Repetitive magnetic stimulation promotes the proliferation of neural progenitor cells via modulating the expression of miR-106b

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Abstract. Increasing evidence shows that repetitive transcranial magnetic stimulation (rTMS) promotes neurogenesis and the expression of microRNA (miR)-106b. The present study investigated whether rTMS promotes the proliferation of neural progenitor cells (NPCs) and whether the effect is associated with the expression of miR-106b. NPCs were cultured from the rat hippocampus and exposed to rTMS daily, comprising 1,000 stimuli for 3 days at 10 Hz, with 1.75 T output. The proliferation ability of the NPCs was revealed by EdU staining, and the levels of miR-106b and downstream gene p21 in the NPCs were measured by reverse transcription-quantitative polymerase chain reaction and western blot analyses. For analysis of the mechanism, the NPCs were transfected with Lenti-miR-106b or small interfering RNAs prior to rTMS. The results showed that: i) rTMS increased NPC proliferation, as revealed by the increased proportion of EdU-positive cells; ii) rTMS was able to upregulate the expression of miR-106b and downregulate the level of p21 in NPCs; iii) overexpression of miR-106b further enhanced the effects of rTMS, whereas knockdown of miR-106b had the opposite effects. Taken together, these data indicated that rTMS can promote NPC proliferation by upregulating the expression of miR-106b and possibly inhibiting the expression of p21.

Introduction

Repetitive transcranial magnetic stimulation (rTMS), as a non-invasive stimulation technique delivering a repetitive pulsed magnetic field, has been widely applied in treating various neurological diseases, including depression (1), pain (2), epilepsy (3), headache (4), insomnia (5) and Alzheimer's disease (6). Although the relevant mechanisms remain to be elucidated, rTMS treatment can induce neural plasticity effects, as evidenced by functional magnetic resonance imaging (fMRI) (7) and positron emission tomography (PET) analyses (8). In addition, rTMS has been demonstrated to influence glucose metabolism (8), long-term potentiation (9), the activity of ion channels (10), and the expression of plasticity-associated genes (11).

Neural progenitor cells (NPCs) in the subgranular zone (SGZ) and subventricular zone (SVZ) of the brain can self-renew, proliferate, migrate and differentiate (12). Following cerebral ischemia, NPCs are activated for proliferation and can migrate to the injured region for neuron repair and regeneration (13). rTMS has been shown to increase NPC proliferation in the SGZ of healthy rats (14) and in the SVZ of focal cerebral ischemia rats (15). However, the underlying mechanism of rTMS remains to be fully elucidated.

MicroRNAs (miRs) are 20-40-bp small non-coding RNAs, which can inhibit the translation of mRNAs involved in various physiological and pathological processes (16). Increasing evidence indicates that miRs modulate the proliferation of NPCs (17,18). Using array analysis, a previous study identified that miR-106b may promote the proliferation of NPCs (17). Brett *et al* (19) demonstrated that overexpressing the entire miR106b~25 cluster enhanced the proliferation of *in vitro* cultured NPCs. According to the analysis of targeting gene prediction (www.targetscan.org, and Kyoto Encyclopedia of Genes and Genomes), p21 of the cyclin-dependent kinase inhibitor (CDKI) family is negatively regulated by miR-106b, which has been shown to contribute to cell proliferation through accelerating the G1-to-S transition (20,21). In addition, the expression of p21 can be regulated by other miRs (22,23) in other types of cells. However, whether miR-106b can modulate the expression of p21 in NPCs has not been investigated.

Our previous study (24) indicated that rTMS was able to directly induce the proliferation of NPCs accompanied with the upregulation of miR-106b. The present study aimed to further investigate the effects of rTMS on cultured NPCs transfected with Lenti-miR-106b or small interfering (si)RNAs to clarify whether rTMS promotes NPC proliferation by upregulating the expression of miR-106b and possibly inhibiting the expression of p21.

