Sevoflurane regulating LncRNA Rik-203 contributes to neural differentiation via microRNA-466l-3p /BDNF pathway

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Research Article

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Abstract

Background: The anesthetics inhibit neural differentiation, induced neuron loss and cognitive impairment in young animals. However, the underlying mechanisms of anesthesia on neural differentiation and are unknown.

Methods: Embryonic stem cells (ESCs) and mice received sevoflurane anesthesia. RNA sequencing; gene expression of mRNAs, LncRNAs and miRNAs; over-expression and RNA interference of genes; flow cytometry; real-time quantity PCR and Western blot were used in the studies. RNA pull-down assay and PCR were employed to detect any miRNA that attached to Rik-203. The binding of miRNA with mRNA of BDNF was presented by the luciferase assay.

Results: Here we found that LncRNA Rik-203 was higher expressed in mice brain than other tissues and increased during neural differentiation. Sevoflurane decreased the level of Rik-203 in mice brain. Knockdown of Rik-203 repressed the neural differentiation derived from mouse embryonic stem cell with the downregulation of the neural progenitor cells markers Sox1 and Nestin. RNA pull-down showed that miR-466l-3p was highly bound to Rik-203 and mediated the function of Rik-203. Inhibition of miR-466l-3p restored the neural differentiation repressed by Rik-203 knockdown. BDNF was directly targeted by miR-466l-3p and also downregulated by sevoflurane. Overexpression of BDNF restored the neural differentiation repressed by miR-466l-3p and Rik-203 knockdown.

Conclusion: Our study suggested that sevoflurane related LncRNARik-203 facilitates neural differentiation by inhibiting miR-466l-3p's ability to reduce BDNF levels.

Keywords: Anesthesia; sevoflurane; Rik-203; miR-466l-3p; BDNF; neural differentiation.

Background

The widespread and growing use of anesthesia in children makes its safety a major health issue of interest [1], reviewed in [2]. It has become a matter of even greater concern as evidence shows that multiple exposures to anesthesia and surgery may induce cognitive impairment in children [3-8], and that anesthetics may induce neurotoxic damage and cognitive impairment in young animals [1, 9-13]. Several clinical studies on anesthesia/surgery-induced cognitive impairment in children have been reported [3-8]. However, different reports also exist [8], [14, 15], and single and short time exposure to anesthesia and surgery is not associated with cognitive impairment in children [16, 17]. Nevertheless, these findings suggest that children who have undergone anesthesia and surgery may not develop to their full cognitive potentials as they would have had they not undergone anesthesia and surgery. However, the causes and pathogenesis associated with the gene signaling regulatory pathways are still unknown.

Long non-coding RNAs (LncRNAs) are defined as transcripts that are longer than 200 nucleotides and also reported to be critically involved in the regulation of neural differentiation [18] and associated with sevoflurane anesthesia[19]. LncRNAs, e.g., NBAT-1 and Pnky, may regulate cell differentiation and

development [[20-22], reviewed in [23]]. LncRNA Rik-201 plays important role in gliomagenesis[24]. 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 neurotoxicity [25], remains largely unknown.

One of the functions of LncRNAs is to attach to microRNAs (miRNAs) as a sponge to prevent the miRNA from binding to mRNA 3'UTR, thus inhibiting miRNA's ability to bind to target mRNA and prevent the translation[26]. miRNAs are important to regulate embryonic development[27, 28], especially the neural development[29, 30] , disease occurrence[31-33] and other physiological processes. However, whether miRNA mediate the LncRNA regulating neural differentiation and anesthesia neurotoxicity and downstream target remain largely unknown.

Aberrant neural differentiation has been shown to contribute to cognitive impairment and neurogenesis inhibition in young and offspring rodents[34]. Sevoflurane regulates neurogenesis in offspring rats by down-regulated the expression of brain-derived neurotrophic factor (BDNF) that is the critical neural development and disease related gene [19, 35-37] and induces neurotoxicity and cognitive impairment in young mice[38, 39]. But, the underlying mechanism by which sevoflurane regulates the expression of BDNF remains largely unknown, which impedes further research into anesthesia neurotoxicity in the developing brain. Thus, in this present study, we set out to determine the effects of sevoflurane on neural differentiation and the underlying mechanisms of regulating the expression of BDNF.

We thus identified a novel LncRNA (Rik-203: C130071C03Riken) and systematically investigated its interaction with the anesthetic sevoflurane, miRNA and the brain-derived neurotrophic factor (BDNF). The objective of these studies was: (1) to elucidate the LncRNA-associated underlying mechanisms of anesthesia neurotoxicity; and (2) to use anesthesia as a tool to investigate the clinically relevant effects of LncRNA on neural differentiation. The hypothesis in the present studies was that the anesthetic sevoflurane decreased LncRNA levels, which diminished the function of miRNA and decreased the action of BDNF, consequently leading to inhibition of neural differentiation.

Methods

Cell culture

46C mESCs were cultured in knockout DMEM medium (Hyclone, USA) with 15% fetal bovine serum (Gibco, USA), 55 μM β-mercaptoethanol (Thermo, USA), final concentration 1:10000 leukemia inhibitory factor (Millipore, USA), 1% nonessential amino acids (Thermo, USA), 1% L-glutamine (Thermo, USA), 1% sodium pyruvate (Thermo, USA) at 37°C, 5% CO_2 atmosphere.

Inducible Rik-203 knockdown 46c cell lines.

We constructed two shRNA vectors for targeting different sites by using plko-tet-on vector. The sequence of shRNA is as follows: shRNA-1, 5'-GGTGTTGGGCCAGTTCCTTAT-3'; shRNA-2, 5'-

GCTTGAATTCAGGCTGCTTGA-3’.

For knockdown of the Rik-203, mESCs were dissociated with 0.05% trypsin and infected with rtTA lentivirus supplemented with 8×10^{-3}μg/mL polybrene (final concentration 1:1000). After 48 hours later, cells were treated by final concentration 200ug/ml geneticin G418 (Thermo, USA) to select stable transfected cell line. Selected cells then were infected by plko-tet-on lentivirus for 48 h with 8×10^{-3} μg/mL polybrene before selection with 5μg/mL puromycin (Sigma-Aldrich, USA).

Neural differentiation from mESCs.

mESCs were dissociated to single cells with 0.05% trypsin (Hyclone, USA) and then neutralized with DMEM medium (Gibco,USA) with 10% fetal bovine serum (Gibco). mESCs were washed with GMEM medium (Gibco, USA). Cells were resuspended and cultured into a low adsorption petri dish (Thermo) with neural differentiation medium at the density of $2.5\text{x }10^4$ cells/mL. The neural differentiation medium contains GMEM medium with 8% knockout serum replacement (Gibco), 1% L-glutamine, 1% sodium pyruvate, and 55 μM β-mercaptoethanol. The medium was changed every days until 7 days. The single-cell clone could be identified under microscope.

