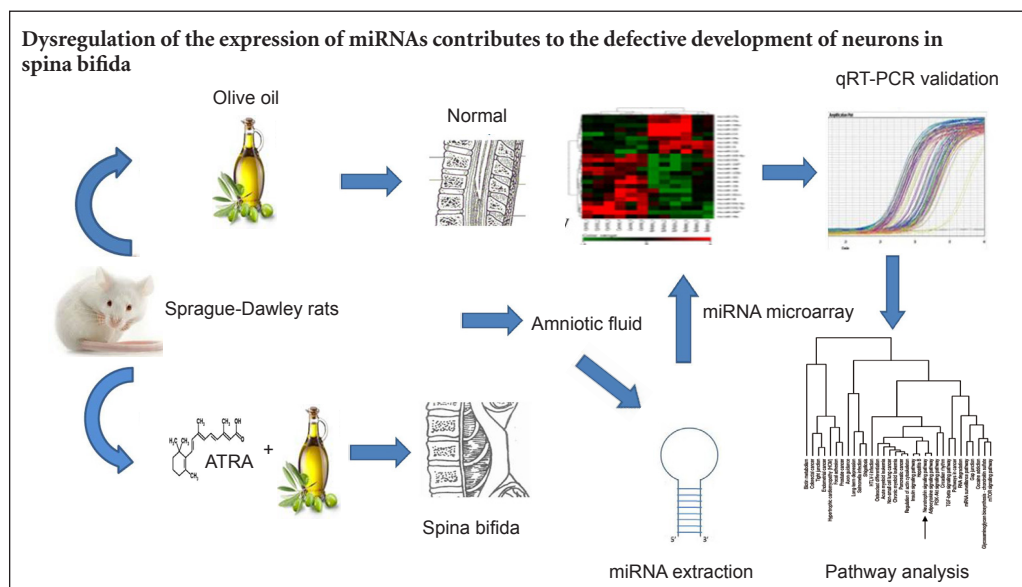
Dysregulation of the expression of miRNAs contributes to the defective development of neurons in spina bifida

Abstract

MicroRNAs (miRNAs) are dynamically regulated during neurodevelopment, yet few reports have examined their role in spina bifida. In this study, we used an established fetal rat model of spina bifida induced by intragastrically administering olive oil-containing all-trans retinoic acid to dams on day 10 of pregnancy. Dams that received intragastric administration of all-trans retinoic acid-free olive oil served as controls. The miRNA expression profile in the amniotic fluid of rats at 20 days of pregnancy was analyzed using an miRNA microarray assay. Compared with that in control fetuses, the expression of miRNA-9, miRNA-124a, and miRNA-138 was significantly decreased (> 2-fold), whereas the expression of miRNA-134 was significantly increased (> 4-fold) in the amniotic fluid of rats with fetuses modeling spina bifida. These results were validated using real-time quantitative reverse-transcription polymerase chain reaction. Hierarchical clustering analysis of the microarray data showed that these differentially expressed miRNAs could distinguish fetuses modeling spina bifida from control fetuses. Our bioinformatics analysis suggested that these differentially expressed miRNAs were associated with many cytological pathways, including a nervous system development signaling pathway. These findings indicate that further studies are warranted examining the role of miRNAs through their regulation of a variety of cell functional pathways in the pathogenesis of spina bifida. Such studies may provide novel targets for the early diagnosis and treatment of spina bifida.

Key Words: nerve regeneration; spina bifida; amniotic fluid; all-trans retinoic acid; microarray; microRNA; reverse transcription-polymerase chain reaction; MAPK; neural regeneration

