Sevoflurane represses the self-renewal ability by regulating miR-7a,7b/Klf4 signalling pathway in mouse embryonic stem cells

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Abstract

Sevoflurane is a frequently-used clinical inhalational anaesthetic and can cause toxicity to embryos during foetal development. Embryonic stem cells (ESCs) are derived from the inner cell mass of blastospheres and can be used as a useful model of early development. Here, we found that sevoflurane significantly influenced self-renewal ability of mESCs on stemness maintenance and cell proliferation. The cell cycle was arrested via G1 phase delay. We further found that sevoflurane upregulated expression of miR-7a,7b to repress self-renewal. Next we performed rescue experiments and found that after adding miR-7a,7b inhibitor into mESCs treated with sevoflurane, its influence on self-renewal could be blocked. Further we identified stemness factor Klf4 as the direct target of miR-7a,7b. Overexpression of Klf4 restored self-renewal ability repressed by miR-7a,7b or sevoflurane. In this work, we determined that sevoflurane repressed self-renewal ability by regulating the miR-7a,7b/Klf4 signalling pathway in mESCs. Our study demonstrated molecular mechanism underlying the side effects of sevoflurane during early development, laying the foundation for studies on safe usage of inhalational anaesthetic during non-obstetric surgery.

1 | INTRODUCTION

Sevoflurane, the commonly used inhalational anaesthetic, is widely used in pregnancy non-obstetric surgery. Besides anaesthesia and sedation, recently, people have increasingly focused on side effect of anaesthetics. More and more research proved that inhalational anaesthetic can cause development problems. Inhalational anaesthetic could readily cross the placental barrier, so that it increased the risk of foetus development during the non-obstetric surgery in pregnant women.¹ Previous study showed that inhalational anaesthetic for pregnancy can cause damage to children's cognitive academic skills.² Especially, exposure to sevoflurane inhibits neurogenesis in the brain of foetal mice and impairs subsequent learning and memory ability.³ Isoflurane, another inhalational anaesthetic, decreases the self-renewal of neuron

stem cells and reduces the survival, proliferation and differentiation of human neural progenitor cells.^{4–6} These studies suggested that inhalational anaesthetic could lead significant effect to development and cause serious problems. Additionally, exposure to inhalational anaesthetic during the early pregnancy may cause more serious embryotoxicity and lead to abnormal development than during late pregnancy or other stages. However, there still remains largely unknown about the mechanism of the embryotoxicity side effect of the inhalational anaesthetic on early pregnancy during non-obstetric surgery.

Embryonic stem cells (ESCs) derived from the inner cell mass of mammalian embryos possess the self-renewal ability and pluripotency which means the potential of further development and become important clinical research model for embryonic development and diseases.⁷ The ability of self-renewal determines the further development significantly. Self-renewal implies rapid proliferation under maintenance of stemness. Small changes in numbers of stem cells may

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lead to large differences in further development.⁸ As we know that many protein-coding gene, especially many stemness genes including STAT3,⁹ OCT4,¹⁰ NANOG, SOX2,¹¹ can regulate the self-renewal ability of ESCs. Klf4 is also one of the most important transcription factors, which is related to maintain the self-renewal of ESCs. There are many signalling pathways that can regulate the expression and function of Klf4, and play key roles in cell cycle regulation, somatic cell programming and pluripotency.¹² Study showed that Klf4 prevented differentiation of ESCs by regulating Nanog gene expression.¹³ While, whether Klf4 can candidate into or be regulated by certain signalling pathway to mediate the influence of inhalational anaesthetic, such as sevoflurane, on self-renewal and pluripotency remains largely unclear.

As the non-coding RNA, microRNAs (miRNAs) are post-transcriptional modulators of gene expression. Many studies verified that miRNAs can inhibit the downstream target genes to participate in regulating cell cycle, stemness maintenance, genomic stabilization and so on.^{7,14,15} Some papers reported that miRNAs can regulate the expression of Klf4 in different biological process. There is also research determined that miR-7a can regulate the expression of Klf4 through β -catenin signalling pathway, and finally promote self-renewal.¹⁶ But whether it will candidate into the signalling pathway of sevoflurane influencing ESCs is still unknown.

