LncRNA Rik-203 contributes to anesthesia neurotoxicity via microRNA-101a-3p and GSK-3β-mediated neural differentiation

Lei Zhang1, Jia Yan2, Qidong Liu2, Zhongcong Xie3 & Hong Jiang1

The mechanism of anesthesia neurotoxicity remains largely to be determined. The effects of long noncoding RNAs (LncRNAs) on neural differentiation and the underlying mechanisms are unknown. We thus identified LncRNA Rik-203 (C130071C03Rik) and studied its role on neural differentiation and its interactions with anesthetic sevoflurane, miRNA and GSK-3β. We found that levels of Rik-203 were higher in hippocampus than other tissues and increased during neural differentiation. Sevoflurane decreased the levels of Rik-203. Rik-203 knockdown reduced mRNA levels of Sox1 and Nestin, the markers of neural progenitor cells, and decreased the count of Sox1 positive cells. RNA-RNA pull-down showed that miR-101a-3p was highly bound to Rik-203. Finally, sevoflurane, knockdown of Rik-203, and miR-101a-3p overexpression all decreased GSK-3β levels. These data suggest that Rik-203 facilitates neural differentiation by inhibiting miR-101a-3p's ability to reduce GSK-3β levels and that LncRNAs would serve as the mechanism of the anesthesia neurotoxicity.

The widespread and growing use of anesthesia in children makes its safety a major health issue of interest [1, reviewed in2]. It has become a matter of even greater concern as evidence shows that multiple exposures to anesthesia and surgery may induce cognitive impairment in children3–8, and that anesthetics may induce neurotoxic damage and cognitive impairment in young animals9–13. These findings suggest that children who have undergone anesthesia and surgery may not develop to their full cognitive potential as they would have if they had not undergone anesthesia and surgery.

Sevoflurane, the most commonly used anesthetic in children, induces neurotoxicity and cognitive impairment in young mice14 and may regulate neurogenesis in vitro15,16. But, the underlying mechanism by which sevoflurane induces cognitive impairment remains largely unknown, which impedes further research into anesthesia neurotoxicity in the developing brain. Neural differentiation has been shown to contribute to cognitive impairment in young rodents17. Thus, in the present study, we set out to determine the effects of sevoflurane on neural differentiation and the underlying mechanisms.

Long non-coding RNAs (LncRNAs) are defined as transcripts that are longer than 200 nucleotides and are not translated into protein18. One of the functions of LncRNAs is to attach to microRNAs (miRNAs) as a sponge and to prevent the miRNA from binding to 3′UTR of target mRNA, thus inhibiting miRNAs ability19. miRNAs are endogenous short noncoding RNAs and regulate many physiological processes by targeting mRNA 3′UTR20–22. LncRNAs, e.g., NBAT-1 and Pnky, may regulate cell differentiation and development [21–23, reviewed in24]. However, the role of LncRNAs on neural differentiation, the process where Embryonic Stem Cells (ESCs) mature into specialized Neural Progenitor Cells (NPCs), which is crucial for cognitive function, neural development and

1Department of Anesthesiology, Shanghai Ninth People’s Hospital, Shanghai Jiao Tong University School of Medicine, Center for Specialty Strategy Research of Shanghai Jiao Tong University China Hospital Development Institute, Shanghai, P.R. China. 2Shanghai Tenth People’s Hospital, Anesthesia and Brain Research Institute, Tongji University School of Medicine, Shanghai, P.R. China. 3Department of Anesthesia, Critical Care and Pain Medicine, Massachusetts General Hospital and Harvard Medical School, 149 13th Street, Room, 4310, Charlestown, MA, USA. Lei Zhang and Jia Yan contributed equally. Zhongcong Xie and Hong Jiang jointly supervised this work. Correspondence and requests for materials should be addressed to Z.X. (email: zxie@mgh.harvard.edu) or H.J. (email: jianghongjiuyuan@163.com)
neurotoxicity, remains largely unknown. Therefore, we used the anesthetic sevoflurane as a tool to determine the clinically relevant function of specific LncRNA and the underlying mechanism.

We identified a novel LncRNA (Rik-203: C130071C03Rik) and systematically investigated its interaction with the anesthetic sevoflurane, miRNA, the mRNA and protein of Glycogen Synthase Kinase-3β (GSK-3β). The objective of these studies was: (1) to elucidate the LncRNA-associated underlying mechanisms of anesthesia neurotoxicity; and (2) to investigate the pathway by which Rik-203 regulated neural differentiation via miR-101a-3p and GSK-3β. The hypothesis in the present studies was that the reduction of Rik-203 by sevoflurane released miR-101a-3p, which then acted on the 3′UTR of mRNA of GSK-3β, leading to reduction of mRNA of GSK-3β and consequent inhibition of neural differentiation.

Methods
RNA sequencing and analysis of the gene expression profiles of mRNAs, LncRNAs and miRNAs.