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Materials and methods

Reagents. The primary antibodies and reagents used were as follows: Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 (DMEM/F12; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), B-27® Supplement (Invitrogen; Thermo Fisher Scientific, Inc.), basic fibroblast growth factor (b-FGF; Peprotech, Inc., Rocky Hill, NJ, USA), epidermal growth factor (EGF; Peprotech, Inc.), TrypLE™ Express Enzyme (Gibco; Thermo Fisher Scientific, Inc.), poly-L-lysine (Sigma; Merck KGaA, Darmstadt, Germany), β-actin antibody (cat. no. BM0627; Wuhan Boster Biological Technology Co., Ltd., Wuhan, China), EdU (Ruibo Biological Technology Co., Ltd., Guangzhou, China), mouse anti-rat nestin (cat. no. 556309; BD Biosciences, Franklin Lakes, NJ, USA), FITC-labeled rabbit anti-mouse IgG (cat. no. 315-005-003; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA).

Preparation of proliferation medium. For the production of 100 ml of proliferation medium, 98 ml DMEM/F12 medium, 2 ml B-27® without vitamin A, 2 μg b-FGF and 2 μg EGF were mixed, sterilized using a 0.22-μm filter in a laminar flow hood, and stored in a 4˚C refrigerator.

Culture of NPC neurospheres. The NPC neurospheres were cultured as previously described (25). In brief, bilateral hippocampal tissues were rapidly dissected from the brains of 10-15 neonatal Sprague-Dawley rats within 3 days of birth for each experiment. The neonatal rats (weight, 5-6 g) were provided by Tongji Medical College Experimental Animal Center of Huazhong Technology University (Huazhong, China). Rooms were maintained at 20-24˚C (50% relative humidity) and a 12-h light/dark cycle. The hippocampal tissues were placed into cold Hank's Buffered Salt Solution (HBSS; Sigma-Aldrich; Merck KGaA), Following enzyme digestion with TrypLE™ Express (Gibco; Thermo Fisher Scientific, Inc.) in a 5% CO_2 incubator (37˚C for 2 min), the tissues were mechanically dissociated using a pipette several times, and centrifuged (300 x g 5 min, 4˚C). The cells were suspended in the proliferation medium, as described above, and were seeded (10^{4-5} cells/ml, passage one) in dishes for culture with DMEM/F12 in a 5% CO_2 incubator at 37˚C. The neurospheres were subcultured every 5 days. The second generation of NPCs was prepared for rTMS. All experimental procedures were approved by the ethics committee of the Wuhan Sports University (Wuhan, China).

Experimental design. The experimental design is outlined in Fig. 1A. The NPCs were used for rTMS and miR overexpression/downregulation experiments. For the overexpression of miR-106b, the NPCs were transfected with lentivirus (Lenti)-null, or Lenti-miR-106b for 48 h prior to rTMS. For the downregulation of miR-106b, the NPCs were transfected with miR-106b siRNA using Lipofectamine™ 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) for 6 h prior to rTMS. Following 3 days of stimulation, the NPCs were used for EdU staining or miR/protein analyses. In the sham group, the NPCs were treated with rTMS without stimuli output. An empty lentivirus or Lipofectamine 2000 without siRNA was used for the respective negative control groups (Lenti-null + sham: LN; negative control + sham: NC). The groups were named as follows: Lenti-miR-106b + sham: L106b; Lenti-miR-106b + rTMS: L106bS; anti-miR-106b + sham: A106b; anti-miR-106b + rTMS: A106bS.

Transfection of the NPCs with Lenti-miR-106b or miR-106b siRNA. The pLVX-ZsGreen-Puro-rno-miR-106b vector (Wuhan Biofavor Co., Ltd., Wuhan, China) was transfected into 293T cells (Wuhan Biofavor Co., Ltd.) to generate high-titer lentivirus (biological titer, $1.0x10^8$ TU/ml) containing miR-106b. The NPCs were infected with the lentivirus based on the equation that MOI=30. The cells were re-suspended in 2 ml of complete medium, and incubated with $1.5x10^7$ TU lentivirus at 37˚C with 5% CO_2 for 48 h. Subsequently, the medium containing the NPCs was replaced with fresh medium to obtain 80% confluence. The siRNAs for miR-106b-5p (5'-UAAAGUGCUGACAGUGCAGAU-3') were synthesized by GenePharma Co., Ltd. (Shanghai, China). The NPCs were re-suspended at 10^5 cells/ml in Opti-MEM medium (Invitrogen; Thermo Fisher Scientific, Inc.), and transferred into flasks to culture for 2 h. According to the manufacturer's protocol, the miR-106b siRNAs were transiently transfected into NPCs using siRNA-Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) and cultured for 48 h at 37˚C with 5% CO_2. The NPCs were then treated with rTMS.