Overexpression of miR-466l-3p.

The plvx-puro-miR-466l-3p vector (Biogot technology,co,Ltd,China) lentivirus was infected the 46c mESCs to establish the miR-466l-3p overexpressing cell line.

Inhibition of the miR-466l-4p

Lipofectamine 2000 (Thermo) was used tansfect the miR-466l-3p inhibitor , a chemically modified RNA single chain competing with mature miR-446l-3p , or control inhibitor (Ribobio ,China) following the instructions to inhibit the function of miR-466l-3p at day 3 and day 5 during the neural differentiation.

Overexpression of BDNF

The whole RNA was isolated by using the RNAiso plus (TaKaRa, China) and inversed transcription to cDNA by cDNA Synthesis Kit (TaKaRa). BDNF CDS fragments were amplified and inserted into the Fugw vector. The primers sequence is as follows: PF: 5’-GGCGGATCCATGACCATCCTTTTCCTTACTATGG-3’(BamH1 site); 5’-GGCGAATTCCTATCTTCCCCTTTTAATGGTCAGT-3’(EcoR1 site). The vector was packaged to be lentivirus and transfected into the cells by using Lipofectamine 2000 (Thermo) and the instructions for the reagent.

Sevoflurane treatment of mice

C57BL/J6 mice at postnatal day 6 (P6) (Shanghai SLAC Laboratory Animal, Zhangjiang, Shanghai, P. R. China) were used for sevoflurane treatment. The protocol was approved by the Standing Committee on Animals at Shanghai Ninth People’s Hospital, Shanghai, China. The mice received the sevoflurane

anesthesia as described in our previous studies[40, 41]. The mice were allowed to totally recover from the anesthesia. Each of the mice was euthanized via decapitation at the end of the sevoflurane administration on P8 and the hippocampus tissue were then harvested.

Quantitative Real-time PCR (qRT-PCR)

Total RNA was isolated using RNAiso Plus (TaKaRa). For miRNA, cDNA inverse transcription was carried out with the TIANScript RT Kit (Tiangen, China). qRT-PCR primers of miRNA were purchased (RiboBio, China). mRNA was inverse transcribed to cDNA using cDNA Synthesis Kit (TaKaRa). The primers for detecting mRNA or LncRNA level are as follows:

Rik-203:PF:5'-CATCACTTGGACCATGGACACTAAT-3', RF:5'-GAATCCTATACACATGAATGCAGAA-3'; Nestin:PF:5'-GAATGTAGAGGCAGAGAAAACT-3', RF:5'-TCTTCAAATCTTAGTGGCTCC-3'; Sox1:PF:5'-GTTTTTTGTAGTTGTTACCGC-3', RF:5'-GCATTTACAAGAAATAATAC-3';

GAPDH:PF: 5'-ATGACATCAAGAAGGTGGTG-3',

RF: 5'-CATACCAGGAAATGAGCTTG-3'.

Nucleus and cytoplasm extraction

In order to detect the distribution of LncRNA Rik-203 in the NSCs, we performed the nucleus and cytoplasm extraction studies according to the previous study[42]. Purification and analysis of cytoplasmic and nucleus RNA was performed by using qRT-PCR.

Luciferase reporter assays

pGl3-cm vector was used to construct the 3'UTR luciferase reporter. BDNF 3'UTR fragment was amplified from mESCs DNA.

The PCR primers are as follows:

For miR-466l-3p binding sites UTR region: PF:5'-GGCGTCGACTGAACTGCATGTATAAATGAAGTTT-3'; PR:5'-GGCTCTAGAAATTGGTACACTTAAATAGAACCTG-3'.

Mutant UTR reporter vector was further obtained from the 3' UTR luciferase reporter by replacing the 6 base pair (bp) miRNA seed sequence by using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, USA).

For analysis, 3T3 cells (2×10^4 cells/well of 48 wells plate) were transfected with 200 ng of the 3'UTR luciferase reporter or mutant one, 5 ng Renilla vector, and 20 pmol of miR-466l-3p mimics or miRNA control mimics (Ribobio). 48 hours later cells were harvested to perform the luciferase level using the Dual Luciferase Assay kit (Promega, USA) and SpectraMax M5 microplate reader (Molecular Devices, USA).

Western blot

Cells or tissues were lysed by RIPA buffer to obtain the protein for electrophoresis. Protein was transferred onto the PVDF membrane (BioRed, USA). Then incubated the primary antibodies: GAPDH (ab8245, Abcam) uased for normalizing whole protein levels, and BDNF (ab10505, Abcam). Enhanced chemiluminescence (ECL) substrate (Thermo) was used to visualize the protein expression signaling.

RNA pull-down assay

1×10^8 mESc-derived NSCs 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 Desthiobiotinylation 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 50μL 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 microRNAs were washed and eluted, and the binding microRNAs were detected using qRT-PCR. Primers for the qRT-PCR analysis of miRNA include the following list. For miR-466l-3p: Primer of Stem-loop reverse transcription:

5'-GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACTCTTATG -3', Primers for qRT-PCR: PF: 5'- ACGCAGATACATACACGCACA -3', RF: 5'- AGTGCGTGTCGTGGAGTCG -3'.

Statistics analysis

The data were presented as mean ± standard deviation (SD) with more than three independent experiments. The significance of statistics was determined by a Student's t-test or one-way ANOVA. * and # $p < 0.05$, ** and ## $p < 0.01$, *** and ### $p < 0.001$. We used the Graph Pad (Software Inc., San Diego, California, USA) to evaluate all of the study data.

Results

Rik-203 decreased by sevoflurane and is critically involved in the neural differentiation.

LncRNA Rik-203 expression level was significantly lower in the hippocampus of mice exposure with 3% sevoflurane 2 hours daily for 3days (Fig 1A). We then identified that Rik-203 was especially enriched in the mouse brain and much less expressed in other tissues, such as limb, heart and liver (Fig 1B). The level of Rik-203 was upregulated significantly during the neural differentiation from mouse embryonic stem cells (mESCs) to neural stem cells (NSCs) (Fig 1C). In the contrary we repressed the process of neural differentiation derived from 46C mESCs , the Sox1-promoter GFP transgenic ESCs[43], by treating

sevoflurane which is a widely used anesthetic and induce neural development neurotoxicity in developing brain [44-46] . In order to investigate whether Rik-203 could regulate the neural differentiation or just be a concomitant expression, we downregulated the Rik-203 (Fig 1D) and found that the neural differentiation was inhibited by Rik-203 knockdown (Fig 1E). Flow cytometry assay showed the proportion of Sox1-GFP positive cells was repressed by Rik-203 knockdown (Fig 1F). Additionally, the expression of Nestin, Sox1, and N-cadherin which are critical neural development marker genes was significantly lower in Rik-203 knockdown groups (Fig 1G).

