MicroRNA-29b Regulates Ethanol-induced Neuronal Apoptosis in the Developing Cerebellum through SP1/RAX/PKR Cascade*

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Background: We investigated the role of microRNAs in ethanol neurotoxicity in the developing cerebellum.

Results: MiR-29b regulates ethanol-induced apoptosis of cerebellar granule neurons in the developing cerebellum via SP1/RAX/PKR cascade.

Conclusion: MiR-29b plays an important role in ethanol neurotoxicity in the developing cerebellum.

Significance: MiR-29b may be a new preventive/therapeutic target for fetal alcohol spectrum disorders.

Fetal alcohol spectrum disorders, defects in the fetus due to maternal drinking during pregnancy, are the leading cause of mental retardation. Neuronal loss is a prominent pathological effect of fetal alcohol exposure. The vulnerability of neurons to alcohol differs among brain regions as well as developmental stages. The cerebellum is one of the areas in the brain that is most sensitive to alcohol, especially during the temporal window of ethanol vulnerability. MicroRNAs are small, non-coding RNAs capable of regulating diverse cellular functions including apoptosis. Ethanol exposure has been shown to interfere with the expression of microRNAs. However, the role of microRNAs in ethanol neurotoxicity is still not clear. In the present study, we identified a particular microRNA, miR-29b, as a novel target of ethanol in the developing cerebellar granule neurons. We discovered that ethanol exposure suppressed miR-29b and induced neuronal apoptosis. Overexpression of miR-29b rendered neurons protection against ethanol-induced apoptosis. Furthermore, our data indicated that miR-29b mediated ethanol neurotoxicity through the SP1/RAX/PKR cascade. More importantly, the expression of miR-29b is developmentally regulated, which may account for, at least partially, the temporal window of ethanol sensitivity in the developing cerebellum.

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§ The abbreviations used are: PD, postnatal day(s); miRNA, microRNA; miR-29, microRNA-29; CGN, cerebellum granule neuron; SP1, specificity protein 1; RAX, PKR activator X.

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MiR-29b and Ethanol Neurotoxicity

arsenite, oxidative stress, and inflammation (12). The murine protein RAX or its human homologue, PACT, is the endogenous activator of PKR. RAX is a stress response protein that activates PKR under various stressful conditions (13). Activation of PKR by ethanol has been shown to induce neuroblastoma cell apoptosis through enhancing the binding of RAX and PKR (14). Overexpression of Rax increases cellular sensitivity to ethanol by further activating PKR. In contrast, mutated Rax inhibits ethanol-promoted PKR activation as well as cell apoptosis.

In the present study, using primary culture of cerebellum granule neurons (CGNs) isolated from C57BL/6 mouse at PD 7, we identified miR-29b as a target of ethanol in the developing cerebellum. Our data indicated that ethanol exposure suppressed the miR-29b level, which in turn activated PKR and induced CGN apoptosis through the specificity protein 1 (SP1)/RAX/PKR cascade. Furthermore, the expression of miR-29b is developmentally regulated, which may account for, at least partially, the temporal window of ethanol sensitivity in the developing cerebellum.

EXPERIMENTAL PROCEDURES

Materials—The following materials were used: ethanol (Sigma-Aldrich, E7023); ethanol assay kit (Sigma-Aldrich, MAK076); anti-actin antibody (Sigma-Aldrich, A5441); anti-cleaved caspase-3 antibody (Cell Signaling Technology, 9661); anti-SP1 antibody (Santa Cruz Biotechnology, sc-59); anti-PKR antibody (Santa Cruz Biotechnology, sc-709); anti-phospho-PKR antibody (Santa Cruz Biotechnology, sc-101784); anti-PACT antibody (Santa Cruz Biotechnology, sc-377103); miRNA mimic negative control (Invitrogen, 4464058); miRNA mimics for miR-9, miR-29b, and miR-133a (Invitrogen, MC10022, MC10218, and MC10413); miRNA inhibitor negative control (Invitrogen, 4464076); miRNA inhibitors for miR-29b, miR-34a, and miR-181a (Invitrogen, MH10218, MH11030, and MH10421); mouse Sp1 siRNA (Origene, SR420087); ketamine (023061, Butler Schein Animal Health); and xylazine (033197, Butler Schein Animal Health).