Here we found that sevoflurane significantly repressed the maintenance of stemness and cell proliferation by upregulating the level of miR-7a,7b in mouse embryonic stem cells (mESCs). Klf4 was the direct target of miR-7a,7b and could restore the self-renewal ability repressed by miR-7a,7b or sevoflurane. We determined that sevoflurane represses the self-renewal ability through miR-7a,7b/Klf4 signalling pathway in mESCs. These results suggested the underlying molecule mechanism of embryotoxicity side effect of sevoflurane and provided an insight into the safe usage of inhalational anaesthetic during non-obstetric surgery.

2 | MATERIALS AND METHODS

2.1 | Cell culture

E14 mESCs, purchased from the Institute of Biochemistry and Cell Biology (Shanghai, China; SCSP-204), were seeded in plates pre-coated by 0.1% gelatin and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA, USA) containing 15% ESC-foetal bovine serum (FBS; Gibco), glutamine (1:100; Invitrogen, Life Technologies, CA, USA), β -mercaptoethanol (1:550; Invitrogen) and leukaemia inhibitory factor (1:10 000; Invitrogen) at a density of 7×10^4 cells per well in six-well plates and cultured at 37°C in a humidified atmosphere containing 5% CO₂. NIH3T3 cells were cultured in DMEM containing 10% FBS.

2.2 | Cell treatment

Cells of the sevoflurane group were exposed to 4.1% sevoflurane, 5% CO₂ plus 21% O₂ for 6 hours, whereas the control group conditions includes 5% CO₂ plus 21% O₂. A DragerVamogas analyzer

(DragerVamos, Lubeck, Germany) was used to monitor the concentration of CO₂, O₂ and sevoflurane.

2.3 | Construction of luciferase reporter gene

Fragments of miR-7a,7b 3'-UTR were amplified from the DNA of mESCs by the polymerase chain reaction (PCR), with the following primers: forward, 5'-GGCGAGCTCATCCCACGTAGTGATGTGACC-3', with a SacI restriction site; reverse, 5'-GGCTCTAGAGAAACCACAGTGCATAACAGACTG-3' with an XbaI restriction site. The product was inserted into the luciferase reporter vector pGL3-cm (Promega, Madison, WI, USA). The PCR fragment was digested by SacI and XbaI (Fermentas, Waltham, MA, USA) in 1× buffer Tango (Fermentas) at 37°C for 5 hours. Digested DNA fragment was collected the by using TIANGel Midi Purification Kit (TIANGEN, Beijing, China) and linked to the pGL3-cm vector by using T4 ligase (Fermentas).

2.4 | Construction of luciferase reporter expressing mutated UTR

The miR-7a,7b 3'-UTR mutation was generated by replacing the miRNA-binding site sequence by the miRNA seed sequences and by inserting the mutated sequence into the wild-type pGL3cm-klf4 3'UTR luciferase reporter vector by using the QuikChange Lightning Multi Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA).

2.5 | Quantitative real-time PCR

Total RNA was extracted by using Trizol reagent (Sigma, St. Louis, MO, USA) and reverse-transcribed with M-MLV Reverse Transcriptase (Promega) and primers published in a previous study.¹⁷ Real-time quantitative PCR included 40 cycles of amplification. Expression of target genes ($2^{-\Delta\Delta C_t}$) was normalized against the gene for endogenous D-glyceraldehyde-3-phosphate dehydrogenase (Gapdh). Quantitative reverse transcription with PCR (RT-PCR) was carried out in an Mx3000P system (Stratagene).

2.6 | Quantitative real-time PCR for miRNA

miRNA was reverse-transcribed to cDNA by using a stem-loop reverse transcription primer (RiboBio, Guangzhou, China). Quantitative RT-PCR was carried out in an Mx3000P system (Stratagene). Small nuclear RNA U6 was used as an internal control. Target gene expression ($2^{-\Delta\Delta C_t}$) was normalized against endogenous U6 RNA. The miRNA quantitative PCR primers were synthesized by RiboBio.

2.7 | Transfection of miRNA or inhibitor

mESCs were transfected with chemically synthesized miRNA inhibitor (Biolend, China) with Lipofectamine 2000. miR-7a,-7b mimics (RiboBio), the synthetic RNA nucleic acids, were used for overexpression of miRNAs. Cells were transfected with a miR-7a,7b mimics (50 nM), miR-7a,7b inhibitor (100 nM) or both, 12 hours prior to sevoflurane exposure.

2.8 | Luciferase assay

Mouse embryonic stem cells (1×10^5 per well of 24-well plates) were transfected with 200 ng wild-type UTR or mutant UTR reporter, 10 ng Renilla vector and 50 nM chemically synthesized miR-7a,7b (Biolend) with Lipofectamine 2000. Cell lysates were harvested 24 hours after transfection for the dual luciferase assay (Promega).