rTMS. The NPCs with or without miR modification were treated by sham or rTMS using a CCy-I type transcranial magnetic stimulation instrument (Wuhan Yiruide Medical Equipment Co., Ltd., Wuhan, China) according to a previous study (24). In brief, the culture dish was placed in the cross-center of an '8'-shaped magnetic coil which had a stimulus distance of rTMS of <1 cm between the cells and the coil (Fig. 1B). The rTMS was performed daily at 1,000 stimuli for 3 days at 10 Hz, with 1.75 T output. The neurospheres were examined under a light microscope (Fig. 1C).

Immunofluroscence and EdU staining. Following 3 days of rTMS, the cells were stained with nestin, which is a common marker of NPCs. The resuspended neurospheres were seeded into the 24-well glass slides coated with polylysine, and fixed with -20˚C methanol for 20 min. Subsequently, for the immunostaining of nestin, each coverslip was incubated with 20 μl mouse anti-rat nestin antibody (1:100) at 4˚C overnight. The cells were then incubated with secondary FITC-labeled rabbit anti-mouse IgG (1:400) for 2 h at room temperature, protected from the light. DAPI was added for nuclear staining for 15 min at room temperature.

EdU staining was used to determine the proliferative NPCs. The re-suspended NPCs in each 24-well contained 500 μl solution which was diluted with the culture medium at a ratio of 1,000:1 (reagent A) and cultured for 2 h. The medium was the discarded and 500 μl of pre-cooling pure methanol was added for fixation at room temperature for 20 min. The slides were then stained with 1X Apollo® staining reaction solution and 1X Hoechst 33342 reaction solution for 30 min respectively at room temperature (Fig. 1D).

Immunofluorescence images were observed using the Olympus Bx51 fluorescence microscope. A total of five

randomly-selected fields were counted in a blinded-manner using image processing software (ImageJ, v.1.6.0; National Institutes of Health, Bethesda, MD, USA) for quantification.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. According to the manufacturer's protocol, the total RNA of the cells was isolated using TRIzol reagent (Thermo Fisher Scientific, Inc.) and RNA concentration was measured using a spectrophotometer. The reverse transcription of RNA was performed using a TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) at 70˚C for 5 min, 42˚C for 60 min, and 95˚C for 5 min. To quantify the expression of miR-106b, a 20-μl reaction system included 100 μM/l rno-miR-106b forward and rno-miR-106b reverse primer, 10 μl SYBR Green/Flourescein qPCR Master mix (2X; Takara Bio, Inc., Otsu, Japan) and 4 μl cDNA (10X). The conditions were as follows: A cycle of 50˚C for 2 min, a 95˚C for 10 min, followed by 40 cycles of 95˚C for 30 sec and 60˚C for 30 sec. The $2^{-\Delta\Delta Cq}$ method was used to analyze the relative change in the expression of miR-106b (26). The primer sequences were as follows: U6, forward 5'-CGC TTCGGCAGCACATATAC-3 and reverse 5'-AAATATGGA ACGCTTCACGA-3'; rno-miR-106b, forward 5'-TGCGCT AAAGTGCTGACAGTG-3' and reverse 5'-CTCAAGTGT CGTGGAGTCGGCAA-3'.

Western blot analysis. The lysates of NPCs were extracted using a RIPA buffer (Beyotime Institute of Biotechnology, Shanghai, China) and were centrifuged at 12,000 x g for 10 min at 4˚C. Then 400 μl the supernatant mixed with 100 μl Laemmli buffer and was heated at 100˚C for 10 min. The protein concentration was determined by using the Protein Assay kit for bicinchoninic acid (Thermo Fisher Scientific, Inc.). Electrophoresis was performed with 50 μg of total protein. Protein was resolved on a 15% SDS PAGE and transferred on to polyvinylidene difluoride membranes. Membrane transfer of the p21 protein was achieved under 200 mA for 1 h. The membrane was then immersed in 5% tris-buffered saline and tween (TBST) and incubated at room temperature for 2 h. The primary antibody rabbit anti-rat p21 (1:500; cat. no. sc-397; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) was incubated overnight at 4˚C for 16 h. The membrane was then fully washed with TBST, and the goat anti-rat IgG secondary antibody (1:50,000; cat. no. BA1054; Wuhan Boster Biological Technology Co., Ltd.) conjugated to HRP was used for incubation of the membrane at room temperature for 2 h. The Gene Genius Bio-Imaging system gel imager was used to capture images, and BandScan version 5.0 software (Glyko Inc., Novato, CA, USA) was used to analyze the optical density signal strips.