Animals and Treatment—C57BL/6 mice were obtained from Harlan Laboratories and maintained at the animal facility of the University of Kentucky Medical Center. All procedures were performed in accordance with the guidelines set by the National Institutes of Health and the Animal Care and Use Committee of the University of Kentucky. A binge ethanol exposure paradigm (15), which has been shown to induce robust neuronal apoptosis in mouse brain of early postnatal days, was used. Briefly, mice at PD 3, 6, 9, 12, 15, and 18 were injected subcutaneously with saline or ethanol (2.5 g/kg; 20% solution in saline) twice at time at 0 and 2 h. The cerebella were collected from five pups of each treatment group 8 h after ethanol injection and processed for immunoblotting or immunohistochemistry analysis.

Primary Culture of Cerebellar Granule Neurons—CGNs were isolated from 7-day-old mice and cultured as described previously (14, 16). Briefly, mouse pups at PD 7 were decapitated, and cerebella were carefully removed. The tissue was minced with a sterile razor blade and suspended in 10 ml of trypsin (0.025%) solution at 37 °C. After incubation for 15 min, an equal volume of solution containing DNase (130 Kunitz units/ml) (Sigma-Aldrich, D4527) and trypsin inhibitor (0.75 mg/ml) (Sigma-Aldrich, T9003) were added, and the tissue was sedimented by a brief (5-s) centrifugation. The tissue was dissociated by trituration, and the cell suspension was centrifuged through a 4% bovine serum albumin solution, which is a critical step for neuronal viability. The cell pellet was resuspended in medium consisting of Neurobasal-A medium (Invitrogen, 10888-022) containing B-27 serum-free supplement (Invitrogen, 17504-044), 1 mM l-glutamine (Invitrogen, 25030-081), 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, 15140-148). Sufficient medium was added to obtain a cell suspension containing 1.5 × 10⁶ cells/ml. Cells were plated in poly-D-lysine-coated cell culture plates or dishes. Cells were incubated at 37 °C in a humidified environment containing 5% CO₂ for 24 h before ethanol treatment.

Transfection of Cerebellar Granule Neurons—Transfection of siRNAs, miRNA mimics, or miRNA inhibitors into primary cultured neurons has been reported before (17, 18). In this study, the Neon transfection kit (Invitrogen, MPK10025) was used according to the manufacturer’s instructions. Briefly, cerebellar granule neurons in 24-well plates at a final concentration of 3 × 10⁵/well in 24-well plates were transfected with 100 nM specific miRNA mimics, miRNA inhibitors, or Sp1 siRNA, respectively, by electroporation at 1350 V with two 20-ms pulses.

In Vitro Ethanol Exposure Protocol—The method for ethanol treatment in vitro has been described previously (14, 16). Because of the volatility of ethanol, a method utilizing sealed containers was used to maintain ethanol concentrations in the medium. With this method, ethanol concentrations in B-27-containing serum-free medium can be accurately maintained and were confirmed by using an ethanol assay kit from Sigma-Aldrich. A physiologically relevant concentration of 400 mg/dl was used in this study. In addition, different time points were used in the study because the time needed to show the optimal effects of ethanol on miRNA levels, protein levels, and cell death was different.

Determination of Cell Viability—Cell viability was quantified using Cell Counting Kit-8 (Dojindo) according to the manufacturer’s instructions. Cerebellar granule neurons were cultured in a 96-well microplate at a density of 3 × 10³ cells/well for 24 h. The cells were then treated with ethanol at 200, 400, or 800 mg/dl for 24, 48, or 72 h. Untreated CGN cells were used as a negative control. There were three wells in a microplate for each treatment. After treatment, Cell Counting Kit-8 solution (10 μl) was added to each well of the plate, and the cells were incubated at 37 °C for 2 h. The optical density at a wavelength of 450 nm was measured with a SpectraMax M2e microplate reader (Molecular Devices). The results were expressed as the mean ± S.E. from three independent experiments.