2.9 | Western blotting analysis

Cell lysates were resuspended in $5\times$ loading lysis buffer (250 mM Tris-HCl pH 6.8, 5% dithiothreitol, 10% SDS, 0.5% bromophenol blue, 50% glycerin). The membrane was then incubated with a primary antibody against KLF4 (Abcam, Cambridge, MA, USA) or GAPDH (Cell Signaling Technology, Danvers, MA, USA). Signals were visualized by enhanced chemiluminescence (Thermo Scientific, Waltham, MA, USA). Protein level quantification was normalized to GAPDH by using Image J software (National Institutes of Health, USA).

2.10 | Cell apoptosis assay

AnnexinV-FITC Cell Apoptosis Detection kit (Keygentec, Nanjing, China) was used for cell apoptosis assay.

2.11 | Bromodeoxyuridine (BrdUrd) incorporation assay

Cells were cultured with BrdUrd incubation for 2 hours. The cells were then fixed by phosphate-buffered saline (PBS) solution with 4% paraformaldehyde for 18 minutes. Cells were washed by PBS and treated by DNase (TIANGEN) for 30 minutes. The BrdUrd primary antibody (Abcam) was then added and incubated at room temperature for 4 hours. Later, the cells were incubated with secondary

antibody at room temperature for 1 hour. Cell nucleus was dyed with 4',6-diamidino-2-phenylindole (DAPI) (Sigma). Then, the BrdUrd-positive cells in each clones per microscopic field were counted.

2.12 | Statistical analyses

Results are presented as means $\pm$ standard deviation (SD). We used GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA) for all statistical analyses. Student's *t*-test or one-way ANOVA was used for statistical analyses. Statistical significance was defined as follows: * $P < .05$; ** $P < .01$; *** $P < .001$.

3 | RESULTS

3.1 | Sevoflurane inhibited the self-renewal of mESCs

In order to identify the mechanism underlying the effect of sevoflurane on mESCs, we treated the cells with the gradient concentration of sevoflurane (1%, 2%, 4.1%) for 6 hours to stimulate the long time non-obstetric surgery and found that the self-renewal was significantly influenced by sevoflurane 2% and 4.1%. mESCs tend to differentiate in advance for further 24-hour culture with normal cell culture medium. The inhibition of self-renewal was dependent on sevoflurane concentration (Fig. 1a). Additionally, because the concentration of 4.1% is clinically relevant concentration and has been shown to induce neurotoxicity and neuroinflammation in H4 human neuroglioma cells, neurons and microglia cells in previous studies.^{2,17,18} We then chose the concentration of 4.1% of sevoflurane to perform the further experiments. We detected the expression of the core stemness markers Oct4, Sox2 and Nanog which are essential in maintaining stemness, and found the significant downregulation in mESCs treated with sevoflurane (Fig. 1b). Flow cytometry analysis of cell cycle

FIGURE 1 Sevoflurane reduces self-renewal ability of mESCs. (a) Representative micrograph shows the morphology of mESCs differentiation by treating with sevoflurane for 6 h (Ctrl means control group with no sevoflurane). The scale bar represents 100 μ m. (b) Detection of mRNA level of stemness markers in the group treated with sevoflurane compared with control group by qRT-PCR (Ctrl means control group treated without sevoflurane). Data shown are means $\pm$ SD ($n=5$), * $P < .05$, ** $P < .01$. (c) Increased G1 phase population in sevoflurane-treated mESCs sorted after 6 h, analysed by flow cytometry. Data shown are means $\pm$ SD ($n=5$). (d) BrdUrd incorporation assay of mESCs treated with sevoflurane for 6 h. Data shown are means $\pm$ SD ($n=5$), * $P < .05$

FIGURE 2 miR-7a,7b was upregulated in sevoflurane treated mESCs. (a) Detection of mRNA level of miR-7a,7b in the group treated with sevoflurane compared with control group by qRT-PCR (Ctrl means the control group treated without sevoflurane). Data shown are means $\pm$ SD (n=3), * P <.05, ** P <.01. (b) Micrograph shows that differentiation of mESCs overexpressed with miR-7a/7b (Ctrl means control group transfected with miRNA control). The scale bar represents 100 μ m. (c) Detection of the mRNA level of stemness markers Oct4, Sox2 and Nanog, in the group treated with miR-7a or 7b for 48 h compared with control group by qRT-PCR (Ctrl means control group transfected with miRNA control). Data shown are means $\pm$ SD (n=4) * P <.05, ** P <.01, *** P <.001. (d) Increased G1 phase population in miR-7a/7b overexpressed mESCs, analysed by flow cytometry. Data shown are means $\pm$ SD (n=4). (e) BrdUrd incorporation assay of mESCs overexpressed with miR-7a or 7b for 48 h. Data shown are means $\pm$ SD (n=3), ** P <.01, *** P <.001