Introduction

Neural tube defects (NTDs) are a group of severe congenital malformations of the central nervous system resulting from failure of the neural tube to close during neurulation between 21 and 28 days post conception (Botto et al., 1999). NTDs are a significant cause of fetal mortality and morbidity globally, with an approximate incidence of 0.5–2 per 1,000 births (Greene et al., 2009). In the rural areas of the north-ern provinces of China, the prevalence of NTDs at birth is approximately 6 per 1,000 births (Moore et al., 1997). NTDs are multi-factorial in origin, arising from a combination of genetic and environmental factors (Frey and Hauser, 2003; Padmanabhan, 2006). One of the most common NTDs is spina bifida, a malformation of the spinal column characterized by herniation or exposure of the spinal cord through incompletely closed vertebrae. Even after fetal surgery to repair
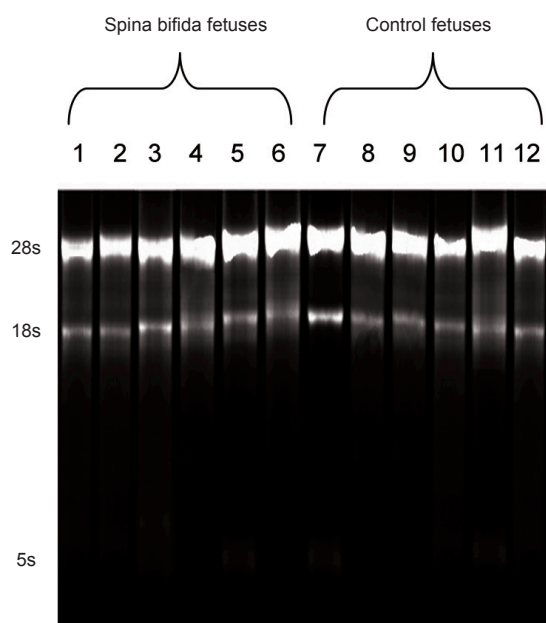
Figure 1 Total RNA electrophoresis.
Lanes 1–6 are results from rats with fetuses modeling spina bifida, and lanes 7–12 are from control rats with normal fetuses.

Table 1 miRNA differentially expressed in the amniotic fluid of rats with fetuses modeling spina bifida and control rats with normal fetuses

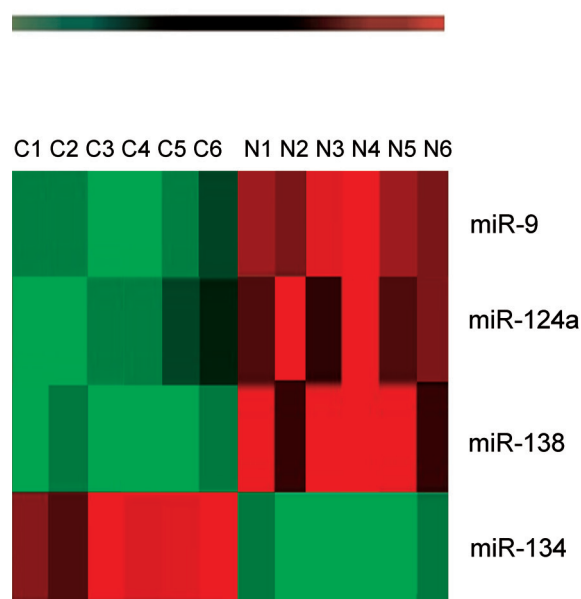| No.	miRNA	Mean fold change (log2 fold change)	P value
1	miR-134	4.09	0.0151
2	miR-221	1.87	0.0069
3	miR-222	1.68	0.0051
4	miR-34a	1.31	0.0068
5	miR-9	−3.49	0.0394
6	miR-124a	−2.75	0.0178
7	miR-138	−2.52	0.0076
8	miR-21	−1.4	0.0020
9	miR-181c	−1.27	0.0096
10	miR-182	−1.25	0.0411
11	miR-130a	−1.12	0.0260

Figure 2 Hierarchical clustering of miRNAs differentially expressed in the amniotic fluid of rats with fetuses modeling spina bifida and control rats with normal fetuses based on results of a microarray analysis.
Each row represents an miRNA, and each column represents a sample. The miRNA clustering tree is shown on the left, and the sample clustering tree appears across the top. The color scale shown at the top illustrates the relative expression level of an miRNA, in which red represents a high expression level and green represents a low expression level. C1–C6 identify rats with fetuses modeling spina bifida and N1–N6 identify control rats with normal fetuses.

the defect, patients show neurological deficits of varying degrees, such as sensory and motor weakness in the legs as well as bowel and bladder incontinence, and they often require long-term care and assistance (Date et al., 1993). Despite years of epidemiologic and experimental studies, the mechanisms responsible for spina bifida remain largely unknown.

