A novel regulatory network among LncRpa, CircRar1, MiR-671 and apoptotic genes promotes lead-induced neuronal cell apoptosis

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Introduction

Lead is a metal that has toxic effects on the developing nervous system. However, the mechanisms underlying lead-induced neurotoxicity are not well understood. Non-coding RNAs (ncRNAs) play an important role in epigenetic regulation, but few studies have examined the function of ncRNAs in lead-induced neurotoxicity. We addressed this in the present study by evaluating the functions of a long non-coding RNA (named LncRpa) and a circular RNA (named CircRar1) in a mouse model of lead-induced neurotoxicity. High-throughput RNA sequencing showed that both LncRpa and CircRar1 promoted neuronal apoptosis. We also found that LncRpa and CircRar1 induced the upregulation of apoptosis-associated factors caspase8 and p38 at the mRNA and protein levels via modulation of their common target microRNA miR-671. This is the first report of a regulatory interaction among a lncRNA, circRNA, and miRNA mediating neuronal apoptosis in response to lead toxicity.

Electronic supplementary material

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Abstract Lead is a metal that has toxic effects on the developing nervous system. However, the mechanisms underlying lead-induced neurotoxicity are not well understood. Non-coding RNAs (ncRNAs) play an important role in epigenetic regulation, but few studies have examined the function of ncRNAs in lead-induced neurotoxicity. We addressed this in the present study by evaluating the functions of a long non-coding RNA (named LncRpa) and a circular RNA (named CircRar1) in a mouse model of lead-induced neurotoxicity. High-throughput RNA sequencing showed that both LncRpa and CircRar1 promoted neuronal apoptosis. We also found that LncRpa and CircRar1 induced the upregulation of apoptosis-associated factors caspase8 and p38 at the mRNA and protein levels via modulation of their common target microRNA miR-671. This is the first report of a regulatory interaction among a lncRNA, circRNA, and miRNA mediating neuronal apoptosis in response to lead toxicity.

Keywords CircRNA · LncRNA · MiRNA · Cell apoptosis · Lead · Neurotoxicity

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sponges, thereby indirectly regulating target gene expression (Hansen et al. 2013a; Wilusz and Sharp 2013). There have been no reports to date of interactions between lncRNAs and circRNAs.

The present study investigated the functions of lncRNAs and circRNAs in lead-induced neurotoxicity. We first carried out an RNA screen in a mouse model. A quantitative real-time polymerase chain reaction (qRT-PCR) analysis revealed that the pro-apoptotic lncRNA (named *lncRpa*) and the apoptosis-related circRNA (named *circRar1*) were upregulated in the hippocampus and cerebral cortex of mice with lead-induced neurotoxicity. A similar upregulation of *lncRpa* and *circRar1* was observed in N2a cells treated with lead acetate (PbAc). We found that *lncRpa* and *circRar1* acted via the common target miR-671 to promote neuronal apoptosis. These findings highlight the regulatory roles of lncRNAs and circRNAs in lead-induced neurotoxicity and provide the first evidence of these ncRNAs modulating a cellular process by jointly targeting a specific miRNA.

**Materials and methods**

**Lead-induced neurotoxicity models**

The mouse model of lead-induced neurotoxicity has been previously described, and the animal studies were approved by the Animal Care and Use Committee of Guangzhou Medical University (Nan et al. 2016). We also used mouse neuroblastoma N2a cells exposed to PbAc at a concentration of 0.1 µM for 48 h as an in vitro model of lead-induced neurotoxicity (Nan et al. 2016).

**RNA extraction**

RNA was obtained from brain tissue (cerebellum, pons, medulla oblongata, hippocampus, and cerebral cortex) of mice with lead-induced neurotoxicity (2 and 5 weeks of exposure to PbAc) and control mice. Trizol reagent (Invitrogen, Carlsbad, CA, USA) was used according to the manufacturer’s instructions to extract total RNA from tissues and cells. For quantitation of circRNAs, RNase R (Invitrogen, Carlsbad, CA, USA) was added to degrade linear RNAs. RNA quality and concentration were measured with a NanoDrop1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

**High-throughput RNA sequencing**

The HiSeq 2000 sequencing platform (Illumina, San Diego, CA, USA) was used for high-throughput RNA sequencing. The protocol involved removal of rRNA, followed by synthesis of double-stranded cDNA and end repair. After linking sequencing adaptors and selecting fragments, the second strand of cDNA was degraded and the remaining strand was enriched by PCR. The quality of the library was confirmed by sequencing. A bioinformatic analysis of the raw sequencing data was carried out. Differentially expressed ncRNAs were searched in the NCBI database (http://www.ncbi.nlm.nih.gov/) to determine their genome loci.