Rik-203 targeted the miR-466l-3p during the neural differentiation

Next we examined the downstream mediator of Rik-203 in neural differentiation. There're more Rik-203 distributing in cytoplasm than in nucleus (Fig 2A), which indicated the competing endogenous RNAs (ceRNA) function of Rik-203 in cytoplasm. Bioinformatics assay and RNA pull down analysis showed that mmu-miR-466l-3p could bind with the Rik-203 (Fig 2B). Additionally, sevoflaurne treatment could not induce the change of miR-466l-3p expression level (Fig 2D) which further suggested the Rik-203 regulatory function of ceRNA. Overexpression of miR-466l-3p significantly repressed the neural differentiation (Fig2D, 2E). The expression of NSCs markers, Sox1, Nestin, N-cadherin were also inhibited (Fig 2F). We further performed the rescue experiment and found that inhibition of miR-466l-3p by transfecting miRNA inhibitor could restored the repression of neural differentiation cause by Rik-203 knockdown(Fig 2G,2H) and also the NSCs related genes expression level (Fig 2I).

BDNF is directly targeted by miR-466l-3p to mediate neural differentiation.

Next, we addressed which downstream target gene could be regulated by miR-466l-3p. Brain derived neurotrophic factor (BDNF) is critical involved in the differentiation of iPSCs to NSCs [47] and also promotes growth of neurons and NSCs[48]. Sevoflurane also resulted in significant inhibition BDNF expression and cognitive impairment[45]. We detected the downregulation of the BDNF in cells treated by sevoflurane during the neural differentiation (Fig 3A). miR-466l-3p is predicted to target BDNF(Fig 3B) by TargetScan[49], miRBase[50]. To investigate whether BDNF could be directly targeted by miR-466l-3p, we engineered luciferase reporters with either the wild-type 3' UTRs or mutant UTRs with deletion of 6 base pair (bp) miRNA seed sequence binding sites. A scrambled control with no homology to the mouse genome was used to control the nonspecific effects of endogenous miRNA expression. Overexpression of miR-466l-3p repressed the luciferase expression. In contrast, mutant reporter was not repressed by miR-466l-3p (Fig 3C). Overexpression in the NSCs also induced the downregulation of BDNF (Fig 3D). This data indicate that BDNF 3'UTR was directly targeted by miR-466l-3p and mediated the expression level repression. Knockdown of the BDNF could inhibit the neural differentiation (Fig 3E, 3F) and also repress the expression level of Sox1, Nestin, N-cadherin (Fig 3G), which was similar with the phenomenon of the overexpression of miR-466l-3p. We further performed the rescue experiment to study whether the regulatory pathway of miR-466l-3p/BDNF function as the regulator of neural differentiation. Overexpression of BDNF significantly blocked the repression of neural differentiation cause by miR-466l-

3p overexpression (Fig 3F, 3I). The expression of NSCs markers Sox1, Nestin, N-cadherin expression was also restored by BDNF in the miR-466l-3p overexpressing cells (Fig 3J).

BDNF restored the neural differentiation repression caused by Rik-203 downregulation

Knockdown of BDNF induced the neural differentiation repression, which was similar with the phenomenon of Rik-203 knockdown. miR-466l-3p has also been confirmed to be the downstream target of Rik-203. Next we found that overexpression of BDNF could be the downstream target of RIk-203 to rescue the repression of neural differentiation cause by Rik-203 (Fig 4A, 4B) and also the expression of NSCs marker genes expression (Fig 4C).

Discussion

In the current studies, we were able to show for the first time that the anesthetic sevoflurane decreased levels of LncRNA Rik-203 in the brain tissues of the mice. Such reductions resulted in the inhibition of neural differentiation via the cascade action of miRNA (miR-466l-3p) /BDNF signaling. These data also suggest that Rik-203 would serve as the underling mechanism for sevoflurane anesthesia neurotoxicity.

LncRNAs are known to function as epigenetic modulators to orchestrate epigenetic processes [51]. LncRNAs have been found to play crucial roles in developmental and neurodegenerative diseases[52]. LncRNA Riken has 5 transcripts (splice variants). A recent study identified a novel LncRNA Rik-201 and demonstrated its functional role in gliomagenesis [24].Previous studies have shown that anesthetics may regulate LncRNAs expression, but their function in mediating anesthesia-induced neurotoxicity is largely unknown. Our findings from the current study showed, for the first time, that Rik-203 was upregulated gradually during the neural differentiation and downregulated by sevofalurne treatment in mice brain. This indicated the critical role during the neural differentiation and also suggested the relationship between sevoflurane treatment and Rik-203 expression level.

Sevoflurane increased the level of LncRNA Gadd45a in the rat hippocampus neural stem cells and induced neurotoxicity [53]. However, it did not provide sufficient mechanistic studies. Our studies showed that Rik-203 was downregulated by sevoflurane treatment. Downregulation of Rik-203 significantly reduced the neural differentiation. This result indicated the mechanism of sevoflurane repressive regulating mechanism during the neural differentiation. Additionally, several studies found that sevoflurane caused neurotoxicity by regulating the expression of miRNAs [54-56]. Rik-203 mainly located in the cytoplasm to function as the ceRNA to inhibit downstream miR-466l-3p effects of repressing the neural differentiation. These results uncovered that Rik-203/ miR-466l-3p pathway control the balance of the neural differentiation process.

Sevoflurane was also reported to decrease the level of BDNF and induced cognitive impairment in 18 month-old rats[45]. BDNF is critical involved in the differentiation of NSCs from iPSCs [47] and could promote neurons and NSCs growth [48]. We further found that miR-466l -3p directly targets BDNF, leading to the inhibition of the neural differentiation. BDNF over expression could restore the neural

differentiation repressed by the Rik-203 knockdown or miR-466l-3p overexpression. These data indicated that BDNF construct the Rik-203/miR-466l-3p/BDNF signaling pathway regulating the neural differentiation and mediating sevoflurane neurotoxicity.

Conclusions

We identified the functional role of sevoflurane regulating LncRNA Rik-203 in facilitating neural differentiation and elucidated the underlying miR-466l-3p/BDNF associated molecular pathway mechanisms. Moreover, these findings have identified Rik-203 as a potential target for prevention and treatment of anesthesia neurotoxicity in young mice and children.

Abbreviations

Embryonic stem cells (ESCs); Long non-coding RNAs (LncRNAs); Neural progenitor cells (NPCs); Brain-derived neurotrophic factor (BDNF).

Declarations

Ethics approval and consent to participate: The animal protocol was approved by the Standing Committee on Animals at Shanghai Ninth People's Hospital, Shanghai, P.R. China. The mice were purchased from SHANGHAI SLAC LABORATORY ANIMAL CO. LTD and were housed in a temperature-controlled facility (22 ± 2 °C) and kept under a 12-h light/dark cycle. The animals were given free access to food and water.

Consent for publication: Not applicable.

Availability of data and materials: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no competing interests.