showed that there was reduction in proportion of cells in S phase, and an increase in G1 and G2/M phases in mESCs treated with sevoflurane (Fig. 1c). We also performed BrdUrd experiment to determine

the influence on proliferation and found that BrdUrd-positive cell number was lower in mESCs treated with sevoflurane than control (Fig. 1d). Furthermore, we found that the sevoflurane treating mESCs

FIGURE 3 Sevoflurane represses the self-renewal of mESCs by upregulating the level of miR-7a,7b. (a) Representative pictures of mESC transfected with miR-7 inhibitor or miR-7a/7b inhibitor and control group. The scale bar represents 100 μ m. (b) Stemness markers Oct4, Sox2 and Nanog, in mESCs transfected with miR-7a/7b inhibitors by qRT-PCR (control group treated without miR-7a,7b inhibitors). Data shown are means $\pm$ SD (n=5), * P <.05, ** P <.01, *** P <.001. (c) Increased and rescued G1 phase population in miR-7a/7b overexpressed mESCs after transfected with miR-7a,7b inhibitors, analysed by flow cytometry. Data shown are means $\pm$ SD (n=5). (d) BrdUrd experiment of mESCs transfected with miR-7a,7b inhibitors. Data shown are means $\pm$ SD (n=3), ** P <.01

underwent about 4-fold increase in apoptosis than control group. But the total of apoptosis cell was not a lot (Fig. S1A).

3.2 | The level of miR-7a,7b was upregulated in sevoflurane-treated mESCs

To find the mechanism of the effect of sevoflurane on mESCs, we treated the mESCs with 4.1% sevoflurane for 6 hours, and detected

that the level of miR-7a,7b was upregulated (Fig. 2a). We wanted to know whether miR-7a,7b regulates the self-renewal of mESCs, so we transfected the miR-7a or miR-7b into the mESCs and found that the mESCs tend to differentiate in advance (Fig. 2b). Then we detected that the expression of Oct4, Sox2 and Nanog was decreased in mESCs transfected with miR-7a/7b compared with control group (Fig. 2c). Furthermore, we performed cell cycle assay experiment and found that both miR-7a- and miR-7b-transfected group turn up to

FIGURE 4 Sevoflurane inhibits the self-renewal of mESCs by increasing miR-7a,7b and subsequently repressing Klf4. (a) Prediction of Klf4 3'-UTR-binding site. (b) Luciferase reporter assay performed with vectors containing DNA fragments corresponding to the putative wild-type or mutant target sites for miR-7a,7b in the 3'-UTRs of Klf4 mRNAs. Firefly luciferase activity is normalized to Renilla luciferase activity (WT-UTR luciferase reporter gene vector with wild-type Klf4 3'-UTR, mut-UTR mutant luciferase reporter gene vector with mutant miRNA-binding site). The miR-7a,7b is used as a negative miRNA control in the assay. Data shown are means $\pm$ SD (n=5), **P<.01. (c, d) miR-7a,7b decreases the expression of Klf4, both at the mRNA and protein levels (ctrl means miRNA control). Data shown are means $\pm$ SD (n=5). *P<.05. **P<.01

be arrested *via* G1 phase delay. There was reduction in proportion of cells in S phase, and an increase in G1 and G2/M phases (Fig. 2d). The result of BrdUrd experiment came out similarly with the mESCs treated with sevoflurane (Fig. 2e).

3.3 | Sevoflurane effects the self-renewal of mESCs through miR-7a,7b

To prove that miR-7a,7b are the key miRNA downstream mediator of sevoflurane, we treated mESCs with sevoflurane, and then transfected the mESCs with miR-7a or 7b inhibitors. The micrograph (Fig. 3a) shows that the cells treated with both sevoflurane and miRNA inhibitors restored the clone morphology influenced by sevoflurane alone to be similar with control group. Then we detected the expression level of Oct4, Sox2 and Nanog and found that all the expression of factors can be rescued after

being transfected with miR-7a or 7b inhibitors in sevoflurane-treated mESCs (Fig. 3b). Additionally, the cell cycle of sevoflurane-treated mESCs transfected with inhibitors almost restored as same as control group (Fig. 3c). BrdUrd experiment also consistently determined the rescue of proliferation (Fig.3d). These results showed that sevoflurane influences mESCs by regulating the level of miR-7a,7b in mESCs.