**qRT-PCR**

The Goscript Reverse Transcription System (Promega, Madison, WI, USA) was used to reverse transcribe lncRNAs, circRNAs, and mRNAs to cDNA. Go Taq qPCR Master Mix (Promega) was used for qRT-PCR. All-in-one miRNA qRT-PCR Detection kit (Genecopoeia, Rockville, MD, USA) was used to reverse transcribe and amplify miRNAs. *Glyceraldehyde 3-phosphate dehydrogenase* (GAPDH) was used as an internal control for the relative quantitation of lncRNAs, circRNAs, and mRNAs, whereas *U6* was used for miRNAs. The detection of internal control gene GAPDH would be affected after treating with RNase R; we divided the same RNA sample into two uniform parts when performing the qRT-PCR experiment. One part was treated with RNase R for delinearization; this part was for the further detection of circRNA. The other part was treated with RNase R-free water for finally detecting GAPDH gene. The primer sequences are shown in Supplementary Table 3. The 2−ΔΔCt method was used to determine relative expression levels.

**RNA interference and overexpression**

LncRNA and circRNA expression was suppressed by siRNA-mediated knockdown. Three different siRNAs were designed and tested for both *lncRpa* and *circRar1*. Overexpression vectors for *lncRpa* and *circRar1* were also constructed (BersinBio, Guangzhou, China). CircRNA upstream intron cyclization component (526 bp), circRNA (462 bp) and circRNA downstream intron cyclization component (804 bp) were included in circRNA expression area. BamHI and Hind III were jointly connected to expression vector pcDNA 3.1+ through double enzyme connection. Overexpression and siRNA sequences are shown in Supplementary Table 1. A specific inhibitor and mimic (RiboBio, Guangzhou, China) were used to inhibit or induce miR-671 expression, respectively. Cells were transfected with plasmid using EndoFectin Lenti reagent (Genecopoeia). RibofECT CP Transfection kit (166T) (RiboBio) was used for the *miR-671* inhibitor and mimic.
Detection of cell apoptosis by FCM

The Annexin V-fluorescein isothiocyanate (FITC) apoptosis assay kit (KeyGen Biotech, Nanjing, China) was used according to the manufacturer’s instructions to detect apoptotic cells 48 h after transfection and PbAc treatment. Briefly, 5 x 10⁵ cells were collected and resuspended in 100 μl 1 × binding buffer. Five microliters Annexin V-FITC and 5 μl propidium iodide staining solution were added to the cells, followed by incubation at room temperature (shielded from light) for 10 min. Four hundred microliters 1 × binding buffer was added to the reaction, and cells were analyzed by FCM (BD Biosciences, Franklin Lakes, NJ, USA) within 1 h.

Detection of cell apoptosis by TUNEL assay

A TUNEL kit (Roche Diagnostics, Indianapolis, IN, USA) was used to detect apoptotic cells. Cells were cultured on Lab-Tek chambered slides (Thermo Fisher Scientific, Waltham, MA, USA). Following treatment, the samples were washed twice with phosphate-buffered saline (PBS) and fixed with 4 % paraformaldehyde at room temperature for 20 min, followed by two washes with PBS. Proteinase K (20 μg/ml; Sangon Biotech, Shanghai, China) was added, and the slides were covered with a film and incubated at 37 °C for 20 min and then washed twice with PBS. The TUNEL reaction mixture (enzyme and labeling solutions at a 1:9 ratio) was added to the slides, which were covered with film and incubated at 37 °C for 60 min. After three washes with PBS, converter-peroxidase was added at 37 °C for 30 min; after three more washes with PBS, diaminobenzidine reagent (Roche Diagnostics) was added at room temperature for 10 min. The samples were washed three times with PBS and counterstained with hematoxylin for 10 s and then washed with running water. After dehydration in a graded series of alcohol, the samples were dried and mounted with neutral balsam. Nuclei with yellowish brown staining were positive (apoptotic), and hematoxylin-counterstained intact nuclei appeared blue under a light microscope.