Immunoblotting—Cells were washed with phosphate-buffered saline (PBS; pH 7.4) and lysed with radioimmune precipitation assay buffer (150 mM NaCl, 50 mM Tris [pH 8.0], 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.5% deoxycholic acid sodium, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 10 μg/ml aprotinin) on ice for 10 min, solubilized cells were centrifuged, and the supernatant was col-
The immunoblotting procedure has been described previously (14). Briefly, after the protein concentrations were determined, aliquots of the protein samples (20–40 μg) were loaded into the lanes of an SDS-polyacrylamide gel. The protein samples were separated by electrophoresis, and the separated proteins were transferred to nitrocellulose membranes. The membranes were blocked with either 5% BSA or 5% nonfat milk in 0.01 x PBS (pH 7.4) and 0.05% Tween 20 (TPBS) at room temperature for 1 h. Subsequently, the membranes were probed with primary antibodies directed against target proteins for 2 h at room temperature or overnight at 4 °C. After three quick washes in TPBS, the membranes were incubated with a secondary antibody conjugated to horseradish peroxidase diluted in TPBS for 1 h. The immune complexes were detected by enhanced chemiluminescence method (PerkinElmer Life Sciences, NEL105001EA). The blots were stripped and repripped with an anti-actin antibody. Experiments were replicated three times, and results were quantified by densitometry and expressed as the mean ± S.E.

### Quantitative RT-PCR Detection of miRNA Expression

The miRNA expression was analyzed by quantitative real time reverse transcription-PCR, which has been described (19). Briefly, total RNA was extracted by a TRIzol reagent (Invitrogen, 15596-026). RNAs were polyadenylated at 37 °C for 60 min in a 50-μl reaction volume containing 2 μg of RNA and 5 units of poly(A) polymerase (New England Biolabs, M0276). An equal volume of acid-phenol-chloroform was added, and the sample was mixed and centrifuged. The aqueous phase was carefully removed and loaded into a filter cartridge provided in the mirVana miRNA isolation kit (Invitrogen, AM1560), and tailed small RNAs were purified according to the manufacturer’s instructions. The tailed RNAs were reverse transcribed using a primer containing oligo(dT) flanked by an adaptor sequence (RTQ-Primer; see Table 1) with a ProtoScript avian myeloblastosis virus first strand cDNA synthesis kit (New England Biolabs, E6550). PCR was performed on a LightCycler 480 by using a LightCycler 480 SYBR Green I Master kit (Roche Applied Science, 04707516001). A small RNA-specific primer and a universal reverse primer, RTQ-UNIr, were used for amplification of each of the small RNAs (Table 1). The results were analyzed using the software of the LightCycler system. The relative expression level of a given mRNA was assessed by normalizing it to the housekeeping gene snoRNA202 and then compared with controls. The results were expressed as the mean ± S.E. from three replicates.

### Immunohistochemistry

After treatments, the mice were deeply anesthetized with xylazine-ketamine mixture (100 mg/kg ketamine and 10 mg/kg xylazine) and perfused with saline followed by 4% paraformaldehyde (Sigma-Aldrich, P6148) in 0.1 M potassium phosphate buffer (pH 7.4) and 0.05% Tween 20 (TPBS) at room temperature for 1 h. The brains were removed and postfixed in 4% paraformaldehyde (Sigma-Aldrich, P6148) for an additional 24 h and then transferred to 30% sucrose (Sigma-Aldrich, S7903). The brain samples were sectioned at 40 μm with a sliding microtome (Leica Microsystems). The procedure for immunohistochemistry staining has been described previously (14). Briefly, free floating sections were incubated in 0.3% H₂O₂ (Sigma-Aldrich, H0904) in methanol (Sigma-Aldrich, M3641) for 30 min at room temperature and then treated with 0.1% Triton X-100 (Sigma-Aldrich, T9284) for 10 min in PBS. The sections were washed with PBS three times and blocked with 1% BSA and 0.01% Triton X-100 for 1 h at room temperature. The sections were incu-