Statistical analysis. The experimental data are expressed as the mean ± standard deviation. All experiments were repeated at least 3 times. Differences between groups were analyzed by one-way analysis of variance followed by the LSD test. Differences between two groups were analyzed using Student's t-test. SPSS 17.0 statistical software (version 17.0; SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Characterization of NPCs cultured from the hippocampus. The hippocampal tissues were separated from the newborn rats. Following the first passage, the cells started to form neurospheres, which had grown to almost 100 μm on the fifth day. The neurospheres at passage 2 exhibited a smooth shiny surface under light microscopy (Fig. 1C) and positively expressed the NPC-specific marker nestin (Fig. 1D).

rTMS promotes the proliferation of NPCs in vitro. EdU staining was used to analyze NPC proliferation. The results showed that there was a higher proportion of EdU-positive cells in the rTMS group than in the sham group cells (sham, vs. rTMS, 38.1±9.5%, vs. 51.7±25.5%, $P<0.01$; Fig. 2A and B). These results indicated that rTMS promoted the proliferation of NPCs.

rTMS increases miR-106b and decreases p21 levels in NPCs in vitro. The results showed that the treatment of rTMS significantly upregulated the expression of miR-106b (sham, vs. rTMS, 0.87±0.15, vs. 1.18±0.21, $P<0.01$; Fig. 2C). As shown in the results of the western blot analysis, rTMS markedly decreased the level of p21 (sham, vs. rTMS, 0.57±0.15, vs. 0.28±0.09, $P<0.05$; Fig. 2D and E).

Overexpressing miR-106b further enhances the proliferation of NPCs induced by rTMS. In order to illustrate whether miR-106b is involved in the effects induced by rTMS on NPCs, the expression of miR-106b in NPCs was modulated. As shown in Fig. 3A and B, the overexpression of miR-106b increased the number of EdU-positive cells compared with the number in the cells transfected with Lenti-null, the transfection control (L106b, vs. LN, 64.3±8.6%, vs. 28.1±4.7%, $P<0.01$). However, the knockdown of miR-106b reduced the proliferation of NPCs (A106b, vs. NC, 18.4±5.9%, vs. 38.1±9.5%, $P<0.01$). rTMS further increased the proliferation of NPCs in the miR-106b overexpression group (L106bS, vs. L106b, 88.2±4.6%, vs. 64.3±8.6%, $P<0.01$), which was eliminated by miR-106b siRNA (A106bS, vs. A106b, 38.6±6.5%, vs. 18.4±5.9%, $P<0.01$). Together, these data indicate that miR-106 modulated the rTMS-induced proliferation of NPCs.

rTMS upregulates the expression of miR-106b. Subsequently, the present study examined the expression of miR-106b in each group, and found that rTMS increased miR-106b in cells of the overexpression group (L106b, vs. L106bS, 2.09±0.1, vs. 2.43±0.11, $P<0.01$; Fig. 4A) and knockdown group (A106b, vs. A106bS, 0.30±0.02, vs. 0.48±0.02, $P<0.01$; Fig. 4B).

rTMS attenuates the protein expression of p21 in NPCs. Following lentiviral infection and knockdown of miR-106b in NPCs, the protein expression of p21 was assessed by western blot analysis (Fig. 5A and B). The data showed that the level of p21 was significantly decreased by rTMS in the overexpression group (L106b, vs. L106bS, 0.40±0.03, vs. 0.24±0.05, $P<0.05$) and knockdown group (A106b, vs. A106bS, 0.67±0.03, vs. 0.48±0.05, $P<0.05$).