CCK-8 cell viability assay

The CCK-8 assay kit (Beyotime Institute of Biotechnology, Shanghai, China) was used to assess cell viability. Cells were harvested in logarithmic phase, and 100 μl of the suspension (~2000 cells) were seeded in each well of a 96-well plate and incubated overnight at 37 °C and 5 % CO₂. EndoFectin Lenti reagent, plasmids, siRNAs, and serum-free Dulbecco’s Modified Eagle’s Medium (DMEM; HyClone, Logan, UT, USA) equilibrated to 15–25 °C were added to the wells followed by incubation at room temperature for 10–25 min. The medium was changed after 6 h, and 0.1 μM PbAc solution was added for 48 h. Ten microliters CCK-8 solution was added for 1–4 h, and the absorbance at 450 nm was measured using a microplate reader.

Western blotting

Total protein was extracted using a commercial kit (KeyGen Biotech). Protein samples (4–8 μg/μl) were mixed with a 4:1 ratio of 5 × loading buffer and β-mercaptoethanol and stored at −80 °C until use. Proteins (40–60 μg per well) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (100–120 V). A protein marker with a molecular weight range of 16–220 kDa was used as reference. The proteins were transferred to a polyvinylidene difluoride membrane (Merck Millipore, Billerica, MA, USA) at 200 mA using a wet membrane-transfer device (Bio-Rad, Hercules, CA, USA). The membrane was washed with Tris-buffered saline containing 0.1 % Tween-20 (TBST) for 1–2 min and then blocked at room temperature for 60 min with TBST containing 5 % non-fat milk powder. After overnight incubation at 4 °C with primary antibodies, the membrane was washed with TBST three times for 15 min. The membrane was then incubated at room temperature for 60 min with secondary antibody and washed three times with TBST for 15 min each. Protein bands were visualized using the BeyoECLPlus chemiluminescence reagent (Beyotime Institute of Biotechnology) followed by exposure to X-ray film. Primary antibodies against the following proteins were used in this study: caspase3 (Cell Signaling Technology, Danvers, MA, USA), caspase9 (Epitomics, Burlingame, CA, USA), Akt2 (Cell Signaling Technology), caspase 8 (Proteintech, Rosemont, IL, USA), and p38 (Cell Signaling Technology). The secondary antibody was horseradish peroxidase-conjugated IgG (Boster Bio, Pleasanton, CA, USA).

FISH

Cells grown on coverslips were fixed with 4 % paraformaldehyde at room temperature for 15 min, washed twice with 0.1 % diethylpyrocarbonate solution and treated with 0.5 % Triton X-100 at room temperature for 5 min. The samples were dehydrated in a graded series of alcohol and air-dried. After adding probe hybridization solution, the samples were mounted, denatured at 73 °C for 3 min, and hybridized in a humid and dark environment at 37 °C for 12–16 h...
with Cy3-labeled miRNA probe, 6-carboxyfluorescein-labeled circRNA probe, and Cy5-labeled lncRNA probe (BersinBio). The samples were washed three times with a pre-heated (43 °C) solution consisting of 50 % formamide and 2× saline sodium citrate (SSC), and then washed twice with 2× SSC (37 °C). After counterstaining with 4′, 6-diamidino-2-phenylindole, the samples were mounted with fluorescence mounting medium and imaged with a microscope.

Dual luciferase reporter gene assay

Cells were seeded and incubated for 24 h. At 80–90 % confluence, the cells were transfected with firefly and Renilla luciferase plasmids. After washing with PBS, passive lysis buffer (PLB) was added and cells were incubated at room temperature for 15 min, with a micro-oscillator used to lyse the cells. Lysates were centrifuged at 10,000 rpm for 5 min at 4 °C. The supernatants were removed, and 20 μl sample were transferred to a 96-well plate and mixed with 100 μl Dual-Glo Luciferase Assay System (Promega), with cell lysis buffer used as the control. The relative light units were measured before and after adding 100 μl Stop & Glo reagent.

RNA antisense purification (RAP)

The RAP kit (BersinBio) was used for this experiment. RAP employs specific biotinylated probes that hybridize to target RNAs (mRNAs or miRNAs); these can then be pulled down, reverse transcribed to cDNA, and identified by qRT-PCR or sequencing. A total of 107 cells were pulled down, reverse transcribed to cDNA, and identified by qRT-PCR or sequencing. A total of 107 cells were washed three times with a pre-heated (43 °C) solution consisting of 50 % formamide and 2× saline sodium citrate (SSC), and then washed twice with 2× SSC (37 °C). After counterstaining with 4′, 6-diamidino-2-phenylindole, the samples were mounted with fluorescence mounting medium and imaged with a microscope.