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Author contributions: LZ and HJ, study concept and design; LZ, ZX and JY acquisition of data, analysis and interpretation of data; LZ and QL drafted of the manuscript. LZ, JY and HJ obtained funding, administrative, technical, and material support.

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References

1. Rappaport B, Mellon RD, Simone A, Woodcock J: **Defining safe use of anesthesia in children**. *N Engl J Med* 2011, **364**(15):1387-1390.
2. Vutskits L, Xie Z: **Lasting impact of general anaesthesia on the brain: mechanisms and relevance**. *Nat Rev Neurosci* 2016, **17**(11):705-717.
3. DiMaggio C, Sun LS, Kakavouli A, Byrne MW, Li G: **A retrospective cohort study of the association of anesthesia and hernia repair surgery with behavioral and developmental disorders in young children**. *J Neurosurg Anesthesiol* 2009, **21**(4):286-291.
4. Kalkman CJ, Peelen L, Moons KG, Veenhuizen M, Bruens M, Sinnema G, de Jong TP: **Behavior and development in children and age at the time of first anesthetic exposure**. *Anesthesiology* 2009, **110**(4):805-812.
5. Wilder RT, Flick RP, Sprung J, Katusic SK, Barbaresi WJ, Mickelson C, Gleich SJ, Schroeder DR, Weaver AL, Warner DO: **Early exposure to anesthesia and learning disabilities in a population-based birth cohort**. *Anesthesiology* 2009, **110**(4):796-804.
6. Flick RP, Katusic SK, Colligan RC, Wilder RT, Voigt RG, Olson MD, Sprung J, Weaver AL, Schroeder DR, Warner DO: **Cognitive and behavioral outcomes after early exposure to anesthesia and surgery**. *Pediatrics* 2011, **128**(5):e1053-1061.
7. Hu D, Flick RP, Zaccariello MJ, Colligan RC, Katusic SK, Schroeder DR, Hanson AC, Buenvenida SL, Gleich SJ, Wilder RT *et al*: **Association between Exposure of Young Children to Procedures Requiring General Anesthesia and Learning and Behavioral Outcomes in a Population-based Birth Cohort**. *Anesthesiology* 2017, **127**(2):227-240.
8. Warner DO, Zaccariello MJ, Katusic SK, Schroeder DR, Hanson AC, Schulte PJ, Buenvenida SL, Gleich SJ, Wilder RT, Sprung J *et al*: **Neuropsychological and Behavioral Outcomes after Exposure of Young Children to Procedures Requiring General Anesthesia: The Mayo Anesthesia Safety in Kids (MASK) Study**. *Anesthesiology* 2018.
9. Rappaport BA, Suresh S, Hertz S, Evers AS, Orser BA: **Anesthetic neurotoxicity--clinical implications of animal models**. *N Engl J Med* 2015, **372**(9):796-797.
10. Servick K: **Biomedical Research. Researchers struggle to gauge risks of childhood anesthesia**. *Science* 2014, **346**(6214):1161-1162.
11. Jevtovic-Todorovic V, Hartman RE, Izumi Y, Benshoff ND, Dikranian K, Zorumski CF, Olney JW, Wozniak DF: **Early exposure to common anesthetic agents causes widespread neurodegeneration in the developing rat brain and persistent learning deficits**. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 2003, **23**(3):876-882.
12. Lin EP, Soriano SG, Loepke AW: **Anesthetic neurotoxicity**. *Anesthesiology clinics* 2014, **32**(1):133-155.

13. Creeley CE, Dikranian KT, Dissen GA, Back SA, Olney JW, Brambrink AM: **Isoflurane-induced apoptosis of neurons and oligodendrocytes in the fetal rhesus macaque brain**. *Anesthesiology* 2014, **120**(3):626-638.
14. Sprung J, Flick RP, Wilder RT, Katusic SK, Pike TL, Dingli M, Gleich SJ, Schroeder DR, Barbaresi WJ, Hanson AC *et al*: **Anesthesia for cesarean delivery and learning disabilities in a population-based birth cohort**. *Anesthesiology* 2009, **111**(2):302-310.
15. Bartels M, Althoff RR, Boomsma DI: **Anesthesia and cognitive performance in children: no evidence for a causal relationship**. *Twin Res Hum Genet* 2009, **12**(3):246-253.
16. Sun LS, Li G, Miller TL, Salorio C, Byrne MW, Bellinger DC, Ing C, Park R, Radcliffe J, Hays SR *et al*: **Association Between a Single General Anesthesia Exposure Before Age 36 Months and Neurocognitive Outcomes in Later Childhood**. *JAMA* 2016, **315**(21):2312-2320.
17. Davidson AJ, Disma N, de Graaff JC, Withington DE, Dorris L, Bell G, Stargatt R, Bellinger DC, Schuster T, Arnup SJ *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* 2016, **387**(10015):239-250.
18. Cesana M, Cacchiarelli D, Legnini I, Santini T, Sthandier O, Chinappi M, Tramontano A, Bozzoni I: **A long noncoding RNA controls muscle differentiation by functioning as a competing endogenous RNA**. *Cell* 2011, **147**(2):358-369.
19. Lu B, Nagappan G, Guan X, Nathan PJ, Wren P: **BDNF-based synaptic repair as a disease-modifying strategy for neurodegenerative diseases**. *Nature reviews Neuroscience* 2013, **14**(6):401-416.
20. Fatica A, Bozzoni I: **Long non-coding RNAs: new players in cell differentiation and development**. *Nat Rev Genet* 2014, **15**(1):7-21.
21. Pandey GK, Mitra S, Subhash S, Hertwig F, Kanduri M, Mishra K, Fransson S, Ganeshram A, Mondal T, Bandaru S *et al*: **The risk-associated long noncoding RNA NBAT-1 controls neuroblastoma progression by regulating cell proliferation and neuronal differentiation**. *Cancer Cell* 2014, **26**(5):722-737.
22. Ramos AD, Andersen RE, Liu SJ, Nowakowski TJ, Hong SJ, Gertz C, Salinas RD, Zarabi H, Kriegstein AR, Lim DA: **The long noncoding RNA Pnky regulates neuronal differentiation of embryonic and postnatal neural stem cells**. *Cell Stem Cell* 2015, **16**(4):439-447.
23. Briggs JA, Wolvetang EJ, Mattick JS, Rinn JL, Barry G: **Mechanisms of Long Non-coding RNAs in Mammalian Nervous System Development, Plasticity, Disease, and Evolution**. *Neuron* 2015, **88**(5):861-877.
24. Deguchi S, Katsushima K, Hatanaka A, Shinjo K, Ohka F, Wakabayashi T, Zong H, Natsume A, Kondo Y: **Oncogenic effects of evolutionarily conserved noncoding RNA ECONEXIN on gliomagenesis**. *Oncogene* 2017, **36**(32):4629-4640.
25. Sacco R, Cacci E, Novarino G: **Neural stem cells in neuropsychiatric disorders**. *Current opinion in neurobiology* 2017, **48**:131-138.