We harvested the cells by centrifuging at 1000 × g for 2 min in the centrifuge tube. Removed the supernatant and added the RNAiso plus (Takara, China) to suspend and lysed the cells. We also harvested the mouse hippocampus tissues, and sent the cells and tissue samples protected by dry ice to the Beijing Genomics Institute (Beijing, China) for RNA-sequencing as well as the analysis of gene expression profiles of mRNA and LncRNA. In addition, we sent hippocampus tissues of mice to NovelBio (Shanghai, China) for the RNA-sequencing and analysis of gene expression profiles of the miRNA. The RNA sequencing library generation, workflow, data analysis, and enrichment analysis were performed as reported previously34,35. Illumina Hiseq2500/Hiseq3000 platform was used for sequencing. After trimming using sickle.pe (pair-end) (v1.29, https://github.com/najoshi/sickle) with parameters (−q 20, −130), sequenced reads were mapped to genome in mouse(mm10) using Tophat (2.0.7) with the default parameters and Ensembl genome annotation (Mus_musculus.GRCm38.73.gtf)30. Each gene expression level (fragments per kilobase of exon per million fragments mapped) is estimated using Cufflinks (v2.0.2) software41. Differentially expressed genes (DEGs) were detected by Cuffdiff32. False discovery rate (FDR) assay is used for adjusting multiple tests. FDR < 0.05 was chosen to indicated the statistical significance.

Establishment of inducible Rik-203 knockdown mESCs lines. We designed the specific shRNA nucleotides targeting the transcripts of LncRNA Rik-203. The primers are as follows: shRNA-1, 5′-CCGG GGTTTGGCC AGTCTCTATATCGAG AATAAGGAACTGGCC AAACACCTTTTT TGG-3′. PR5′-AATTCAAAAAGGTGTTGGCCAGTCTCTATATCGAGAATAAGGAACTGGCCAAACACCTTTTT TGG-3′. shRNA-2, 5′-CCGG GCCTTTGAATTCAGGCTGACTCAGTGAACGCTGATTCTCA AGCTTTTTTGG-3′. PR5′-AATTCAAAAAGGTGTTGGCCAGTCTCTATATCGAGAATAAGGAACTGGCCAAACACCTTTTT TGG-3′. Each gene expression level (fragments per kilobase of exon per million fragments mapped) is estimated using Cufflinks (v2.0.2) software41. Differentially expressed genes (DEGs) were detected by Cuffdiff32. False discovery rate (FDR) assay is used for adjusting multiple tests. FDR < 0.05 was chosen to indicated the statistical significance.

Neural differentiation of mESCs. We performed the neural differentiation of mESCs by using the methods described in previous studies33,34. The detail of the neural differentiation is as follows: we performed neural differentiation studies by using 46c mouse embryonic stem cells (mESCs). 46c is a Sox1-GFP reporter ESCs line that recapitulates endogenous Sox1 expression when GFP is expressed. 46c mESCs were dissociated into single cells using 0.05% trypsin (Gibco, USA) and then neutralized with DMEM (Gibco, USA) containing 10% FBS. After being counted, mESCs were washed with GMEM (Gibco, USA) and re-suspended in a Petri dish at a density of 25,000–50,000/mL using the neural differentiation medium GMEM with 8% Knockout Serum Replacement (KOSR) (Gibco, USA), 1% L-glutamine, 1% sodium pyruvate, and 0.1 mM β-mercaptoethanol. The medium was changed every day for 7 days until the single-cell clone could be identified under a microscope. Clones were picked up and dissociated with trypsin and plated onto feeder cell-coated 24-well plates.

Sevoflurane anesthesia for treating mice and cells. C57BL/6j mice at postnatal day 6 (P6) (Shanghai SLAC Laboratory Animal, Zhangjiang, Shanghai, P. R. China) were used in the studies. The animal protocol was approved by the Standing Committee on Animals at Shanghai Ninth People's Hospital, Shanghai, China. All experiments were performed in accordance with relevant guidelines and regulations. According to the previous studies, the mice received the 3% sevoflurane anesthesia 2 hours daily at 6, 7, 8 days after birth to mimic the clinical several times anesthesia14,35,36, which is reported to induce the neurotoxicity and further cognitive function defect37. 3% is also the clinical concentration of sevoflurane for anesthesia38. The hippocampus tissues of mice were harvested at the end of the sevoflurane anesthesia administration. Treatment of the cells with 4.1% sevoflurane was similar to that as described in previous studies34,39,40. Specifically, the cells were treated with 4.1% sevoflurane for 2 hours daily at day 4, 5 and 6 after the start of neural differentiation to mimic the clinical several times anesthesia. The cells were harvested at day 7 during the neural differentiation, at which there're many NPCs. In some experiments, the cells were transfected with GSK-3β 12 hours before the sevoflurane treatment.
Flow cytometry studies. The cells were suspended in PBS for flow cytometry analysis by using FACS Calibur (BD Biosciences, USA) operating at 488 nm excitation with standard emission filters. Fluorescence noise baseline was referenced with the 46C mESCs. FlowJo software was used to analyze the results.