Statistical analysis

Data are presented as mean ± SD. All experiments were performed at least three times, and western blotting, TUNEL, FCM, and FISH results are representative of three independent experiments. The unpaired t test was used for statistical analyses. * represents statistically significant difference (p < 0.05). ** represents highly statistically significant difference (p < 0.01). Data were analyzed using SPSS v.19.0 software (IBM, Armonk, NY, USA).

Results

Identification of lncRNAs and circRNAs differentially expressed in lead-induced neurotoxicity

A mouse model of lead-induced neurotoxicity was established by PbAc exposure (Nan et al. 2016), and high-throughput RNA sequencing was carried out using the brain tissues of these mice. Three lncRNAs and two circRNAs showing more significant differences in expression between lead-injured mice and controls—including lncRNA TCONS00001596 (named lncRpa), lncRNA Gm16025 (ENSMUST00000161282), lncRNA Gm14260 (ENSMUST0000125121), and the circRNAs (located at chr1_75418457_75418970_+, named circRar1) and Trerf1 (located at chr17_47315500_47316549_+)—were selected for further analysis. The expression of these five molecules in the cerebellum, pons, medulla oblongata, hippocampus, and cerebral cortex was evaluated by qRT-PCR (Fig. 1a–e). The expression of Gm16025 was downregulated in the cerebellum, while that of the other four RNAs was unaltered (Fig. 1a).

None of the RNAs were differentially expressed in the pons and medulla oblongata between injured and control animals (Fig. 1b, c). LncRpa, Gm14260, and circRar1 levels were markedly upregulated in the hippocampus, while Gm16025 was downregulated and circRNA Trerf1 level showed no change upon injury (Fig. 1d). In the cerebroventricular system, lncRpa, circRar1, and Trerf1 were upregulated while no changes in Gm16025 or Gm14260 were observed (Fig. 1e). LncRpa and circRar1 expression was also upregulated in lead-treated N2a mouse neuroblastoma cells (Nan et al. 2016), with higher levels observed for the former (Fig. 1f). The genomic loci of lncRpa and circRar1 were determined (Fig. 1g, h).

LncRpa and circRar1 promote apoptosis in lead-induced neurotoxicity

We studied lncRpa and circRar1 functions by their overexpression and knockdown in N2a cells. Three short interfering RNAs (siRNAs) as well as overexpression constructs (Supplementary Table 1) were designed for each of lncRpa and circRar1. The efficiency of
knockdown and overexpression was evaluated by qRT-PCR. SiRNA1 and siRNA 2 were more efficient at suppressing lncRpa or circRar1 expression than siRNA 3 (Fig. 2a), and were used in subsequent experiments. Both lncRpa and circRar1 overexpression constructs resulted in higher levels of these two ncRNAs in N2a cells (Fig. 2a). The viability of cells with knockdown or overexpression of lncRpa or circRar1 was evaluated before and after PbAc exposure with Cell Counting Kit-8 (CCK-8)(Fig. 2b), while cell apoptosis was assessed by flow cytometry (FCM) (Fig. 2c, d), terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) (Fig. 2e, f), and detection of caspase9 and caspase3 expression by western blotting (Fig. 2g, h). The rate of apoptosis was higher in the negative control siRNA (NC) + PbAc group than in untreated cells, an effect that was mitigated by lncRpa and circRar1 knockdown prior to PbAc treatment. On the other hand, lncRpa and circRar1 overexpression increased apoptosis in PbAc-treated cells relative to those overexpressing an empty vector as NC. These results suggest that lncRpa and circRar1 promote apoptosis in lead-induced neurotoxicity.