Endogenous, small non-coding RNAs approximately 22 nucleotides in length are called microRNAs (miRNAs). They constitute a class of negative regulators of gene expression, either inhibiting translation or inducing degradation of their target messenger RNA (mRNA), resulting in complete or incomplete binding to the 3′ untranslated region of their target mRNAs. This class of regulators reportedly plays an important role in a vast range of biological processes, such as proliferation, differentiation, and apoptosis (Esquela-Kerscher and Slack, 2006; Gu et al., 2009). A number of miRNA profiling studies have shown the expression of miRNAs during neural stem cell differentiation and morphological development of mammalian brain changes (Miska et al., 2004; Croce and Calin, 2005). However, few studies (Wei et al., 2013; Wu et al., 2013) have reported on the role of miRNA in spina bifida, and none have investigated a mechanism for the miRNA involvement. Additionally, these previous studies are limited to individual miRNA and, hence, lack a global level analysis.

The congenital rat spina bifida model induced by administration of all-trans retinoic acid (ATRA), a metabolic product of vitamin A, is well established. In many respects, this model shows remarkable similarities to spina bifida generated by vitamin A deficiency in humans; thus, it is widely used in research of spina bifida. In the present study, we used miRNA microarray and real-time qRT-PCR analyses to characterize differentially expressed miRNAs in amniotic fluid samples obtained from rats with fetuses modeling spina bifida induced by ATRA and from control rats with normal fetuses. We aimed to clarify whether dysregulation of the expression of miRNAs contributes to the defective development of neurons in spina bifida.

Materials and Methods

Establishment of the spina bifida model
Adult Sprague-Dawley rats (10–12 weeks old; weighing 250–

300 g) were purchased from Henan Provincial Laboratory Animal Center (license No. SYXK 2010-0001) in China. All animal experiments followed the policies and procedures recommended by the committee of animal research and ethics and approved by the ethics committee at Zhengzhou University, China. Female rats were mated with male rats overnight. The appearance of a vaginal plug the morning after mating was considered embryonic day 0 (E0). Spina bifida was induced with a single intragastric injection of ATRA (40 mg/mL in olive oil; 150 mg/kg body weight; Sigma, St. Louis, MO, USA) on E10 (n = 6 rats). Rats receiving the control treatment were injected with 4 mL/kg body weight of ATRA-free olive oil (Sigma) on the same day (n = 6 rats).

Sample collection

Pregnant rats were killed on E20 using an overdose of 10% chloral hydrate injected into the abdominal cavity, and the fetuses were harvested. After examining the fetuses for deformities under a microscope (Nikon SMZ1500 stereoscopic microscope, Nikon, Japan), amniotic fluid samples from both groups of pregnant rats were immediately centrifuged at 1,200 × g for 10 minutes, aliquoted in 1.5 mL Eppendorf tubes, and stored at −80°C for use in RNA extractions.

RNA isolation

Total amniotic fluid RNA was isolated using a mirVana PARIS miRNA isolation kit (Ambion, Foster City, CA, USA) according to the manufacturer's protocol. All RNAs were stored at −80°C. RNA purity was assessed using 260/280 nm and 260/230 nm absorbance ratios, with a 260/280 nm absorbance ratio > 1.8 and a 260/230 nm absorbance ratio > 1.5 considered high purity. RNA integrity was determined using the 28s:18s RNA ratio obtained from electrophoresis analysis with agarose gels. Total RNA from all samples demonstrated strong 28s and 18s bands (**Figure 1**), indicating sufficient quality of total RNA for subsequent miRNA microarray analysis.

miRNA microarray

The expression profiles of miRNAs in amniotic fluid samples from six pregnant rats with fetuses modeling spina bifida were compared with those from six rats with control fetuses using TaqMan Low Density Array according to the manufacturer's protocols. Briefly, total RNA (350 ng) was reverse transcribed using a TaqMan MicroRNA RT kit and Multiplex RT rodent primer pool (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The cDNAs were added to Master Mix (Applied Biosystems) and applied to miRNA TaqMan low-density array rodent panels 2.0 (Applied Biosystems) according to the manufacturer's instructions for the simultaneous quantification of 375 miRNAs. The miRNA TaqMan assays were performed using a 7900 HT Fast Real-Time PCR system (Applied Biosystems). Each sample was screened with two arrays. Differentially expressed miRNA profiles were analyzed with DataAssist software v1.0 (Applied Biosystems). A value for P < 0.05 was considered statistically significant.

Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

We used qRT-PCR to validate the miRNAs differentially expressed in the two groups. Briefly, 10 ng of total RNA was reverse transcribed to cDNA using microRNA specific primers and a TaqMan Reverse Transcription Kit (Applied Biosystems). Diluted cDNA was subjected to qRT-PCR using the TaqMan MicroRNA Assay and TaqMan Universal PCR Master Mix (ABI, Life Technologies, Foster City, CA, USA) with a 7500 Real-Time PCR system (Applied Biosystems). Relative quantification was performed using the ΔΔCt method (Yuan et al., 2006), and the data were normalized to U6 and RNU48 (Applied Biosystems), as endogenous controls. The PCR was performed in triplicate for each sample for both control and each miRNA simultaneously. Relative miRNA expression levels were quantified using the $2^{-\Delta\Delta Ct}$ method.

Identification of miRNA related pathways

The DIANA miRPath web-based computational tool (http://diana.cslab.ece.ntua.gr/) was used to identify potential Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways related to the miRNAs differentially expressed in amniotic fluid samples from control rats and those with fetuses modeling spina bifida. A pathway assigned a value of P < 0.05 was considered significantly enriched.

Western blot analysis

The protein samples were extracted by using a ReadyPrep protein extraction kit (Bio-Rad, Hercules, CA, USA). The total protein sample was diluted using 5× sample buffer at a ratio of 4:1. The analytical sample was denatured at 100°C for 5 minutes and then underwent sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis. The proteins in the gel were subsequently transferred to polyvinylidene fluoride membranes, which were incubated in 5% fat-free milk at room temperature to block nonspecific proteins. The membranes were then incubated in mouse anti-rat MAPK monoclonal antibody (Cell Signaling Technology, Danvers, MA, USA) diluted to a ratio of 1:200 at 4°C overnight. After being washed with Tris-buffered saline containing Tween 20 (TBST: 50 mM Tris base, 0.9% NaCl, 0.05% Tween 20, pH 7.5), the membranes were incubated in fluorescein isothiocyanate-labeled goat anti-mouse IgG (for MAPK; ABI, Life Technologies) diluted to a ratio of 1:8,000 at 37°C for 1 hour. After being washed with TBST, the membranes were processed using enhanced chemiluminescence and exposed to X-ray film for 5 minutes. Beta-actin (mouse anti-rat monoclonal antibody, dilution 1:200, Cell Signaling Technology, Boston, MA, USA) was used as an internal reference. The expression levels of the proteins were compared with those of the β-actin control, based on the relative optical density of the bands. Band density was quantified using Quantity One v4.62 software (Bio-Rad, Hercules, CA, USA).

Statistical analysis

Data are expressed as the mean ± SD. Intergroup comparison was performed by a two-sided Student's t-test using
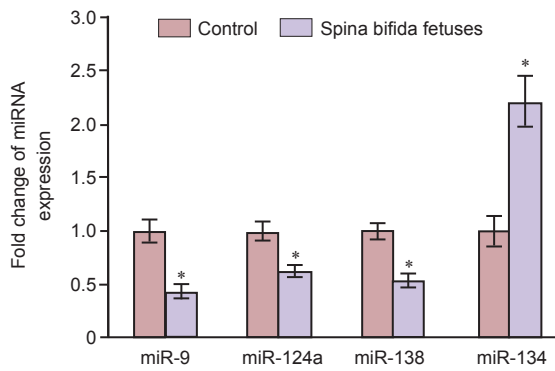
Figure 3 Expression levels of miRNA-9, miRNA-124a, miRNA-138, and miRNA-134 in the amniotic fluid of rats with fetuses modeling spina bifida and control rats with normal fetuses as determined using real-time quantitative reverse transcription-polymerase chain reaction.
Relative miRNA expression is shown as the mean ± SD (n = 6 rats per group). *P < 0.05, $vs.$ control fetuses (two-sided Student's t-test).