### TABLE 1

| Name | Sequence (5’–3’) | Tₘ | Usage |
|------|-----------------|----|-------|
| RTQ-Primer | CGATCTCTACAGGCGGCAGC | 76 | cDNA synthesis |
| RTQ-UNIr | CGATCTCTACAGGCGGCAGC | 59 | qPCR |
| snoRNA202 | GGTATCAGCTTGAAGATGTG | 51 | qPCR |
| let-7a | GAGTTAATAGTTTATAGTGA | 48 | qPCR |
| miR-9 | TCTTTGTGATCTCAGAGTGA | 50 | qPCR |
| miR-16 | TACACAGCCTAATATATCGCG | 53 | qPCR |
| miR-20a | TAAAGGCTTTATATATCGAGTGA | 52 | qPCR |
| miR-21a | TACACCTCTACAGTATGTGGA | 49 | qPCR |
| miR-23a | TACACCTCTACAGTATGTGGA | 51 | qPCR |
| miR-29a | TACACCTCTACAGTATGTGGA | 50 | qPCR |
| miR-29c | TACACCTCTACAGTATGTGGA | 49 | qPCR |
| miR-34a | TGCGAGCTCTAGCTGTGGA | 55 | qPCR |
| miR-101a | TACACCTCTACAGTATGTGGA | 47 | qPCR |
| miR-101b | TACACCTCTACAGTATGTGGA | 49 | qPCR |
| miR-107 | AGCAGCTATTACGCGCTAAC | 55 | qPCR |
| miR-124 | TAAAGGCTTTATATATCGAGTGA | 56 | qPCR |
| miR-128 | TACACCTCTACAGTATGTGGA | 52 | qPCR |
| miR-133a | TTTGCTCCTTTAAACCACTGAG | 57 | qPCR |
| miR-133b | TTTGCTCCTTTAAACCACTGAG | 55 | qPCR |
| miR-153 | TACACCTCTACAGTATGTGGA | 49 | qPCR |
| miR-181a | ACATTTCAACCTGCGCGTGGT | 55 | qPCR |
| miR-181b | ACATTTCAACCTGCGCGTGGT | 55 | qPCR |
| miR-181c | ACATTTCAACCTGCGCGTGGT | 53 | qPCR |
| miR-181d | ACATTTCAACCTGCGCGTGGT | 53 | qPCR |
| miR-206 | TGAAGCTTTAAGGAGGTTGGA | 53 | qPCR |
| miR-302b | TAA AGCCTCTACAGTATGTGGA | 50 | qPCR |
| miR-335 | TCAAGACAGATACAGGGGCTATCA | 48 | qPCR |
| miR-338 | TCAAGACAGATACAGGGGCTATCA | 51 | qPCR |
| miR-497 | TCAAGACAGATACAGGGGCTATCA | 55 | qPCR |
bated with an anti-active caspase-3 antibody (1:1,000 dilution) overnight at 4 °C. Negative controls were performed by omitting the primary antibody. After rinsing in PBS, sections were incubated with a biotinylated goat anti-rabbit IgG (1:200; Vector Laboratories, BA-1000) for 1 h at room temperature. The sections were washed three times with PBS, then incubated in avidin-biotin-peroxidase complex (1:100; Vector Laboratories, SP-2001) for 1 h, and developed in 0.05% 3,3′-diaminobenzidine (Sigma-Aldrich, D3939) containing 0.003% H2O2 in PBS. The images were captured with an Olympus microscope (BX61) equipped with a DP70 digital camera.