26. Tay Y, Rinn J, Pandolfi PP: **The multilayered complexity of ceRNA crosstalk and competition**. *Nature* 2014, **505**(7483):344-352.
27. Lin Y, Zeng Y, Zhang F, Xue L, Huang Z, Li W, Guo M: **Characterization of microRNA expression profiles and the discovery of novel microRNAs involved in cancer during human embryonic development**. *PloS one* 2013, **8**(8):e69230.
28. Liu Q, Wang G, Chen Y, Li G, Yang D, Kang J: **A miR-590/Acvr2a/Rad51b axis regulates DNA damage repair during mESC proliferation**. *Stem cell reports* 2014, **3**(6):1103-1117.
29. Stevanato L, Sinden JD: **The effects of microRNAs on human neural stem cell differentiation in two- and three-dimensional cultures**. *Stem cell research & therapy* 2014, **5**(2):49.
30. Mercado AT, Yeh JM, Chin TY, Chen WS, Chen-Yang YW, Chen CY: **The effect of chemically modified electrospun silica nanofiber on the mRNA and miRNA expression profile of neural stem cell differentiation**. *Journal of biomedical materials research Part A* 2016, **104**(11):2730-2743.
31. Ha J, Kim H, Yoon Y, Park S: **A method of extracting disease-related microRNAs through the propagation algorithm using the environmental factor based global miRNA network**. *Bio-medical materials and engineering* 2015, **26 Suppl 1**:S1763-1772.
32. Ran M, Chen B, Yin J, Yang A, Jiang M: **Advances in miRNA research related to testis development and spermatogenesis**. *Yi chuan = Hereditas* 2014, **36**(7):646-654.
33. Tutar Y: **miRNA and cancer; computational and experimental approaches**. *Current pharmaceutical biotechnology* 2014, **15**(5):429.
34. Cho KO, Lybrand ZR, Ito N, Brulet R, Tafacory F, Zhang L, Good L, Ure K, Kernie SG, Birnbaum SG *et al*: **Aberrant hippocampal neurogenesis contributes to epilepsy and associated cognitive decline**. *Nat Commun* 2015, **6**:6606.
35. Wu Z, Li X, Zhang Y, Tong D, Wang L, Zhao P: **Effects of Sevoflurane Exposure During Mid-Pregnancy on Learning and Memory in Offspring Rats: Beneficial Effects of Maternal Exercise**. *Front Cell Neurosci* 2018, **12**:122.
36. Low WC, Rujitanaroj PO, Wang F, Wang J, Chew SY: **Nanofiber-mediated release of retinoic acid and brain-derived neurotrophic factor for enhanced neuronal differentiation of neural progenitor cells**. *Drug delivery and translational research* 2015, **5**(2):89-100.
37. Chung CY, Lin MH, Lee IN, Lee TH, Lee MH, Yang JT: **Brain-Derived Neurotrophic Factor Loaded PS80 PBCA Nanocarrier for In Vitro Neural Differentiation of Mouse Induced Pluripotent Stem Cells**. *International journal of molecular sciences* 2017, **18**(3).
38. Zhang MQ, Ji MH, Zhao QS, Jia M, Qiu LL, Yang JJ, Peng YG, Yang JJ, Martynyuk AE: **Neurobehavioural abnormalities induced by repeated exposure of neonatal rats to sevoflurane can be aggravated by social isolation and enrichment deprivation initiated after exposure to the anaesthetic**. *Br J Anaesth* 2015, **115**(5):752-760.
39. Lu Z, Sun J, Xin Y, Chen K, Ding W, Wang Y: **Sevoflurane-induced memory impairment in the postnatal developing mouse brain**. *Exp Ther Med* 2018, **15**(5):4097-4104.

40. Shen X, Dong Y, Xu Z, Wang H, Miao C, Soriano SG, Sun D, Baxter MG, Zhang Y, Xie Z: **Selective anesthesia-induced neuroinflammation in developing mouse brain and cognitive impairment**. *Anesthesiology* 2013, **118**(3):502-515.
41. Lu H, Liufu N, Dong Y, Xu G, Zhang Y, Shu L, Soriano SG, Zheng H, Yu B, Xie Z: **Sevoflurane Acts on Ubiquitination-Proteasome Pathway to Reduce Postsynaptic Density 95 Protein Levels in Young Mice**. *Anesthesiology* 2017, **127**(6):961-975.
42. Zhang L, Zhang J, Yang L, Dong Y, Zhang Y, Xie Z: **Isoflurane and sevoflurane increase interleukin-6 levels through the nuclear factor-kappa B pathway in neuroglioma cells**. *British journal of anaesthesia* 2013, **110 Suppl 1**:i82-91.
43. Ying QL, Stavridis M, Griffiths D, Li M, Smith A: **Conversion of embryonic stem cells into neuroectodermal precursors in adherent monoculture**. *Nature biotechnology* 2003, **21**(2):183-186.
44. Zheng H, Dong Y, Xu Z, Crosby G, Culley DJ, Zhang Y, Xie Z: **Sevoflurane anesthesia in pregnant mice induces neurotoxicity in fetal and offspring mice**. *Anesthesiology* 2013, **118**(3):516-526.
45. Wang JY, Feng Y, Fu YH, Liu GL: **Effect of Sevoflurane Anesthesia on Brain Is Mediated by LncRNA HOTAIR**. *J Mol Neurosci* 2018, **64**(3):346-351.
46. Qiu J, Shi P, Mao W, Zhao Y, Liu W, Wang Y: **Effect of apoptosis in neural stem cells treated with sevoflurane**. *BMC anesthesiology* 2015, **15**:25.
47. Chen SQ, Huang M, Liu CL, Shen YY, Cai Q, Wang PJ: **[Differentiation of induced pluripotent stem cells into neural stem cells induced by brain-derived neurotrophic factor via Wnt/beta-catenin and extracellular signal-regulated kinase/mitogen-activated protein kinases signal pathway]**. *Zhonghua yi xue za zhi* 2017, **97**(41):3263-3268.
48. Li XT, Liang Z, Wang TT, Yang JW, Ma W, Deng SK, Wang XB, Dai YF, Guo JH, Li LY: **Brain-derived Neurotrophic Factor Promotes Growth of Neurons and Neural Stem Cells Possibly by Triggering the Phosphoinositide 3-Kinase/ AKT/Glycogen Synthase Kinase-3beta/beta-catenin Pathway**. *CNS & neurological disorders drug targets* 2017, **16**(7):828-836.
49. Lewis BP, Shih IH, Jones-Rhoades MW, Bartel DP, Burge CB: **Prediction of mammalian microRNA targets**. *Cell* 2003, **115**(7):787-798.
50. Griffiths-Jones S, Grocock RJ, van Dongen S, Bateman A, Enright AJ: **miRBase: microRNA sequences, targets and gene nomenclature**. *Nucleic acids research* 2006, **34**(Database issue):D140-144.
51. Mercer TR, Mattick JS: **Structure and function of long noncoding RNAs in epigenetic regulation**. *Nat Struct Mol Biol* 2013, **20**(3):300-307.
52. Wan P, Su W, Zhuo Y: **The Role of Long Noncoding RNAs in Neurodegenerative Diseases**. *Mol Neurobiol* 2017, **54**(3):2012-2021.
53. Lu G, Xu H, Zhao W, Zhang J, Rao D, Xu S: **Upregulation of long noncoding RNA Gadd45a is associated with sevoflurane-induced neurotoxicity in rat neural stem cells**. *Neuroreport* 2018, **29**(8):605-614.
54. Yi X, Cai Y, Zhang N, Wang Q, Li W: **Sevoflurane inhibits embryonic stem cell self-renewal and subsequent neural differentiation by modulating the let-7a-Lin28 signaling pathway**. *Cell Tissue Res*