**LncRpa and circRar1 interact directly with miR-671**

To clarify the mechanism underlying the stimulatory effects of lncRpa and circRar1 in lead-induced apoptosis, we used fluorescence in situ hybridization (FISH) (Supplementary Table 2) to visualize the cellular distribution of these two molecules (Fig. 3a). LncRpa and circRar1 were both expressed in the cytoplasm, suggesting that they
Moreover, and lncRpa the input group with no RAP probe, 671 - miR was present, indicated that only lncRpa was a shared target of purification (RAP) and transcriptome sequencing analyses and assay revealed that these miRNAs interact directly with and (Fig. 3b). The dual luciferase reporter revealed that mashree and Swamy 2015; Hsu et al. 2007; Huang et al. using miRanda, Target Scan, and RegRNA (Pad-circRar1 miRNAs that were predicted to interact with function via post-transcriptional mechanisms. We identified circRar1 lncRpa and circRar1 and associated factor X (MAX), Akt2,caspase8, p38, Myc and and increase in circRar1 levels (Fig. 4c). Similarly, circRar1 suppression increased miR-671 and decreased lncRpa expression whereas circRar1 overexpression had the opposite effect (Fig. 4d). These results suggest negative regulation between lncRpa and circRar1 and a positive regulatory relationship between lncRpa and circRar1.

MiR-671 inhibits apoptosis

To clarify the role of miR-671 in lead-induced neurotoxicity in mice, miR-671 expression was assessed by qRT-PCR. The level of miR-671 was downregulated in the hippocampus and cerebral cortex (Fig. 5a) as well as in N2a cells treated with PbAc (Fig. 5b). Apoptosis in N2a cells overexpressing miR-671 with or without PbAc treatment was assessed by FCM (Fig. 5c, d), TUNEL staining (Fig. 5e, f), and western blot analysis of caspase9 and caspase3 expression (Fig. 5g, h). MiR-671 overexpression inhibited apoptosis according the results of all of the assays. These results indicate that lncRpa and circRar1 promote apoptosis via regulation of miR-671.

MiR-671 regulates apoptosis-associated factors

Target mRNAs of miR-671 were predicted using RegRNA software. Five apoptosis-associated genes including Akt2,caspase8, p38, Myc-associated factor X (MAX), and Ras protein-specific guanine nucleotide releasing factor 1 (RASGRF1) were identified (Fig. 6a). The dual luciferase reporter gene assay was used to determine whether there was a direct interaction between miR-671 and each target (Fig. 6b). MiR-671 overexpression and inhibition in N2a cells resulted in the down- and upregulation of Akt2, caspase8 and p38 levels, respectively (Fig. 6c). We also found that the expression of caspase8 and p38 protein was inversely proportional and that of Akt2 was directly proportion to miR-671 expression (Fig. 6d, e). Previous studies have demonstrated the apoptosis-inhibiting function of miR-671; Akt2 is presumed to inhibit while caspase8 and p38 stimulate apoptosis. Thus, miR-671 inhibits neuronal apoptosis via regulation of apoptosis-associated factors. The discrepancy between Akt2 mRNA and protein function via post-transcriptional mechanisms. We identified miRNAs that were predicted to interact with lncRpa and circRar1 using miRanda, Target Scan, and RegRNA (Padmashree and Swamy 2015; Hsu et al. 2007; Huang et al. 2006). Alignment of these miRNAs with seed sequences revealed that miR-671 and miR-218 interacted with both lncRpa and circRar1 (Fig. 3b). The dual luciferase reporter assay revealed that these miRNAs interact directly with lncRpa and circRar1, with miR-671 showing stronger binding to lncRpa and circRar1 (Fig. 3c). RNA antisense purification (RAP) and transcriptome sequencing analyses indicated that only miR-671 was a shared target of lncRpa and circRar1. The miRNAs identified by RAP were analyzed by qRT-PCR and gel electrophoresis. Compared to the input group with no RAP probe, miR-671 was present, whereas miR-218 was absent in the RAP group (Fig. 3d). Moreover, lncRpa and circRar1 bound miR-671 in the RAP but not the control group (Fig. 3e). These results indicate that lncRpa and circRar1 interact directly and specifically with miR-671.

LncRpa and circRar1 regulate miR-671 expression

The cellular localization of lncRpa, circRar1, and miR-671 was evaluated by FISH using the probes (Supplementary Table 2). All three molecules were co-expressed in the cytoplasm of N2a cells in the same pattern (Fig. 4a). In order to investigate the interaction between the three molecules, miR-671 was overexpressed or knocked down and lncRpa and circRar1 levels were evaluated by qRT-PCR. MiR-671 suppression resulted in the upregulation of lncRpa and circRar1, whereas miR-671 overexpression inhibited their expression (Fig. 4b). We also assessed the interaction between the three molecules by altering the expression levels of lncRpa or circRar1 and observing the effect on the expression of the other two molecules. We found that miR-671 expression was upregulated whereas that of circRar1 was downregulated upon lncRpa knockdown. On the other hand, lncRpa overexpression resulted in a decrease in miR-671 and increase in circRar1 levels (Fig. 4c). MiR-671 expression whereas circRar1 overexpression had the opposite effect (Fig. 4d). These results suggest negative regulation between lncRpa and circRar1 and a positive regulatory relationship between lncRpa and circRar1.
Fig. 3 Identification of IncRpa and circRar1 target miRNAs. 