Statistical Analysis—Differences among treatment groups were tested by analysis of variance. *p < 0.05 was considered statistically significant. In cases in which significant differences were detected, specific post hoc comparisons between treatment groups were examined by Student-Newman-Keuls tests.

RESULTS

Ethanol Exposure Alters the Levels of Some MiRNAs in Primary Cultured CGNs—The most devastating effect of ethanol on the fetal brain is ethanol-induced neuronal loss. Primary culture of CGNs isolated from the murine cerebellum of early postnatal days has been widely used as an in vitro model to study neuronal apoptosis induced by ethanol (20, 21) as well as other drugs (22, 23). As shown in Fig. 1A, ethanol at a concentration of 200, 400, or 800 mg/dl induced CGN apoptosis in a dose- and time-dependent manner. Based on these data and reports from others (20, 21), ethanol treatment at 400 mg/dl

**FIGURE 1.** Ethanol induces apoptosis and alters miRNA levels of CGNs. A, CGNs in primary culture were treated with 200, 400, or 800 mg/dl ethanol for 24, 48, or 72 h. Cell viabilities were determined using Cell Counting Kit-8. Experiments were replicated three times, and data are expressed as the mean ± S.E. (error bars). *, p < 0.05 versus matched control groups. B and C, CGNs were treated with 400 mg/dl ethanol for 24 (B) or 48 h (C). Total RNAs were isolated, and the levels of 26 miRNAs were determined by real time PCR. The miRNA expression ratios between ethanol-treated groups and control groups are shown. Data are expressed as the mean ± S.E. (error bars) of three independent experiments. *, p < 0.05 versus control groups.
was chosen for our following study. This dosage is physiologically relevant and has been justified for in vitro study of ethanol neurotoxicity (24). MiRNAs have been shown to regulate diverse cellular functions including cell survival/death. When we started the study, we performed a literature search and found 26 miRNAs that have been reported to mediate survival/apoptosis in neuronal and glia cells (5, 11, 25–39) (Fig. 1B). We next screened the levels of these survival/apoptosis-related miRNAs upon ethanol exposure in the primary cultured CGNs to determine whether any of these miRNAs may be involved in the action of ethanol in the CGNs. As shown in Fig. 1, B and C, ethanol exposure for 24 and 48 h significantly altered levels of six miRNAs: it significantly decreased the levels of miR-9, miR-29a, miR-29b, and miR-133 and increased the levels of miR-34a and miR-181a.

MiR-29b Mediates Ethanol-induced Apoptosis of CGNs—Because miR-29a, -29b, and -29c have extensive sequence homology, especially at the 5′ seed region that is important for mRNA recognition (40), we focused on miR-29b among these three miR-29 members as it was the most highly expressed, showed the greatest reduction by ethanol, and is found at both genomic loci where miR-29a and -29c are located. To determine whether miR-9, miR-29b, miR-133, miR-34a, or miR-181a was involved in ethanol-induced apoptosis, we overexpressed miR-9, miR-29b, or miR-133 and knocked down miR-34a or miR-181a by transfecting the GCNs with their mimics or inhibitors, respectively (Fig. 2A). We then exposed these cells to ethanol. Among these cells, only the miR-29b mimic rendered cells resistant against ethanol-induced apoptosis (Fig. 2B). The protective effect of miR-29b was further verified by the level of active caspase-3, which was decreased by the miR-29b mimic and increased by the miR-29b inhibitor after ethanol exposure (Fig. 2, C and D).