The results showed that miR-106b, which promoted the proliferation of cells via p21, was upregulated by rTMS.

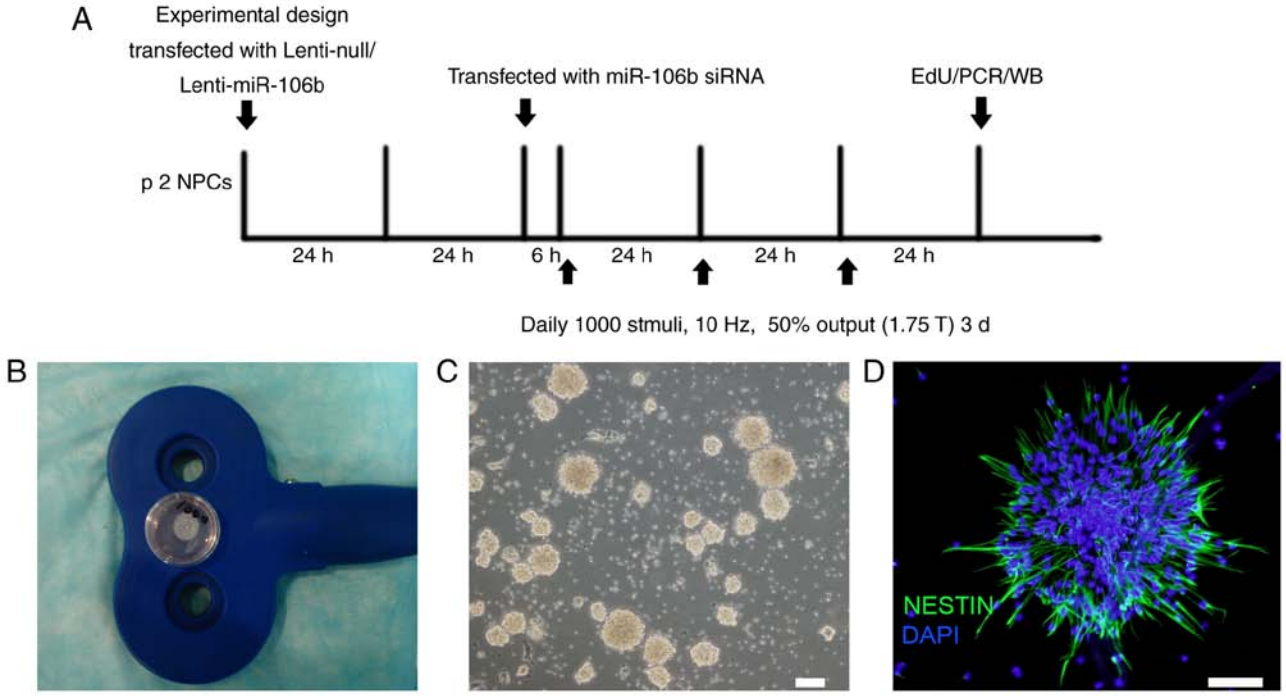

Figure 1. Position of the NPC culture dish and immunocharacterization of NPCs. (A) Outline of the experimental design. (B) Cell culture dish placement on the cross of '8' coil for rTMS. (C) Neurospheres were observed under a light microscope; scale bar=100 μM. (D) Expression of nestin (green) in neurospheres; scale bar=100 μM. NPCs, neural progenitor cells; p2 NPCs, passage 2 NPCs; rTMS, repetitive transcranial magnetic stimulation; miR, microRNA; siRNA, small interfering RNA; PCR, polymerase chain reaction; WB, western blotting.