Figure 4 Significantly enriched signaling pathways associated with differentially expressed miRNAs as determined using KEGG pathway cluster analysis.
The neurotrophin signaling pathway is indicated with an arrow.

SPSS v17.0 (SPSS Inc., Chicago, IL, USA), and a P value < 0.05 was considered statistically significant.

Results

miRNA expression altered in samples derived from rat fetuses modeling spina bifida

Hierarchical clustering analysis of the microarray data based on fold change and probability values revealed that there were 11 significantly altered miRNAs (**Table 1**). Only miRNAs showing expression level changes greater than 2-fold (log2
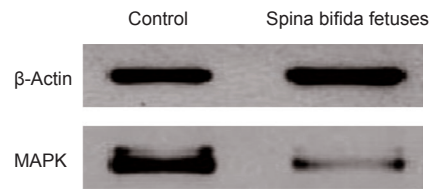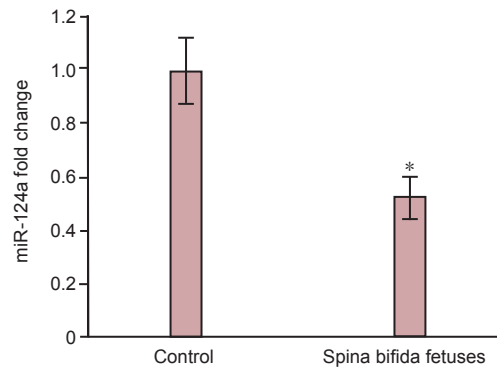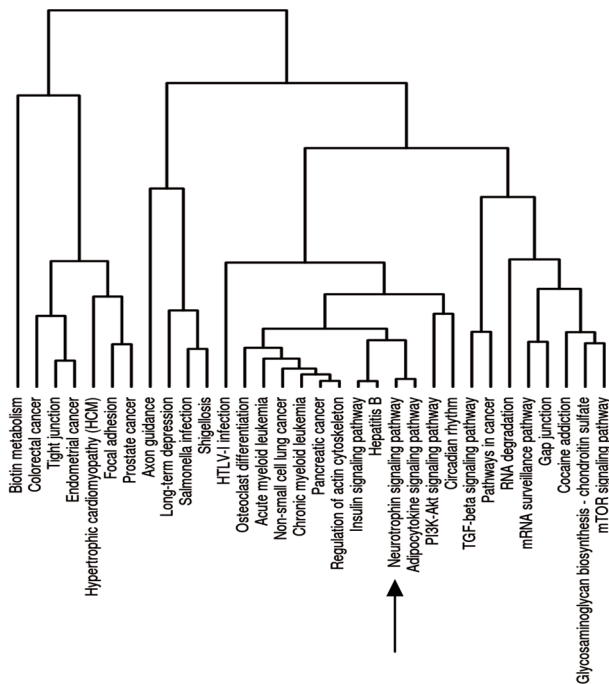

Figure 5 Parallel reductions in miRNA-124a and MAPK expression in the amniotic fluid of rats with fetuses modeling spina bifida fetuses as determined using western blot analysis.
(A) miRNA-124a expression in rats with fetuses modeling spina bifida and control rats with normal fetuses. Data are expressed as the mean ± SD (n = 6 rats per group). *P < 0.05, $vs.$ control fetuses (two-sided Student's t-test). (B) MAPK protein and loading control (β-actin) expression levels in amniotic fluid obtained from rats with fetuses modeling spina bifida and control rats with normal fetuses.

fold change) were further investigated. Among them, three significantly downregulated miRNAs (miRNA-9, miRNA-124a, and miRNA-138) that changed more than 2-fold (P < 0.05) and one significantly upregulated miRNA (miRNA-134) that changed more than 4-fold in expression (P < 0.05) were detected in the amniotic fluid of rats with fetuses modeling spina bifida as compared with control rats (**Figure 2**).