2016, **365**(2):319-330.

55. Zhou X, Xian D, Xia J, Tang Y, Li W, Chen X, Zhou Z, Lu D, Feng X: **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* 2017, **15**(4):2204-2212.
56. Lv X, Yan J, Jiang J, Zhou X, Lu Y, Jiang H: **MicroRNA-27a-3p suppression of peroxisome proliferator-activated receptor-gamma contributes to cognitive impairments resulting from sevoflurane treatment**. *J Neurochem* 2017, **143**(3):306-319.

Figures

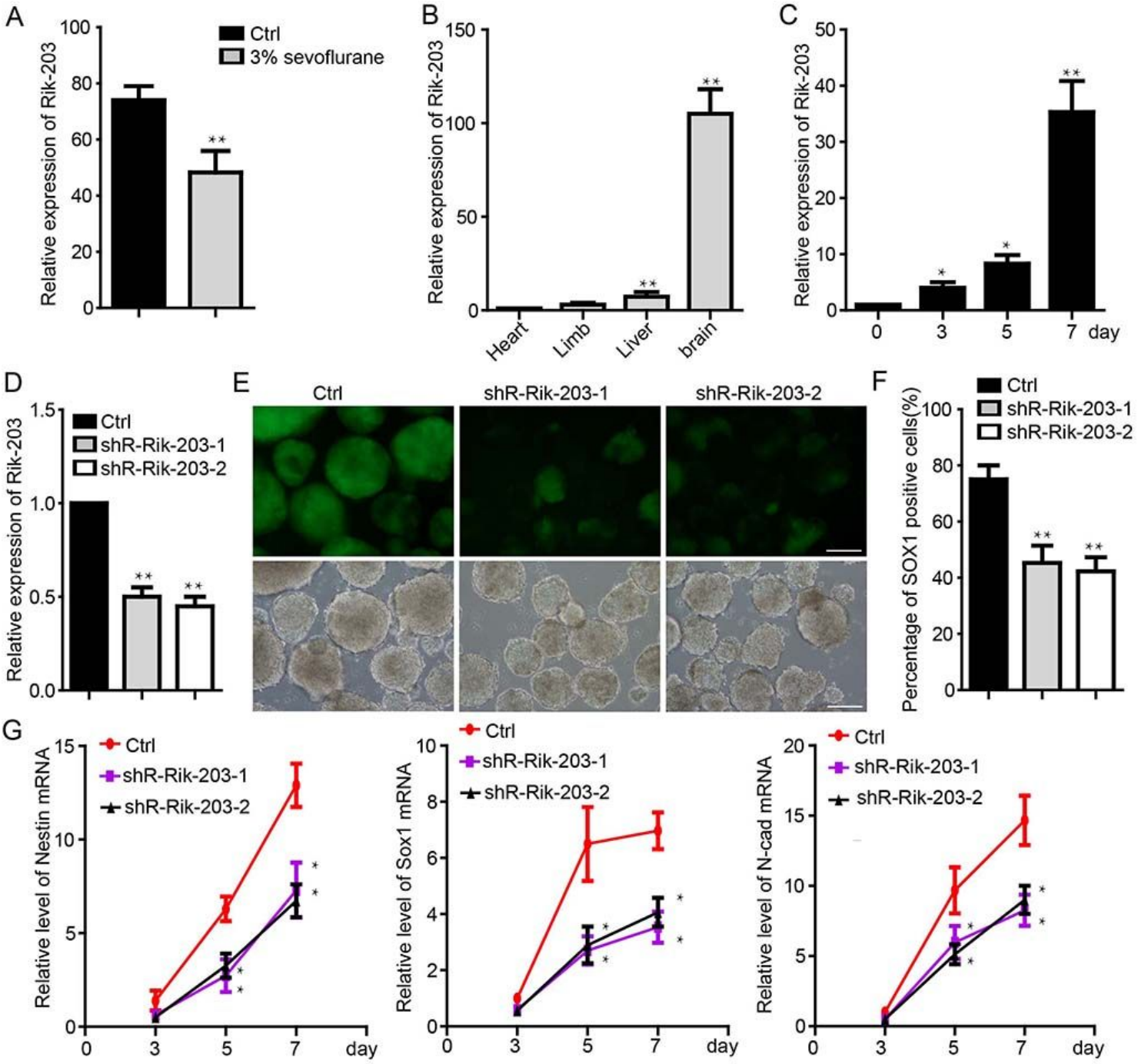

Figure 1

Rik-203 regulates neural differentiation. A. Sevoflurane decreased the Rik-203 level in the mice hippocampus. B. qRT-PCR indicated that Rik-203 level is highest in brain compared with other tissues of mice. C. Level of Rik-203 expression during the neural differentiation from ESCs to NSCs. D. Knockdown of the Rik-203 expression by shRNAs and repressed the neural differentiation (E). F.Flow cytometry assay showed the proportion of Sox1-GFP positive cells was repressed by Rik-203 knockdown. G.qRT-PCR showed that levels of Sox1 ,Nestin,N-cadherin were decreased through Rik-203 knockdown. The scale bar represents 100 um. Ctrl means control; * $p < 0.05$, ** $p < 0.01$, *** $P < 0.001$.