Reverse transcription PCR and real-time quantity PCR. RNA was extracted with RNAiso Plus (TaKaRa, China). Inverse transcription of mRNA to cDNA was performed by using a cDNA Synthesis Kit (TaKaRa, China). Inverse transcription of miRNAs to cDNA was carried out with the TIANScript RT Kit (Tiangen, China). The PCR primers of miRNA were purchased (Ribobio, China). Primers for the qRT-PCR analysis of mRNA are as following sequences: Rik-203: PF: 5'-CATCATTGGACATGGAACATA-3', RF: 5'-GAACTCATACATGGAAGCAT-3'; Sox1: PF: 5'-GTGGTTTGATGTGGTGACC-3', RF: 5'-GCATTAAAGAATAATAC-3'; Nestin: PF: 5'-GAATGTCAGGCAGAAAAACT-3', RF: 5'-TCTTCAAACCTTGAGCCTC-3'; and GAPDH: PF: 5'-ATGACATCAAGAGGTTGGT-3', RF: 5'-CATACCAGGAAATGAGCTTG-3'.

Nuclear and cytoplasm RNA extraction. We carried out the nuclear and cytoplasm extraction studies using the methods described previously. Specifically, 1 × 10^5 mESCs-derived NPCs were prepared for this assay. The cells were washed 3 times with phosphate buffered saline (PBS) and centrifuged at 1,000 × g for 5 minutes. Then, lysis buffer working reagent (Tris (10 mM, pH 8.0), NaCl (140 mM), MgCl2 (1.5 mM) 0.5% Nonidet P-40 (NP-40)) was added to the cells and then placed into an icebox and shaken at 200 rpm on a platform for 2 hours. The samples were centrifuged at 12,000 × g for 5 min at 48 °C, and finally the nuclear and cytoplasm extract was obtained. Then, RNAiso plus (Takara) was used for RNA purification. The RNA level from cytoplasmic and nuclear was detected using quantitative RT-PCR.

RNA pull-down assay. 1 × 10^5 mESC-derived NPCs were used for the studies. Full-length C130071C03Rik and the antisense RNA were transcribed into the cells using T7 RNA polymerase. 50 pmol of C130071C03Rik, or C130071C03Rik's antisense RNA, was labeled using desthiobiotin and T4 RNA ligase via a PierceTM RNA 3'End Desthiobiotinylaton Kit (Thermo). The RNA pull-down assay was performed according to the PierceTM Magnetic RNA-Protein Pull-Down Kit (Thermo) and parts of the experiments were performed in the core facilities in Yingbiotech (Shanghai, China). In addition, the cells were briefly lysed with Pierce IP Lysis Buffer, and incubated on ice for 5 minutes. The lysates were centrifuged at 13,000 × g for 10 minutes, and the supernatant was transferred to a new tube for further analysis. The labeled RNA was added to 50 μL of beads, and incubated for 30 minutes at room temperature with agitation. The RNA-bound beads were incubated with the lysates for 60 minutes at 4°C. The RNA-Binding miRNAs were washed and eluted, and the binding miRNAs were detected using qRT-PCR. Primers for the qRT-PCR analysis of miRNA include the following list. Primer list of Stem-loop was transferred to a new tube for further analysis. The labeled RNA was added to 50 μL of beads, and incubated for 60 minutes at 4°C. The RNA-Binding miRNAs were washed and eluted, and the binding miRNAs were detected using qRT-PCR.

Luciferase assays. A pGL3-cm vector was used to construct the 3'UTR luciferase reporter. The fragment of 3'UTR was amplified from mESCs DNA by the primers in the following list. For miR-101a-3p binding sites of GSK-3β 3'UTR region: PF: 5'-GGCGTGAGCGGGGAAACACAGGAAACAC-3', PR: 5'-GCTCTAGATTTTTGGCGGC-3'. For miR-101a-3p binding sites of Rik-203 region: PF: 5'-GGGTGTAGACCAAGGCTC-3', PR: 5'-GGGTGTAGACCAAGGCTC-3'. The mutant GSK-3β 3'UTR or Rik-203 reporter vector was obtained by replacing the miR-101a-3p binding site sequences using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, USA).

Western blot. Cells were lysed using SDS buffer (Beyotime, China) to obtain the protein for electrophoresis. The whole protein was transferred onto the PVDF membrane (Whatman, USA). Primary antibodies that were used in incubation include GAPDH (ab8245, Abcam), which was used for normalizing the protein levels, and GSK-3β (#12456, Cell Signaling, USA)42–44. Protein expression signaling was visualized through enhanced chemiluminescence (ECL) substrate (Thermo).

Overexpression of GSK-3β by the pcDNA3.1-GSK-3β vector. The whole RNA was isolated. Inverse transcription to cDNA was performed using the cDNA Synthesis Kit (TaKaRa). GSK-3β CDS fragments were amplified and inserted into the pcDNA3.1 vector. The primer's sequence includes the following list: PF: 5'-GGCGTCTAGATGTGGGAGCGGAGAAACAC-3' (restriction enzyme site, Nhe1), PR: 5'-GGCGTCTAGATGTGGGAGCGGAGAAACAC-3' (restriction enzyme site, Nhe1). The vector was transfected into the cells by using Lipofectamine 2000 (Thermo) and the instructions for the reagent.