3.4 | Klf4 is the downstream target gene of miR-7a,7b

miRNAs perform their function by targeting downstream mRNAs and inhibiting the translation of proteins. Thus, we wanted to identify the target genes of miR-7a,7b. Using the TargetScan, Miranda, and miRBase,^{19–21} we predicted that Klf4 might be directly targeted by miR-7a,7b (Fig. 4a). We then engineered luciferase reporters with

FIGURE 5 Klf4 is the downstream gene of miR-7a,7b in sevoflurane/miR-7a,7b signalling pathway. (a) Micrograph shows that the cellular morphology of mESCs, transfected with miR-7a/7b, and overexpressed Klf4, control group without miR-7a,7b and Klf4. The scale bar represents 100 μ m. (b) mRNA level of stemness factors, including Oct4, Sox2, and Nanog in mESCs transfected with miR-7a/7b and Klf4, (control group without Klf4 and miR-7a/7b) by qRT-PCR. Data shown are means $\pm$ SD (n=5), * P <.05, ** P <.01. (c) BrdUrd experiment of cells in Fig. 5b. Data shown are means $\pm$ SD (n=3), ** P <.01. (d) Increased and rescued G1 phase population in miR-7a/7b overexpressed mESCs after transfected with Klf4, analysed by flow cytometry. Data shown are means $\pm$ SD (n=3), ** P <.01. (e) Micrograph of mESCs treated with sevoflurane and overexpressed with Klf4, control group without Klf4. The scale bar represents 100 μ m. (f) mRNA level of stemness factors, including Oct4, Sox2 and Nanog in mESCs transfected with Klf4 (control group without Klf4) by qRT-PCR. Data shown are means $\pm$ SD (n=5), ** P <.01. (g) The cell cycle analysis of mESCs treated with sevoflurane and transfected with Klf4, analysed by flow cytometry. Data shown are means $\pm$ SD (n=3), ** P <.01. (h) BrdUrd experiment of rescue experiment. Data shown are means $\pm$ SD (n=3), *** P <.001

wild-type 3'-UTRs of Klf4 or mutant 3'-UTRs to perform luciferase assays and confirmed the regulating relationship between Klf4 and miR-7a,7b. In 3T3 cells, the luciferase reporters were cotransfected with miR-7a,7b. The miR-7a,7b significantly reduced the luciferase activity of the wild-type Klf4, compared with the negative control. In contrast, mutant reporters were not repressed by miR-7a,7b. A mutation in the miR-7a,7b-binding seed region in Klf4 3'-UTR abrogated the suppressive effect of miR-7a,7b (Fig. 4b). Furthermore, overexpression of miR-7a or 7b was able to downregulate the expression of Klf4 both on the mRNA and protein levels (Fig. 4c,d). These results show that Klf4 is the downstream target gene of miR-7a,7b.

3.5 | Klf4 is the key target of miR-7a,7b to mediate the sevoflurane/miR-7a,7b signalling pathway

As we have proved that Klf4 is the downstream target of miR-7a,7b, we wanted to confirm whether Klf4 can directly mediate the influence on self-renewal of miR-7a,7b. We first transfected mESCs with miR-7a/7b, and then overexpressed Klf4 into the mESCs. Representative pictures showed that the effect of miR-7a,7b on self-renewal was restored by transfected overexpression of Klf4 in the mESCs (Fig. 5a). We also detected the mRNA level of stemness markers and found that overexpression of Klf4 in the mESCs restored the mRNA level of stemness genes downregulated by miRNAs (Fig. 5b). BrdUrd and cell cycle experiment also verified the rescue result about the cell proliferation (Fig. 5c,d). ES cells can differentiate into cells of three different germ layers (ectoderm, endoderm and mesoderm). We treated mESCs with 4.1% sevoflurane and then cultured cells without leukaemia inhibitory factor (LIF) for differentiation. We examined the expression of lineage-specific markers by qRT-PCR in day 7 and found that mES cells treated with sevoflurane tend to differentiate into endoderm and mesoderm (Fig. S2A). To assess the effect of sevoflurane signalling axis in modulating differentiation, we performed the differentiation of ES cells treated with sevoflurane and transfected with miR-7a and 7b inhibitor in the same time. We found that inhibition of miR-7a and 7b could block the influence of sevoflurane on differentiation detected by qRT-PCR on day 5 (Fig. S2B).