(a) Detection of IncRpa and circRar1 by FISH. Green represents FISH probes of IncRpa and circRar1. Nuclei are counterstained with DAPI (blue). 

(b) Alignment of IncRpa and circRar1 and the seed sequences of miR-671 and miR-218. WT, wild-type sequence; Mut, sequence mutated in the dual luciferase reporter gene assay. 

(c) Dual luciferase assay. WT, wild-type vector; Mut, mutated vector; NC, blank control; miRNA mimic, miR-671 overexpression. 

(d) Identification of target miRNAs by RAP. No RAP probes were used for the input control. 

(e) Percentage of purified miRNAs relative to the input group, as detected by qRT-PCR. NC represents control only with beads (color figure online).
expression may be due to post-transcriptional regulation by factors other than miR-671 (Kim et al. 2007).

LncRpa and circRar1 regulate apoptosis-associated factors in lead-induced neurotoxicity

To confirm the mechanistic basis for the pro-apoptotic function of lncRpa and circRar1 in lead-induced neurotoxicity, the mRNA levels of Akt2, caspase8, and p38 were evaluated in N2a cells exposed to PbAc (Fig. 7a). The transcript levels of all three genes were increased by PbAc treatment, whereas the protein expression of caspase8 and p38 was increased and that of Akt2 was decreased under these conditions (Fig. 7b, c). To investigate the relationship between lncRpa and circRar1 and apoptotic proteins, we knocked down or overexpressed lncRpa and circRar1. The mRNA levels of Akt2, caspase8, and p38 were decreased by lncRpa or circRar1 knockdown, whereas their overexpression increased the transcript levels of the three targets (Fig. 7d). The protein levels of caspase8 and p38 were decreased, whereas that of Akt2 was increased by loss of lncRpa or circRar1 (Fig. 7e, f); the opposite trends were observed upon lncRpa or circRar1 overexpression. These data suggest that joint targeting of miR-671 by lncRpa and circRar1 is not the primary reason for the discrepancy between Akt2 mRNA and protein expression. Moreover, our findings indicate that lncRpa and circRar1 target caspase8 and p38 via miR-671 to induce neuronal apoptosis upon lead toxicity.

Discussion

High-throughput sequencing technology has broadened our understanding of gene regulatory networks. Whole genome sequencing has revealed that about 93% of the genome is transcribed as RNA, but only 2% encode proteins (Birney et al. 2007). Although the total number of nucleotides in the human genome is 30 times that of the nematode genome, the number of protein-coding sequences is comparable, which highlights the importance of ncRNA sequences in the regulation of eukaryotic gene expression (Costa 2008).

Environmental toxins such as lead can adversely affect human health, but the molecular mechanisms of lead-induced neurotoxicity are not well understood. Most research in this area has focused on the role of mRNAs (Soliman et al. 2015; Gao et al. 2016) or miRNAs (Li et al. 2015; Martinez-Pacheco et al. 2014), and there is little, if any, information on how lncRNAs and circRNAs are involved in lead-induced neurotoxicity. The roles of ncRNAs have been extensively investigated in the context of carcinogenesis and cancer development (Cheng et al. 2015). For example, H19 is aberrantly expressed in many types of cancer such as liver and bladder cancers and pancreatic ductal carcinoma (Ma et al. 2014; Luo et al. 2013; Tsang and Kwok 2007), while HOTAIR has been implicated in various aspects of cancer development (Wu et al. 2014). Genome-wide association studies have shown that most cancer risk loci are found in non-coding sequences.
Less is known about circRNAs, despite their prevalence in mammalian cells. These molecules also regulate gene expression at the post-transcriptional level (Memczak et al. 2013), and some have been found to be associated with tumors (Hansen et al. 2013b; Peng et al. 2015; Zhao and Shen 2015). LncRNAs interact in a complex regulatory network (Supplementary Fig. 1). LncRNAs alter chromatin structure via
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activation and transport of relevant proteins (Zhao et al. 2008; Tsai et al. 2010; Yao et al. 2010) and are also involved in the regulation of transcription factors (Hung et al. 2011) and mRNA and protein expression (Gong and Maquat 2011; Yoon et al. 2012). In many instances, lncRNAs carry out their functions by modulating the expression of miRNAs at the level of transcription, post-transcription, or splicing (Poliseno et al. 2010; Augoff et al. 2012; Steck et al. 2012; Wang et al. 2010). MiRNAs play a critical role in this regulatory network by directly targeting mRNAs (Orom et al. 2008). Previous studies have shown that miRNAs can be adsorbed by circRNAs, which act as endogenous miRNA competitors (Hansen et al. 2013a, b; Xu et al. 2015).