MiR-29b Regulates Ethanol-induced Apoptosis of CGNs through SP1/RAX/PKR Cascade—SP1 is a transcription factor regulating many important cellular functions such as proliferation, differentiation, and apoptosis (41). It is a bona fide target of miR-29b (42). Interestingly, RAX is regulated transcriptionally by SP1 (13). In our previous study, we have shown that ethanol induced apoptosis of CGNs by activating PKR through RAX (14). Therefore, we next determined whether the SP1/RAX/PKR axis was responsible for the action of miR-29b. We first tested the effect of miR-29b on the levels of SP1 and RAX in CGNs upon ethanol exposure. As shown in Fig. 3A, ethanol increased the levels of SP1 and RAX, and these increases were inhibited by miR-29b up-regulation. Notably, ethanol enhanced the phosphorylation of PKR, whereas the total PKR remained unchanged. To further confirm that RAX was regulated by miR-29b through SP1, we knocked down Sp1 by transfecting cells with Sp1 siRNA (Fig. 3B). As expected, RAX was down-regulated by Sp1 knockdown (Fig. 3C), indicating that miR-29b regulated RAX through SP1. More importantly, Sp1 knockdown inhibited ethanol-promoted PKR phosphorylation. Consequently, ethanol-induced apoptosis was decreased in Sp1 knockdown cells (Fig. 3D).

MiR-29b May Developmentally Regulate Ethanol Neurotoxicity in the Mouse Cerebellum—The developing cerebellum of rodent has been widely used for investigating ethanol neurotoxicity. PD 4–10 represent a window of vulnerability of the cerebellum to ethanol (1–4). As shown in Fig. 4A, ethanol administration to PD 6 mice via subcutaneous injection induced caspase-3 activation in the cerebellum as determined by immunohistochemistry. This ethanol-induced caspase-3 activation was much more evident before PD 12 but became much weaker after PD 12 as revealed by immunoblotting (Fig. 4B). Using the same paradigm of ethanol treatment, a recent in vivo study confirmed that ethanol administration to PD 4 mice causes widespread neuronal apoptosis in the cerebellum but has much less effect on the cerebellum of PD 12 mice (15). Therefore, we examined the levels of miR-29b from PD 3 to 18. As shown in Fig. 4C, the level of miR-29b was low on PD3 and increased dramatically along the developmental stage. Ethanol treatment significantly decreased miR-29b during PD 3–9 but had less effect on the miR-29b after PD 12 (Fig. 4D). We further determined the levels of SP1, RAX, phosphorylated PKR, and total PKR in the cerebellum of mouse from PD 3–18 with or without ethanol treatment. As shown in Fig. 4E, whereas the levels of total PKR were relatively even, the levels of miR-29b, SP1, RAX, and p-PKR were fairly low during PD 3–9 and significantly increased after PD 12. Ethanol exposure increased the levels of SP1, RAX, and p-PKR during PD 3–9.

DISCUSSION

The discovery of miRNAs provided a powerful approach for exploring many complicated cellular biological functions. From knock-out studies of dicer, an endoribonuclease for the formation of mature miRNAs, it is clear that miRNAs are critical for survival and maturation of neurons (43, 44). In addition, the timing of the specific requirement of miRNAs is essential for the development of embryo and postnatal central nervous system (CNS). For instance, loss of miRNAs hindered the development of several regions in the CNS (45).

Ethanol has been shown to interfere with the expression of miRNAs. Using SH-SY5Y cells, a human neuroblastoma cell line, Yadav et al. (39) reported that ethanol induced cell apoptosis by up-regulation of miR-497 and miR-302b, which inhibited the expression of their target genes, Bcl-2 and cyclin D2. Sathyan et al. (5) used a model of fetal mouse cerebral cortex-derived neurosphere (the neurons were isolated from the cerebrum at gestational day 12.5) to show that ethanol at 320 mg/dl suppressed expression of four miRNAs: miR-21, -335, -9, and -153. Their data further indicated that, among these miRNAs, miR-21 functioned as an antiapoptotic factor, and miR-335 exhibited a proapoptotic property (5). In addition, in alcohol liver diseases, miR-217 and miR-214 have been identified to promote ethanol-induced fat accumulation and oxidative stress, respectively (8, 46). Thus, it appears that ethanol targets miRNAs in tissue-cell type- and developmental stage-dependent manners.