Overexpression of miR-101a-3p. The pLVX-puro-miR-101-3p overexpressed vector (Biogot technology, co, Ltd, China) was transfected into the embryonic bodies derived from 46C mESCs during the neural differentiation at day 3 and day 5 using Lipofectamine 2000 (Thermo) following the instructions given to overexpress the miR-101a-3p.

Overexpression of IncRNA Rik-203. The full length transcripts of IncRNA Rik-203 was cloned into the FUW vector the corresponding primers: 5'-GGCGGATCCCTCTCTCTCTCACAAGCTCAT-3' (BamH1 restriction enzyme), 5'-GGCGGAATTCACGCTGAAATATTTATTGGAAGGTTGGT-3', RF: 5'-CATACCAGGAAATGAGCTTG-3'.
Mutant Rik-203 overexpression vector. Mutant Rik-203 overexpression vector was constructed by replacing the miR-101a-3p seed sequence binding site of the wildtype Rik-203 overexpression vector using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, USA).

Statistics. The data were presented as mean ± standard deviation (SD) with three independent experiments. The significance of statistics was determined by a Student’s t-test or one-way ANOVA. *p < 0.05, **p < 0.01, ***p < 0.001. The studies employed a two-tailed hypothesis and statistically significant p values were < 0.05. We used the Graph Pad (Software Inc., San Diego, California, USA) to evaluate all of the study data.

Results
Rik-203 regulated neural differentiation. A recent study indicated the novel LncRNA ECONEXIN that performed the ceRNA function to promote the gliomagenesis, which suggested its potential role of neural related regulation. Interestingly, we found that Rik-203, the ECONEXIN homologous gene in mouse, was higher expressed in the hippocampus tissues of mice than in their heart, lung, intestine and kidney tissues (Fig. 1A). We then found that there’s an increase of Rik-203 expression on day 3 and 5 after the neural differentiation from embryonic stem cells (ESCs) 46c (Fig. 1B). RT-PCR confirmed these results and demonstrated that such increases in Rik-203 levels were higher on day 7 after the induction of the neural differentiation than on days 3 and 5 (Fig. 1C). These data suggest that Rik-203 was present in higher levels in the hippocampus and that the levels increase during neural differentiation.

We established the Doxycycline (Dox) inducible RNA interference (RNAi) knockdown of Rik-203 (Fig. 1D) in the ESCs, and revealed that knockdown of Rik-203 induced by Dox begin at day 2 during the neural differentiation form the mESCs decreased the number of sex determining region Y-box 1 (Sox1) positive cells (Fig. 1E). We also found that there was an increase of Rik-203 expression on day 3 and 5 after the neural differentiation from embryonic stem cells (ESCs) 46c (Fig. 1B). RT-PCR confirmed these results and demonstrated that such increases in Rik-203 levels were higher on day 7 after the induction of the neural differentiation than on days 3 and 5 (Fig. 1C). These data suggest that Rik-203 was present in higher levels in the hippocampus and that the levels increase during neural differentiation.

The anesthetic sevoflurane decreased Rik-203 levels and the Rik-203-associated neural differentiation. We used the anesthetic sevoflurane to further determine the clinically relevant role of Rik-203 in neural differentiation. RNA-seq analysis showed that sevoflurane decreased the levels of Rik-203 in the hippocampus tissues of mice (Fig. 2A). The clinical effect of anesthesia is dose dependent. We found that sevoflurane also decreased Rik-203 mRNA levels in the hippocampus tissues of mice in a dose-dependent manner (Fig. 2B), and in NPCs (Fig. 2C).

Next, we found that sevoflurane reduced Sox1 positive cells at day 7 after the start of neural differentiation of ESCs into NPCs, and that the overexpression of Rik-203 prevented such reductions (Fig. 2D). FACS also showed that overexpression of Rik-203 mitigated the sevoflurane-induced reduction of Sox1 positive cells (Fig. 2E). Sevoflurane decreased mRNA levels of both Sox1 and Nestin, the markers of NPCs (Fig. 1G). These results suggest the role of Rik-203 in the neural differentiation process where the reduction of Rik-203 levels inhibited neural differentiation.

We established the Doxycycline (Dox) inducible RNA interference (RNAi) knockdown of Rik-203 (Fig. 1D) in the ESCs, and revealed that knockdown of Rik-203 induced by Dox begin at day 2 during the neural differentiation form the mESCs decreased the number of sex determining region Y-box 1 (Sox1) positive cells (Fig. 1E). We then found that there’s an increase of Rik-203 expression on day 3 and 5 after the neural differentiation from embryonic stem cells (ESCs) 46c (Fig. 1B). RT-PCR confirmed these results and demonstrated that such increases in Rik-203 levels were higher on day 7 after the induction of the neural differentiation than on days 3 and 5 (Fig. 1C). These data suggest that Rik-203 was present in higher levels in the hippocampus and that the levels increase during neural differentiation.

We used the anesthetic sevoflurane to further determine the clinically relevant role of Rik-203 in neural differentiation. RNA-seq analysis showed that sevoflurane decreased the levels of Rik-203 in the hippocampus tissues of mice (Fig. 2A). The clinical effect of anesthesia is dose dependent. We found that sevoflurane also decreased Rik-203 mRNA levels in the hippocampus tissues of mice in a dose-dependent manner (Fig. 2B), and in NPCs (Fig. 2C).