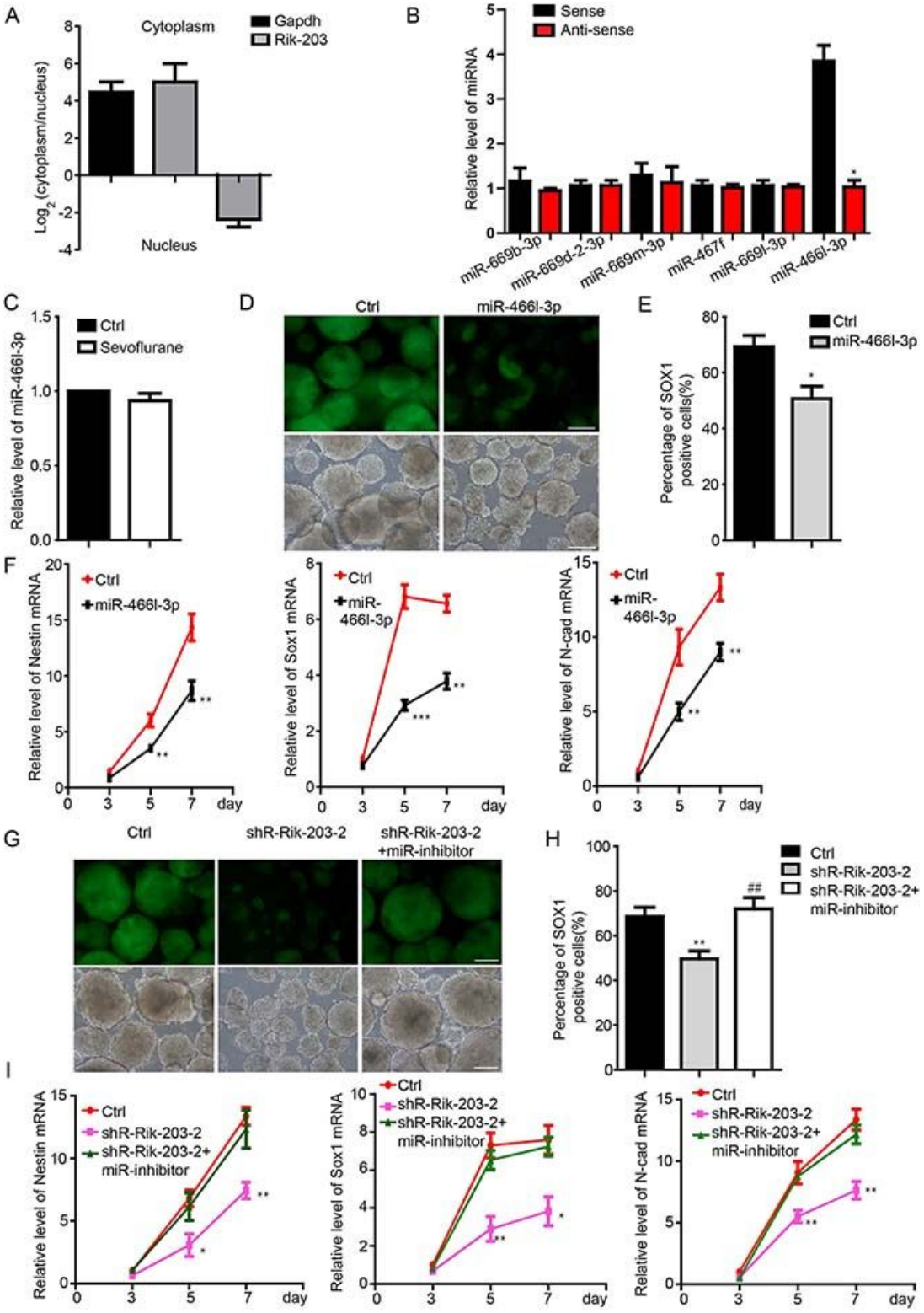

Figure 2

Rik-203 targeted the miR-466l-3p during the neural differentiation. A. Cytoplasmic and nucleus distribution of Rik-203 was detected by RT-PCR and showed that there were higher Rik-203levels in the cytoplasm. B. RNA pull-down assay showed that miR-466l-3p bind to Rik-203. C. Sevoflurane could not affect the level of miR-466l-3p in mice hippocampus. D. Overexpression of miR-466l-3p significantly repressed the neural differentiation. E. Flow cytometry assay also confirmed the inhibition of proportion

of Sox1-GFP positive cells. F. qRT-PCR showed the downregulation of NSCs related genes Sox1, Nestin, N-cadherin by miR-466l-3p. G. rescue experiment showed that inhibition of miR-466l-3p restored the neural differentiation repression caused by Rik-203 knockdwon. miR-inhibitor means the miR-466l-3p inhibitor that is the chemically modified RNA single chain competing with mature miR-446l-3p. Ctrl means the plko-tet-on vector and miR-inhibitor control. H. Flow cytometry assay also indicated the inhibition of mir-466l-3p recued the neural differentiation in G. I. Detection of the NSCs related genes expression in the rescue experiments. The scale bar represents 100 □. * p < 0.05, ** p < 0.01, *** P < 0.001; ** or ## p < 0.01;