Real-time qRT-PCR validation

Retinoic acid induces spina bifida manifesta 20 days post conception. To validate the results from the microarray platform, we assessed the expression of four differentially expressed miRNAs using qRT-PCR. As shown in **Figure 3**, there were significant differences in the expression of these miRNAs. In particular, the levels of expression for miRNA-9, miRNA-124a, and miRNA-138 were significantly downregulated, whereas expression of miRNA-134 was significantly upregulated in the amniotic fluid of rats with fetuses modeling spina bifida compared with those in rats with normal fetuses. These data were consistent with the microarray findings.

Pathway enrichment analysis

Single miRNA can bind to many target genes and function as multiple pathway regulators. Using the web-based pathway analysis system DIANA miRPath, we interpreted the biological and signaling pathways of all altered miRNA. The KEGG results showed these miRNA were involved in multiple significant pathways (**Figure 4**). The neurotrophin signaling pathway was of special interest for spina bifida.

Association of miRNA-124a and MAPK expression and protein levels

Based on the DIANA miRPath analysis results, miRNA-124a had a relationship with the neurotrophin signaling pathway, which involves the MAPK gene (Longo and Massa, 2013). We thus explored the role of miRNA-124a in the regulation of MAPK gene expression. The downregulation of miR-NA-124a paralleled the reduction in MAPK expression in the amniotic fluid of rats with fetuses modeling spina bifida compared with controls (**Figure 5**).

Discussion

Despite the clinical application of fetal surgical treatments available for spina bifida, current therapeutics for spina bifida are still unsatisfactorily compounded by lasting neurological complications. Thus, understanding the mechanisms of neurological dysfunction is essential for developing effective treatments for spina bifida.

A metabolic product of vitamin A, ATRA, is required for normal neuronal differentiation, with the spinal cord being the most active site of ATRA signaling during development (Maden, 2000, 2006). Excessive ATRA is known to cause NTDs in pregnant mammals, spina bifida in particular, and can induce lesions similar to those found in human vitamin A deficiency (Zhao et al., 2008). Maternal administration of ATRA has long been used to induce an experimental model of spina bifida in the fetal rat (Diez-Pardo et al., 1995). No previous studies have provided direct evidence to show which doses of ATRA significantly modify miRNA expression levels. However, the results of some studies showed significantly changed gene expression *in vivo* after treatment with 150 mg/kg ATRA and increased apoptosis percentage in mouse embryo cells induced by 100 and 150 mg/kg ATRT treatment (Stopera and Bird, 1993; Shum et al., 1999). Hence, we used the higher dose, 150 mg/kg, in the present study to induce miRNA expression alteration.

A number of studies have established that miRNAs are critical regulators of cell and tissue differentiation during embryogenesis (Mineno et al., 2006). In mammals, certain miRNAs have been implicated in the maintenance of the pluripotent cell state during early embryogenesis (Houbaviy et al., 2003), and other miRNAs mediate tissue-specific and organ-specific development (Lagos-Quintana et al., 2002). Maintenance and regulation of endogenous miRNA levels are necessary for proper mammalian neurogenesis and neurodevelopment. A previous study of miRNA expression during brain development and function of miRNA biogenesis in neuronal tissues clearly demonstrated their importance in healthy neuronal development and in the pathogenesis of CNS diseases (Vreugdenhil and Berezikov, 2010). Wu et al. (2013) reported the alteration of miRNA in spina bifida and concluded that overexpression of miRNA-451 and miRNA-375, and the consequent upregulation of p53, may further promote apoptosis in spina bifida. With key roles in the regulation of both neural stem cell differentiation and brain development, miRNAs are candidate contributors to the etiopathology of spina bifida. The analysis of miRNA expression based on genome-wide microarrays has been

widely used to rapidly and systematically identify new miRNA markers in human cancers (Lu et al., 2005). Using miRNA microarray analysis in the present study, we demonstrated that the expression levels of miRNA-9, miRNA-124a, and miRNA-138 were significantly downregulated, whereas miRNA-134 expression was significantly upregulated in the amniotic fluid of rats with fetuses modeling spina bifida compared with rats having healthy fetuses. The microarray analysis-identified differentially expressed miRNAs were validated using qRT-PCR. Our results are the first to reveal a unique miRNA expression profile in the amniotic fluid of a rat model of spina bifida and may help identify potential therapeutic targets for spina bifida.