Loss of miR-29b has been shown to contribute to the apoptosis of retinal neurons in diabetic retinopathy (47), pathogenesis of Alzheimer disease (10), and neurodegeneration caused by acute ischemic stroke (9). In addition, miR-29b levels were increased along neuronal maturation, protecting mature neurons against apoptosis induced by diverse stressful conditions such as neurotrophic withdrawal, DNA damage, and endoplasmic reticulum stress (11). It is also worth noting that a study by
Shi et al. (48) demonstrated that up-regulation of miR-29b promotes neuronal cell death after ischemic brain injury. The discrepancy may be due to the different model systems they used, and hence the function of miR-29b may not be universal among different cells or tissues.

A single miRNA may have many potential target genes, and this is also the case for miR-29b. Although several independent groups identified Sp1 as the target of miR-29b (42, 47, 49–51), others found the BCL-2 family as the target (11, 48). In the present study, we found that miR-29b targeted Sp1 but not the...
BCL-2 family upon ethanol exposure because modulation of miR-29b did not alter BCL-2 proteins after ethanol treatment in our system (data not shown). Interestingly, SP1 has previously been found to be up-regulated by ethanol in neural cells (52).

PKR was initially identified as a double-stranded RNA-activated protein in response to virus infection. Later studies have discovered that in addition to double-stranded RNA PKR can also be activated by various physiochemical stresses. In the absence of double-stranded RNA, PKR activation is mediated by direct binding with its protein activator, PACT/RAX. Therefore, PACT/RAX acts as an important stress sensor protein in response to diverse stressful conditions. Upon activation, PKR undergoes homodimerization and autophosphorylation. Following autophosphorylation, PKR catalyzes the phosphorylation of its downstream substrates. Phosphorylated PKR also translocates into the nucleus from the cytosol to regulate the expression of various genes. PKR not only plays an important role in apoptosis under stress conditions (53, 54), but recently PKR has also been verified as an activator of the inflammasome to regulate cellular inflammatory response (55).

Ethanol is a neuronal teratogen, and its action is complicated. Ethanol-induced neuronal apoptosis has been suggested as the major cause of ethanol neurotoxicity. PKR is a critical mediator of ethanol-induced apoptosis and is activated upon binding with RAX. Overexpression of Rax enhances PKR activation as well as cellular sensitivity to ethanol. In contrast, blocking the binding of RAX and PKR by Rax mutation inhibits ethanol-induced PKR activation as well as cell apoptosis. More impor-
tantly, the expression of Rax in the cerebellum is developmentally regulated. A rapid increase of the RAX level is observed during PD 4–10, which falls in the temporal window of ethanol vulnerability (14).

In the present study, we identified miR-29b as a potential novel regulator of ethanol neurotoxicity in the cerebellum granule neurons. Ethanol exposure dramatically suppressed miR-29b, which in turn up-regulated SP1 as well as its target, RAX, resulting in enhancement of PKR activation and neuronal apoptosis. Thus, we have provided a mechanism for ethanol-promoted RAX/PKR interaction and PKR activation. More importantly, our data indicated that ethanol suppressed miR-29b to a much greater degree during PD 3–9, which coincides with the temporal window of ethanol vulnerability in the cerebellum, compared with that after PD 12. Our results are in agreement with a previous observation by Kole et al. (11) showing that miR-29b is developmentally regulated in mouse sympathetic neurons and that the increased miR-29b protects neuronal cells against apoptosis induced by multiple stressful stimuli. Thus, miR-29b may developmentally regulate ethanol neuronal toxicity in the cerebellum. Currently, further in vivo and behavioral studies of miR-29b/RAX/PKR in regulating ethanol neurotoxicity are ongoing in our laboratory. Finally, because PKR is also activated in response to other stress conditions, the miR-29b/SP1/RAX/PKR pathway may not only account for ethanol neurotoxicity in fetal alcohol spectrum disorders but also may be the mechanism underlying other diseases induced by different stimuli.

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