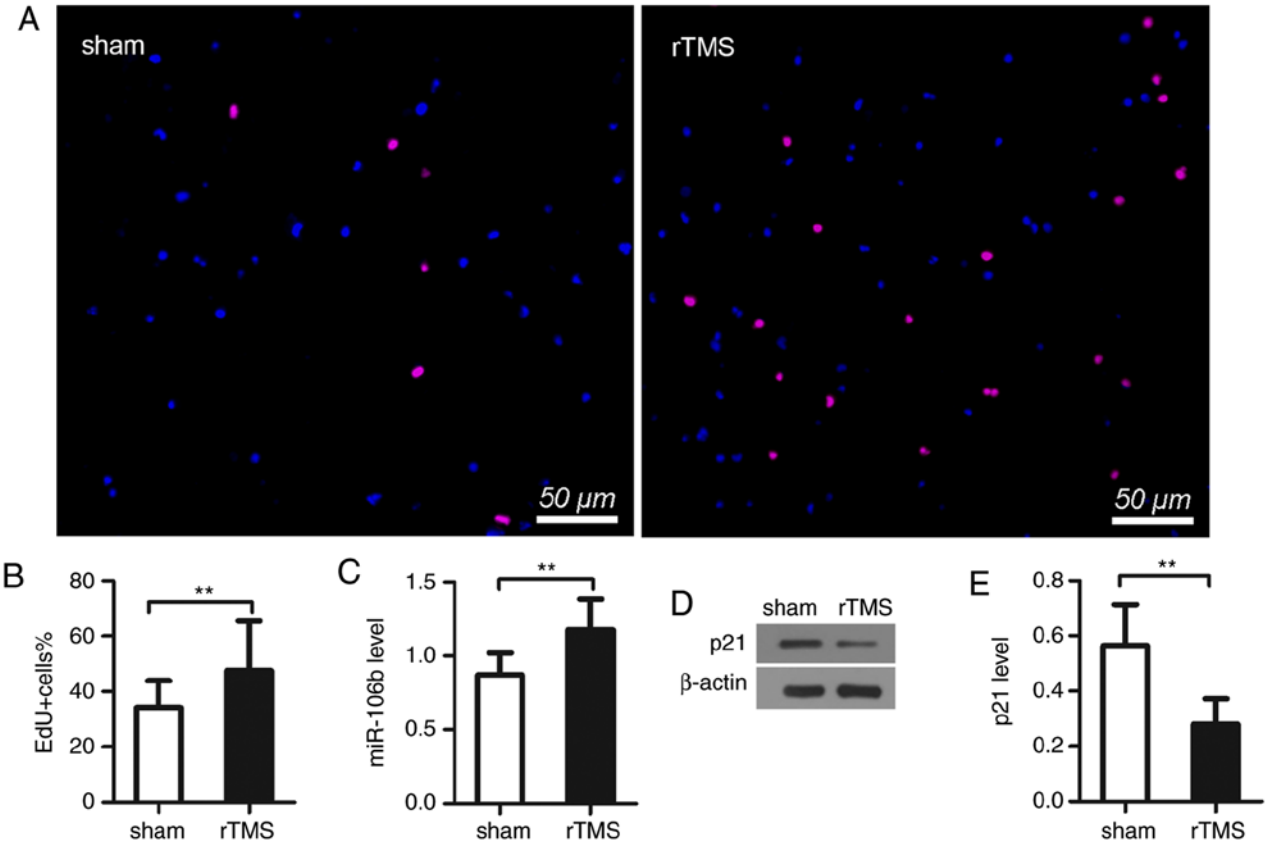

Figure 2. rTMS promotes the proliferation of neural progenitor cells via increasing the expression of miR-106b and decreasing p21. (A) EdU (purple) and DAPI (blue) staining in each group, with (B) statistical data shown in the graph. The proportion of positive cells is expressed as the mean ± standard deviation. Scale bar=50 μm. (C) Relative expression of miR-106b was assessed by reverse transcription-quantitative polymerase chain reaction analysis. Relative expression is expressed as the mean ± standard deviation. (D) Expression of p21 was measured by western blot analysis. (E) Statistical data of the protein expression of p21. Relative expression is expressed as the mean ± standard deviation. **P<0.01. NPCs, neural progenitor cells; rTMS, repetitive transcranial magnetic stimulation; miR, microRNA.