The highly conserved, brain-enriched miRNA-9 is strongly expressed at the fifth stage of embryogenesis, which is a critical period in neural differentiation. Repressed expression of miRNA-9 in neural progenitors could reduce the number of neurons by 30%. Another brain-specific miRNA, miR-NA-124a, is enriched in neural cells, including embryonic stem cell-derived neurospheres. It has been reported that expression levels of miRNA-9 and miRNA-124a are reduced in human anencephaly (Zhang et al., 2010, 2014) and in E19 rat embryos modeling spina bifida (Wei et al., 2013). Significantly reduced expression of miRNA-124a in the spinal cord of a rat model of spina bifida occurred at an earlier developmental stage (from E12 to E18) compared with the expression in control rats, and downregulation of miRNA-9 was observed on E12, suggesting that the change in miRNA expression occurred during an early stage of embryonic development in a rat model of spina bifida (Wei et al., 2013). Both miRNA-9 and miRNA-124a have a binding site for repressor element silencing transcription factor (REST), which suppresses their expression in neuronal cells. As the effector of REST, miR-NA-124a has been reported to suppress the repression of BAF53a and therefore participate in neuronal differentiation in the developing neural tube (Conaco et al., 2006). It is thus reasonable to postulate that the reduced expression of these two microRNAs observed in the amniotic fluid of rats with fetuses modeling spina bifida in our study is due to an increase in REST expression. Indeed, it has been shown that in the defective spinal cords of a rat model of spina bifida with downregulated miRNAs, the expression of REST was upregulated at all stages (Wei et al., 2013).

Similar to miRNA-124a, miRNA-134 is confined to brain cells, where it positively regulates the development of dendrites and spines as well as synaptic integration (Christensen et al., 2010). The upregulation of miRNA-134 has been shown to reduce cell migration in neural progenitors (Gaughwin et al., 2011), a process that is crucial for neural tube closure. Due to the observed upregulation of miR-NA-134 in amniotic fluid of rats with fetuses modeling spina bifida in our study, miRNA-134 overexpression may be considered a high risk factor for NTDs. To date, no functional studies have reported the role of miRNA-138 in brain development and in NTDs. Thus, the biological effects of miRNA-138 should be further explored.

The neurotrophin pathway has been identified as a target of miRNA-124a. Neurotrophins are a family of closely related

proteins that were identified initially as survival factors for sensory and sympathetic neurons (Reichardt, 2006; Wang et al., 2012). Previous studies (Bernd, 2008) reported the effects of neurotrophins during the early developmental stage. One of the component genes in this pathway is MAPK, a downstream signal in neural tube development (Chi et al., 2005; Krupp et al., 2012). Our results revealed a parallel decrease in miRNA-124a and MAPK protein expression, with miRNA-124a previously shown to have a regulatory function in spina bifida pathology.

In summary, we provided an overview of differentially expressed miRNAs in the amniotic fluid of rats with fetuses modeling spina bifida compared with control rats. However, the result of this investigation into the mechanisms associated with this defect are preliminary, and future studies with larger sample sizes are needed to validate the screened differentially expressed miRNAs and to investigate their potential target genes, which may contribute to therapeutic strategies with wide clinical implications for the treatment or prevention of spina bifida.

Author contributions: *PQ and LL contributed to all experiments conducted in the laboratory and wrote the paper. DZ and QLL were responsible for data analysis. XRC, HYY and YZF contributed to the molecular experiments. JXW was responsible for the study design and paper editing. All authors approved the final version of this paper.*
Conflicts of interest: *None declared.*
Plagiarism check: *This paper was screened twice using Cross-Check to verify originality before publication.*
Peer review: *This paper was double-blinded and stringently reviewed by international expert reviewers.*

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