Next, we found that sevoflurane reduced Sox1 positive cells at day 7 after the start of neural differentiation of ESCs into NPCs, and that the overexpression of Rik-203 prevented such reductions (Fig. 2D). FACS also showed that overexpression of Rik-203 mitigated the sevoflurane-induced reduction of Sox1 positive cells (Fig. 2E). Sevoflurane decreased mRNA levels of both Sox1 and Nestin, the markers of NPCs, while Rik-203 overexpression prevented sevoflurane from inducing such effects (Fig. 2F). RNA-seq analysis illustrated that 29.4% and 30.6% overlap of the down- and up-regulation of genes following knockdown of Rik-203 and sevoflurane treatment, respectively, in mESCs (Fig. 2G). Taken together, these data demonstrated the role of LncRNA Rik-203 in anesthelia neurotoxicity where the most commonly used inhalation anesthetic sevoflurane was able to regulate the levels of Rik-203 and the Rik-203-regulated neural differentiation. These data suggest that LncRNAs could be a potential novel target for research revolving the molecular mechanisms of the anesthesia neurotoxicity.

Rik-203 regulated the function of miR-101a-3p level through a ceRNA mechanism. LncRNA often has different mechanisms based on its localization in cells. We thus compared the levels of Rik-203 in the cytoplasm and nucleus by using RT-PCR, and found that there were higher levels of Rik-203 in the cytoplasm than in the nucleus (Fig. 3A). We found that miR-101a-3p could bind with the Rik-203 (Supplemental Fig. 1A) and then we performed a RNA pull-down assay and revealed that miR-138-2-3p, miR-101a-3p and miR-467a-3p were highly bound to Rik-203 (Fig. 3B). We also performed luciferase reporter assay to detect the direct interaction of miR-101a-3p and Rik-203 and found that that overexpression of miR-101a-3p by mimics significantly repressed the luciferase activity of the reporter gene containing Rik-203 binding site, but could not influence the luciferase activity of reporter with mutant Rik-203 binding site (Supplemental Fig. 1B). These data suggest that Rik-203 within the cytoplasm may attach to miRNA. We also found that there were higher levels of Rik-203 in the cytoplasm than those of miR-138-2-3p and miR-467a-3p. Specifically, miR-101a-3p was ranked the 26th among the 1915 expressions of miRNAs in the mice hippocampus tissues (Fig. 3C). We also found that sevoflurane did not affect the levels of miR-101a-3p (Fig. 3D). These results suggest that sevoflurane likely acts on Rik-203, but not on miR-101a-3p, to decrease neural differentiation. Collectively, these findings support the competing endogenous RNA (ceRNA) hypothesis that Rik-203 may serve as a "spouse" to tie with miRNAs and prevent the binding of miRNAs to their target mRNAs. By overexpressing miR-101a-3p (Fig. 3E), we found that miR-101a-3p decreased the Sox1 positive cells whereas in contrast the overexpression of Rik-203 mitigated such decreases (Fig. 3F). FACS studies further indicated that miR-101a-3p reduced Sox1 positive cells, and that overexpression of Rik-203 mitigated such reductions (Fig. 3G). Furthermore, overexpression of miR-101a-3p reduced the miRNA levels of the NPC markers Sox1 and Nestin, which were mitigated by the overexpression of Rik-203 (Fig. 3H). These findings suggest that Rik-203 can bind to and interact with miR-101a-3p, leading to the facilitation of neural differentiation. We also found that miR-467a-3p inhibited neural differentiation (Supplemental Fig. 1C). Given the fact that miR-101a-3p has higher levels in the hippocampus than miR-467a-3p, we focused solely on determining the effects of miR-101a-3p on neural differentiation and its interaction with Rik-203.