Furthermore, we study the correlation of sevoflurane and Klf4. Representative micrograph showed that overexpression of Klf4 rescued the mESCs differentiation caused by treating sevoflurane. And then we detected the stemness markers in these two mESCs to prove the rescue function of Klf4 in mESCs treated with sevoflurane (Fig. 5f). Also, overexpression of klf4 blocked the function of sevoflurane on inducing G1 phase delay (Fig. 5g) and the inhibition of cell proliferation (Fig. 5h).

4 | DISCUSSION

In summary, we found the underlying mechanism of sevoflurane toxicity of repressing the self-renewal ability by regulating miR-7a,7b/Klf4 signalling pathway in mESCs. Many pregnant women accepted the non-obstetric surgery and have to be exposed to inhalational

anaesthetics, such as sevoflurane, isoflurane, etc. Sevoflurane is one of the most commonly used inhalational anaesthetics; previous studies have verified that sevoflurane induces neurotoxicity and causes learning and memory impairment in neonatal mice.² Recent researchers showed that sevoflurane inhibits the proliferation of mouse NPCs and decreases the self-renewal capacity of neural stem cell.⁴ However, the toxicity of sevoflurane on embryonic development, especially the early development, remains largely unknown. In our study, we used the mESCs as the early development model to suggest the development in early pregnant. We found that sevoflurane could dose-dependently damage the clone morphology and induce the differentiation of mESCs in advance, which suggested the toxicity of sevoflurane. Self-renewal ability is important for mESCs because only the cells with self-renewal ability can rapidly proliferate under maintenance of stemness, besides, a few changes in numbers of stem cells may lead to totally different result in development.⁸ Regulation of the cell cycle of human embryonic stem cells (hESCs) regulates pluripotency and self-renewal.²² We found that both the cell proliferation and stemness maintenance were influenced by sevoflurane, which suggested that sevoflurane influenced the self-renewal of mESCs. Additionally, we detected whether the repression of self-renewal and differentiation in advance were conducive to the further development of mESCs. We examined the expression of lineage-specific markers and found that mES cells treated with sevoflurane tend to differentiate more into endoderm and mesoderm, but less ectoderm. These results suggested that sevoflurane might induce the development disorder in foetus. Previous studies showed the influence of sevoflurane on neural differentiation³ or increased the risk of the learning disabilities of children.²³ The repression of ectoderm differentiation caused by sevoflurane suggested the neural development obstacle. Our study revealed the problems that sevoflurane might cause in early development, and uncovered the mechanism of how sevoflurane influences the self-renewal ability in mESCs. Additionally, the study gives an insight into the proper use of sevoflurane for pregnant women and suggest its potential toxicity.

During the maintenance of self-renewal, miRNAs play an important role. miRNAs are key regulators of the self-renewal ability in mESCs.^{24,25} Also, previous study found that miR-125b promotes early germ layer specification in human embryonic stem cells.²⁶ In our study, sevoflurane inhibited the self-renewal ability of mESCs, and miR-7a,7b were upregulated by sevoflurane. miR-7a,7b directly target 3'-UTR region of Klf4, which is an important stemness factor for maintaining the self-renewal ability in mESCs. Moreover, the expression of Klf4 was downregulated by sevoflurane, and the transfection of miR-7a,7b inhibitors and overexpression of Klf4 both restored the mESCs self-renewal repressed by treating sevoflurane. Recent study showed that CHIR 99021 that induce activation of Wnt/ β -catenin signalling enhances Klf4 expression by repressing miR-7a expression.¹⁶ The sevoflurane showed the opposite function of activation of Wnt/ β -catenin signalling, which suggest the drug combination of sevoflurane and Wnt/ β -catenin signalling agonist could be used for pregnant women. This result about the sevoflurane inhibition on mESCs self-renewal

through the miR-7a,7b/Klf4 signalling pathway might provide a new idea for the development of novel therapeutics.

Our study demonstrates that sevoflurane inhibits mESCs self-renewal by regulating the miR-7a,7b pathway. Also, it may lead to a better and safer usage of inhalational anaesthetics for early pregnant women, and provide a helpful theory for finding out new therapeutics to prevent the toxicity of inhalational anaesthetics on foetal development in clinical settings.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.