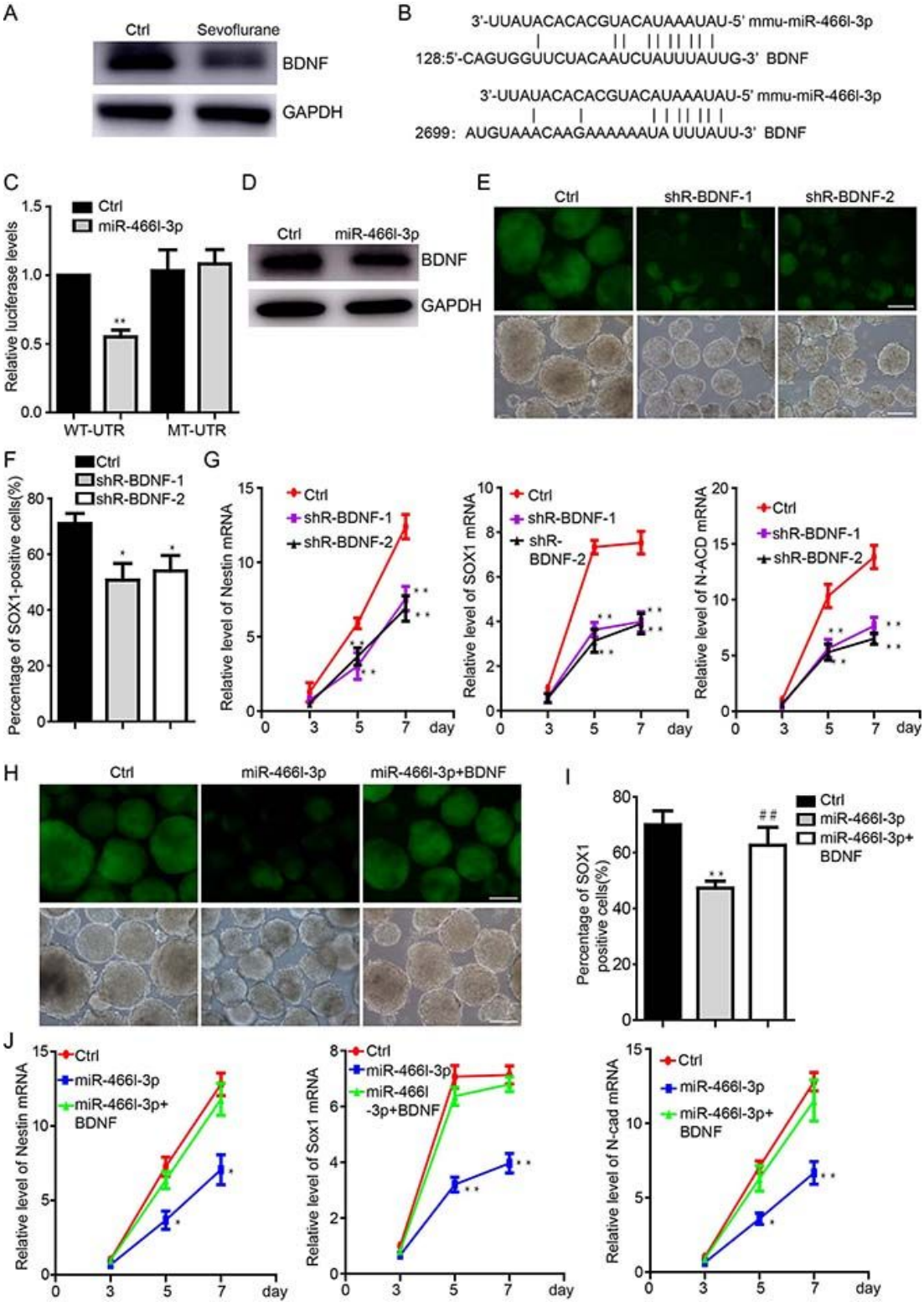

Figure 3

MiR-466l-3p targeted BDNF to mediate neural differentiation. A. Western blot indicated the downregulation of BDNF cause by sevoflurane treatment in mice hippocampus. B. Target validation of the binding of BDNF 3'UTR by miR-466l-3p. C. Luciferase report assay indicated that miR-466l-3p targeted wild-type BDNF 3'UTR but not mutant UTR.D. Overexpression of miR-466l-3p decreased the protein level of BDNF. E. Knockdown of the BDNF inhibited the neural differentiation. F. Flow cytometry assay confirmed the downregulation of BDNF repressed the proportion of Sox1-GFP positive cells. G. Detection of the NSCs related genes Sox1, Nestin, N-cadherin expression by qRT-PCR. H. Overexpression of BDNF blocked the neural differentiation caused by miR-466l-3p, which was also confirmed in I. J. Detection of the expression of Sox1, Nestin, and N-cadherin in the H. The scale bar represents 100 □. * p < 0.05, ** p < 0.01; ** or ## p < 0.01;

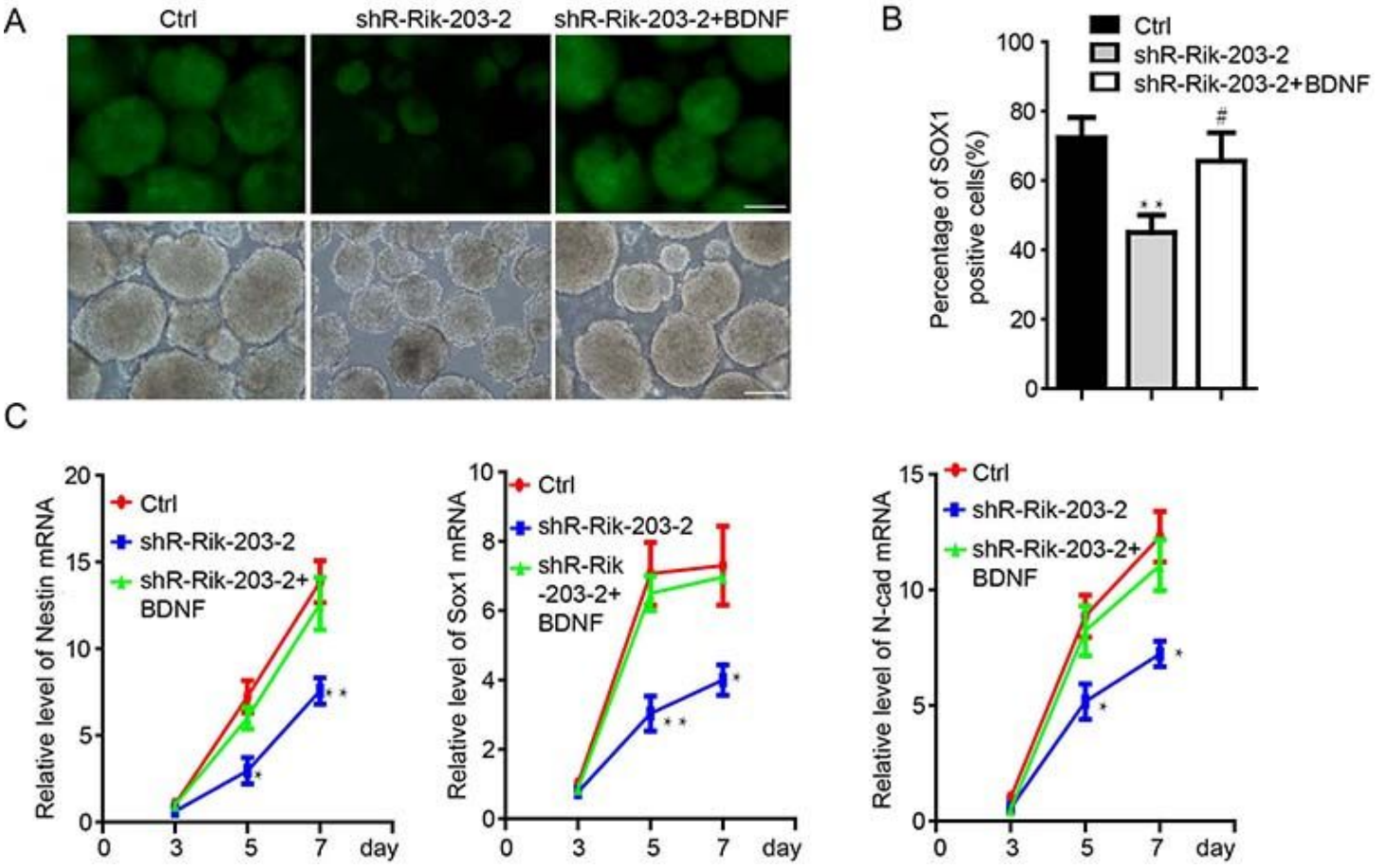

Figure 4

BDNF restored the repression of neural differentiation caused by Rik-203 downregulatio. A. Overexpression of BDNF restored the neural differentiation repressed by Rik-203 knockdown. B. Flow cytometry assay showed that BDNF rescue the proportion of Sox1-GFP positive cells repressed by Rik-203 knockdown. C. The expression level of Sox1, Nestin and N-cadherin is also restored by BDNF in Rik-203 knockdown cells. The scale bar represents 100 □. * p < 0.05, ** p < 0.01, *** P < 0.001; ** or ## p < 0.01;