Fig. 5 Analysis of miR-671 function. a, b Downregulation of miR-671 expression in the hippocampus and cerebral cortex in a mouse model of lead-induced neurotoxicity (a) and in PbAc-treated N2a cells (b). Controls were tissue from mice and N2a cells that were not treated with PbAc. c Apoptosis of N2a cells expressing miR-671 mimic, as determined by FCM using annexin V-FITC/PI. NC, transfection reagent negative control group. d Quantitative analysis of apoptosis rate, calculated as the sum of UR % and LR %. e Apoptosis of N2a cells overexpressing miR-671, as detected with TUNEL. f Apoptotic index calculated from results in panel (e). g Caspase9 and caspase3 expression in N2a cells overexpressing miR-671, as determined by western blotting. Control, solvent control; NC, transfection reagent control; control + PbAc, PbAc treatment; mimic + PbAc, PbAc treatment after miR-671 overexpression; NC + PbAc, transfection reagent control with PbAc treatment. h Quantitative analysis of results shown in panel (g).

Fig. 6 MiR-671 target mRNAs and proteins. a Alignment of miR-671 and seed sequences of Akt2, caspase 8, p38, myc-associated factor (MA)X, and Ras protein-specific guanine nucleotide releasing factor (RASGRF1). WT, wild-type sequences; Mut, sequence mutated in the dual luciferase reporter gene assay. b Luciferase reporter gene assay. WT, wild-type vector; Mut, mutated vector; NC, blank control; miRNA mimic, mRNA overexpression. c MRNA expression of Akt2, caspase 8, and p38. NC, transfection reagent negative control; mimic, miR-671 overexpression; inhibitor, miR-671 knockdown. d Expression of Akt2, caspase 8, and p38 protein. Control, blank control; mimic, miR-671 overexpression; mimic NC, control for miR-671 overexpression; inhibitor, miR-671 knockdown; inhibitor NC, control for miR-671 knockdown. e Quantitative analysis of results shown in panel (d).
In this study, we found that lncRNA lncRpa and circRNA circRar1 were differentially expressed in lead-induced neurotoxicity and directly regulated miR-671 expression to promote neuronal apoptosis via upregulation of the pro-apoptotic proteins caspase8 and p38. We also found that miR-671 negatively regulate circRar1 and lncRpa. Thus, lncRpa and circRar1 jointly target miR-671 to modulate the expression of apoptosis-associated proteins in lead-induced neuronal apoptosis. These findings highlight a new mechanism of lead-induced neurotoxicity and provide a insight for the future investigations of the pathological process.

Fig. 7 Apoptotic proteins regulated by lncRpa and circRar1 in lead-induced neurotoxicity. a Expression of Akt2, caspase 8, and p38 mRNA, as determined by qRT-PCR. NC, cells not treated with PbAc. b Expression of Akt2, caspase 8, and p38 protein, as determined by western blotting. NC, cells not treated with PbAc. c Quantitative analysis of results shown in panel (b). d Changes in Akt2, caspase 8, and p38 mRNA expression after knockdown and overexpression of lncRpa and circRar1, as determined by qRT-PCR. NC, transfection reagent negative control. e Akt2, caspase 8, and p38 protein expression after knockdown and overexpression of lncRpa and circRar1, as determined by western blotting. Control and NC represent transfection reagent and empty vector negative control groups, respectively. f Quantitative analysis of results shown in panel (e).

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T. Y. carried out the Luciferase Reporter Gene Assay and analyzed the data. A. R. N. performed all other experiments and analyzed the data. A. R. N. and Y. G. J. wrote the manuscript. All authors read and approved the final manuscript.

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