**MiR-101a-3p targeted GSK-3β to mediate the Rik-203-associated neural differentiation.** GSK-3β has been shown to be regulated by the anesthetic sevoflurane and linked to miR-101a-3p. Thus, we
Figure 1. Rik-203 regulates neural differentiation. (A) Rik-203 levels in different tissues of mice, detected by RT-PCR. The hippocampus has the highest level of Rik-203. (B) Microarray studies revealed the increase of Rik-203 expression during the neural differentiation from ESCs to NPCs. (C) The increase of Rik-203 mRNA levels during the neural differentiation from ESCs to NPCs was confirmed by RT-PCR at day 3, 5 and 7 after the induction of neural differentiation. (D) knockdown of Rik-203 decreased levels of Rik-203. (E) Measurement of Sox1 positive cells indicated that knockdown of Rik-203 decreased the number of Sox1 positive cells. (F) The quantification of Sox1 positive cells using FACS showed that knockdown of Rik-203 decreased the number of Sox1 positive cells. (G) RT-PCR showed that the mRNA levels of Sox1 and Nestin were decreased through knockdown of Rik-203. The scale bar represents 100 um. FACS: Fluorescent-activated cell sorting. Rik-203: C130071C03Rik; ESCs: Embryonic Stem Cells; NPCs: Neural Precursor Cells; shRNA: Short hairpin RNA; GFP: Green Fluorescent Protein; Dox: Doxycycline. Ctrl: control; Sox1: SRY (sex determining region Y)-box 1. The data were presented as mean ± standard deviation (SD) with three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001; by one-way ANOVA (A,C,D,F,G).
assessed the interactions of sevoflurane, Rik-203, miRNA, and GSK-3β. We employed a RNA-seq in the experiments and found that sevoflurane decreased mRNA levels of GSK-3β (Fig. 4A). We performed the doxycycline (Dox) inducible knockdown of Rik-203 at day 2 after the Dox induction and found that Gsk-3β was downregulated during the neural differentiation detected at day 4 (Fig. 4B). Using the online miRNA target prediction...
Figure 3. Rik-203 regulates miR-101a-3p level by sequestering miRNA through a ceRNA mechanism. (A) Detection of cytoplasmic and nuclear distribution of Rik-203 by fractionation. RT-PCR showed that there were higher levels of Rik-203 in the cytoplasm than in the nucleus. (B) RNA-RNA pull-down assay showed that miR-138-2-3p, miR-101a-3p and miR-467-3p were highly bound to Rik-203. (C) miR-101a was highly expressed in the hippocampus tissue. (D) Sevoflurane did not affect the mRNA level of miR-101a-3p in mice hippocampus tissues. (E) qPCR showed the ectopic expression of miR-101a effect in the NPCs. (F) Overexpression of Rik-203 mitigated the miR-101a-3p-induced reduction in Sox1 positive cells. (G) FACS analysis showed that miR-101a-3p reduced the number of Sox1 positive cells, which was mitigated by the overexpression of Rik-203. (H) Overexpression of Rik-203 restored the expression level of neural differentiation. The scale bar represents 100 µm. The data were presented as mean ± standard deviation (SD) with three independent experiments.* or *p < 0.05; ** or **p < 0.01; by t-test (B,H,I) and one-way ANOVA (E,F). CeRNA: Competing endogenous RNA; NPCs: Neural Precursor Cells.
software TargetScan and miranda, we predicted that GSK-3β might be targeted by miR-101a-3p (Fig. 4C).

These data revealed the role of Rik-203 in the metabolism of GSK-3β and suggest that sevoflurane may regulate GSK-3β levels by acting on Rik-203.

Next, we examined the interaction of miRNA with sevoflurane, Rik-203 and GSK-3β. We engineered luciferase reporters that had the wild-type 3'UTRs of GSK-3β or the mutant UTRs without the miRNA seed sequence-binding site. The luciferase report assay indicated that miR-101a-3p targeted wild-type GSK-3β 3'UTR but not mutant UTR (deletion of the miRNA binding seed sequence). Overexpression of miR-101a-3p decreased the protein level of GSK-3β. Overexpression of GSK-3β mitigated the miR-101a-3p-induced reduction in Sox1 positive cells. FACS analysis showed that overexpression of GSK-3β mitigated the knockdown of Rik-203-induced decrease in Sox1 positive cells. FACS analysis showed that overexpression of GSK-3β mitigated the knockdown of Rik-203-induced decrease in the levels of Sox1 positive cells. The scale bar represents 100µm.

The data were presented as mean + standard deviation (SD) with three independent experiments.* or **p < 0.05; ** or ***p < 0.01; by t-test (D,E) and one-way ANOVA (H,I,L). GSK-3β: Glycogen synthase kinase-3β; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; NPCs: Neural Precursor Cells.

**Figure 4.** miR-101a-3p targets GSK-3β to mediate the Rik-203-associated neural differentiation. (A) qRT-PCR detection showed that sevoflurane decreased the expression of GSK-3β. (B) Knockdown of Rik-203 decreased the protein levels of GSK-3β in NPCs. (C) Target validation of the binding of GSK-3β 3'UTR by miR-101a-3p. (D) Luciferase report assay indicated that miR-101a-3p targeted wild-type GSK-3β 3'UTR but not mutant UTR (deletion of the miRNA binding seed sequence). (E) Overexpression of miR-101a-3p decreased the protein level of GSK-3β. (F) Overexpression of GSK-3β mitigated the miR-101a-3p-induced reduction in Sox1 positive cells. (G) FACS analysis showed that overexpression of GSK-3β mitigated the miR-101a-3p-induced decrease in the levels of Sox1 positive cells. (H) Overexpression of GSK-3β mitigated the knockdown of Rik-203-induced reduction in Sox1 positive cells. (I) FACS analysis showed that overexpression of GSK-3β mitigated the knockdown of Rik-203-induced decrease in the levels of Sox1 positive cells. The scale bar represents 100µm.
of GSK-3β. Additionally, we mutant the Rik-203 overexpression vector by replacing the miR-101a-3p binding site with the sequence that was the same as miR-101a-3p seed sequence. Then we found that overexpression of wild type but not mutant Rik-203 could restore the GSK-3β downregulated by miR-101a-3p. (Supplementary Fig. 2D). Overexpression of GSK-3β mitigated the knockdown of Rik-203-induced decrease of mRNA levels of GSK-3β (Supplemental Fig. 2E) and the Sox1 positive cells (Fig. 4H,I, Supplementary Fig. 2F), and reduced mRNA levels of Sox1 and Nestin (Supplemental Fig. 2G).

Discussion
Sevoflurane has extensive regulation effect to tissues by different physiological processes. Previous studies showed that sevoflurane impairs insulin secretion, to induce insulin resistance27. Administration of sevoflurane before cardiopulmonary bypass induced cardioprotection in patients undergoing coronary artery bypass graft surgery28. However, sevoflurane also was reported to inhibit cardiac function in pulmonary fibrosis mice29. These studies suggested the toxicity of sevoflurane is systematically and complex. Previous study indicated that infants received multiple but not single anesthesiology have higher increased risk of further cognitive impair40,41. In the current studies, we mimic the clinical interval multiple anesthesiology operation to treated the mouse with sevoflurane (3%) plus 60% oxygen (balanced with nitrogen) 2 h daily for 3 consecutive days as performed in previous studies40,41. We showed for the first time that the anesthetic sevoflurane decreased levels of LncRNA Rik-203 in the hippocampus tissues of the mice. Such reductions resulted in the inhibition of neural differentiation via the cascade action of miRNA (miR-101a-3p) and GSK-3β. These data showed the clinical and physiological relevance effects of Rik-203 and suggest that LncRNA Rik-203 would serve as the underling mechanism for anesthesia neurotoxicity.

The mechanics insight of the current studies was that Rik-203, a hippocampus rich LncRNA located in the cytoplasm, interacted with miR-101a-3p and served as a “sponge” to compete with downstream target mRNAs for the binding with miR-101a-3p. The reduction of Rik-203, by knockdown or by sevoflurane, released miR-101a-3p, which then acted on the 3′UTR of mRNA of GSK-3β, leading to reduction of mRNA of GSK-3β and consequent inhibition of neural differentiation (Supplemental Fig. 3A,B).

LncRNA C130071C03Rik has 5 transcripts (splice variants). A recent study identified a novel LncRNA Rik-201 and demonstrated its functional role in gliomagenesis43. The findings from the current study showed, for the first time, that Rik-203 contributed to neural differentiation through acting on miRNA and GSK-3β. Although other studies have reported that miR-101a-3p regulates GSK-3β activity in the glioblastoma44, the role of miR-101a-3p in regulating neural differentiation was first reported in this study.

LncRNAs are known to function as epigenetic modulators to orchestrate epigenetic processes45. Although LncRNAs have been found to play crucial roles in developmental and neurodegenerative diseases46, their function in anesthesia-induced influence is not very clear. Some LncRNAs expression has been reported to be associated with the sevoflurane. LncRNA Gadd45a upregulation is associated with sevoflurane-induced neurotoxicity in rat neural stem cells47. LINC00652 reduce the protective effect of sevoflurane on myocardial ischemia–reperfusion injury in mice48. These studies suggested the complex regulation of sevoflurane to LncRNAs and indicated the potential different signaling pathway of sevoflurane /LncRNAs axis. Our studies showed that sevoflurane decreased the levels of Rik-203, which is mainly located in the cytoplasm, and inhibited neural differentiation via its downstream effects on miR-101a-3p and GSK-3β.

Lu et al. also reported that sevoflurane was able to increase the level of LncRNA Gadd45a in the rat hippocampus neural stem cells49. However, the studies to determine the downstream effects and the underlying mechanisms were not performed. Our studies specifically showed that sevoflurane decreased Rik-203 levels, leading to miRNA- and GSK-3β–regulated inhibition of neural differentiation.

Several studies found that sevoflurane caused neurotoxicity by directly regulating the expression of miRNAs,60,61. In the present study, we showed that sevoflurane might still regulate the function of miRNA without directly affecting the levels of miRNA (Fig. 3). Rather, sevoflurane decreased the levels of Rik-203, which led to the release of the miR-101a-3p from Rik-203. The released miR-101a-3p then decreased the levels of GSK-3β, leading to the inhibition of neural differentiation. Additionally, miR-9 is widely studied in the neural related physiological progress and reported to be necessary for neural differentiation42,64. Here we found that full length of LncRNA C130071C03Rik has interaction with miR-9, but the variant Rik-203 has no interaction with miR-9.

Specifically, the reduction of Rik-203, by knockdown or by sevoflurane, inhibited the “sponge” function of Rik-203, which then released miR-101a-3p to reduce GSK-3β levels and led to the inhibition of neural differentiation (Supplemental Fig. 3). Moreover, both miR-101a-3p and sevoflurane46,67 have been reported to increase interleukin-6 (IL-6) levels. These findings suggest that sevoflurane may induce other effects, e.g., neuroinflammation, via regulating miRNA, through LncRNAs, e.g., like Rik-203. Future studies to test this hypothesis are warranted.

There are several limitations in the studies. First, we did not perform in vivo relevance studies on the in vitro findings of the cascade of “sevoflurane, Rik-203, miR-101a-3p, GSK3β and neural differentiation”. However, the data from the present studies demonstrated that sevoflurane could inhibit neural differentiation via LncRNAs and mRNA. Second, Rik-203 bound to miR-138-2-3p, miR-101a-3p and miR-467a-3p. However, we did not perform downstream studies of miR-138-2-3p and miR-467a-3p.

In conclusion, we identified the functional role of LncRNA Rik-203 in facilitating neural differentiation and elucidated the underlying miRNA-GSK-3β-associated molecular mechanisms, which could promote further studies of the role of LncRNA on neural differentiation.
44. Shi, M., Duan, G., Nie, S., Shen, S. & Zou, X. Elevated FAM3C promotes cell epithelial-mesenchymal transition and cell migration.

43. Liu, W.

55. Davidson, A. J.

48. Tian, T., Mingyi, M., Qiu, X. & Qiu, Y. MicroRNA-101 reverses temozolomide resistance by inhibition of GSK3beta in glioblastoma.

49. Lewis, B. P., Shih, I. H., Jones-Rhoades, M. W., Bartel, D. P. & Burge, C. B. Prediction of mammalian microRNA targets.

50. Lewis, B. P., Burge, C. B. & Bartel, D. P. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 120, 19–20, https://doi.org/10.1016/j.cell.2004.12.035 (2005).

51. Hoyer, K. F., Nielsen, T. S., Risis, S., Treebak, J. T. & Jessen, N. Sevoflurane Impairs Insulin Secretion and Tissue-Specific Glucose Uptake In Vivo. Basic Clin Pharmacol Toxicol 123, 732–738, https://doi.org/10.1111/bcpt.13087 (2018).

52. Lemoine, S., Zhu, L., Gerard, J. L. & Hanouz, J. L. Sevoflurane-induced cardioprotection in coronary artery bypass graft surgery: Randomised trial with clinical and ex-vivo endpoints. Anaesth Crit Care Pain 37, 217–223, https://doi.org/10.1016/j.accp.2017.05.009 (2018).

53. Cao, Y. N. et al. Sevoflurane inhibits cardiac function in pulmonary fibrosis mice through the TL4 signaling pathway. Pulm Circ 8, https://doi.org/10.1177/2045894018800702 (2018).

54. Sprung, J. et al. Attention-deficit/hyperactivity disorder after early exposure to procedures requiring general anesthesia. Mayo Clinic proceedings 87, 120–129, https://doi.org/10.1016/j.mayocp.2011.11.008 (2012).

55. Davidson, A. J. et al. Neurodevelopmental outcome at 2 years of age after general anaesthesia and awake-regional anaesthesia in infancy (GAS): an international multicentre, randomised controlled trial. Lancet 387, 239–250, https://doi.org/10.1016/S0140-6736(15)00608-X (2016).

56. Mercer, T. R. & Mattick, J. S. Structure and function of long noncoding RNAs in epigenetic regulation. Nat Struct Mol Biol 20, 300–307, https://doi.org/10.1038/nsmb.2480 (2013).

57. Wan, P., Su, W. & Zhou, Y. The Role of Long Noncoding RNAs in Neurodegenerative Diseases. Mol Neurobiol 54, 2012–2021, https://doi.org/10.1007/s12052-016-9793-6 (2017).

58. Lu, G. et al. Upregulation of long noncoding RNA Gadd45a is associated with sevoflurane-induced neurotoxicity in rat neural stem cells. Neuroreport 29, 605–614, https://doi.org/10.1097/WNR.0000000000000980 (2018).

59. Zhang, S. B. et al. Suppression of Long Non-Coding RNA LINC00652 Restores Sevoflurane-Induced Cardioprotection Against Myocardial Ischemia-Reperfusion Injury by Targeting GLP-1R Through the cAMP/PKA Pathway in Mice. Cellular physiology and biochemistry: international journal of experimental cellular physiology, biochemistry, and pharmacology 49, 1476–1491, https://doi.org/10.1007/s00973-017-0635-2 (2018).

60. Zhou, X. et al. MicroRNA-34c is regulated by p53 and is involved in sevoflurane-induced apoptosis in the developing rat brain potentially via the mitochondrial pathway. Mol Med Rep 15, 2204–2212, https://doi.org/10.3892/mmr.2017.6268 (2017).

61. Lv, Y. et al. MicroRNA-27a-3p suppression of peroxisome proliferator-activated receptor-gamma contributes to cognitive impairments resulting from sevoflurane treatment. J Neurochem 143, 306–319, https://doi.org/10.1111/jnc.14208 (2017).

62. Shibata, M., Nakao, H., Kiyonari, H., Abe, T. & Aizawa, S. MicroRNA-9 regulates neurogenesis in mouse telencephalon by targeting PP2A dependent manner. Nutrition & metabolism 12, 206, https://doi.org/10.1186/s13059-017-1348-2 (2017).
Additional Information

Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-019-42991-4.

Competing Interests: L.Z., J.Y., Q.L. and H.J. declare no competing interests. Dr. Zhongcong Xie provides consulting service to Baxter (invited speaker), Hengrui, Novartis, Tongji University, Shanghai Jiao Tong University, and Central South University.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2019