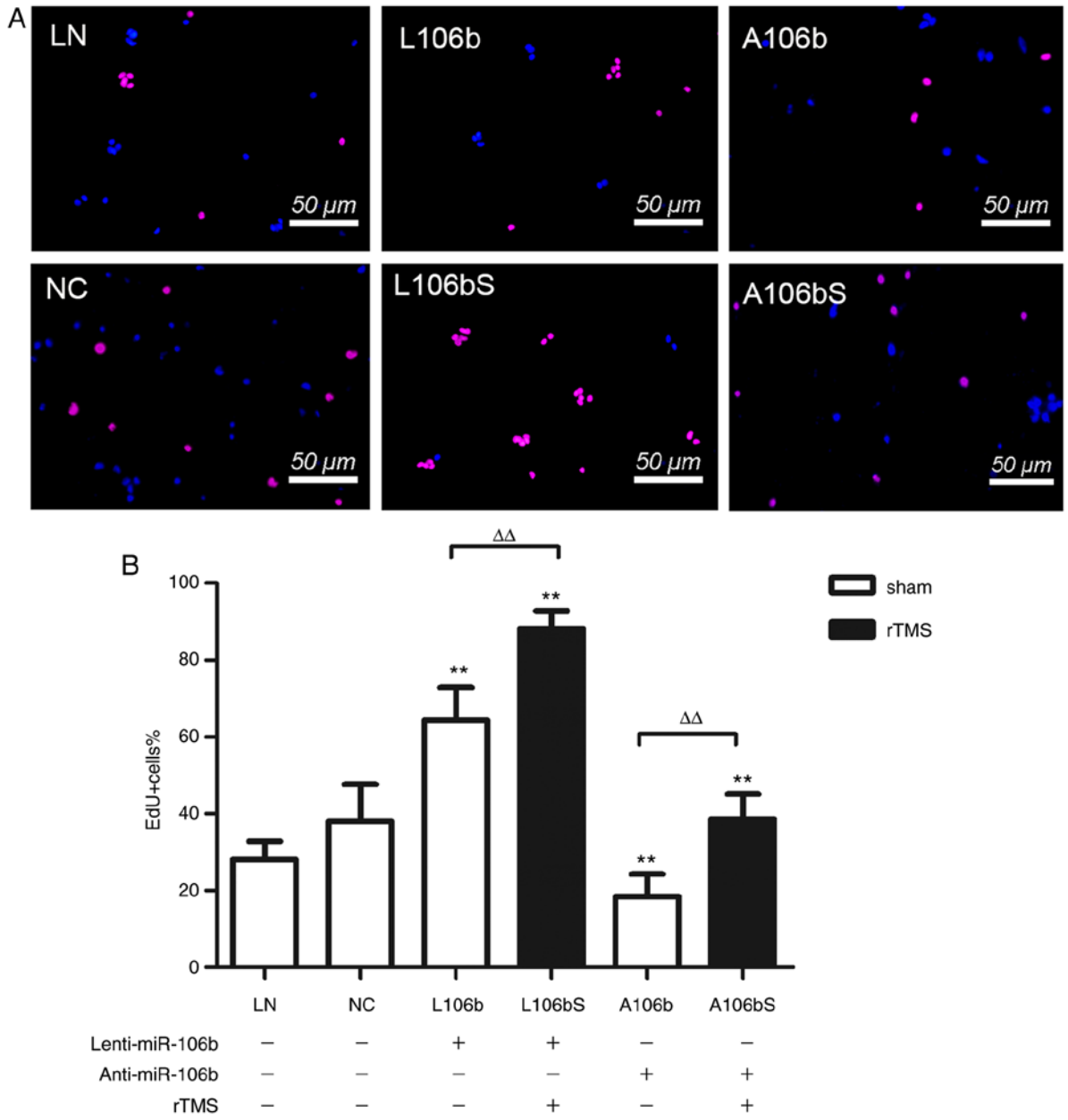

Figure 3. rTMS enhances the proliferation rate of neural progenitor cells in miR-106b overexpression and inhibition. (A) EdU (purple) and DAPI (blue) staining for each condition. Scale bar=50 μm. (B) Statistical graph of the staining results. The proportion of positive cells are expressed as the mean ± standard deviation. **P<0.01, vs. sham group; $^{\Delta\Delta}$P<0.01. miR, microRNA; rTMS, repetitive transcranial magnetic stimulation; LN, Lenti-null + sham; NC, negative control + sham; L106b, Lenti-miR-106b + sham; L106bS, Lenti-miR-106b + rTMS; A106b, anti-miR-106b + sham; A106bS, anti-miR-106b + rTMS.

These results suggested that rTMS promotes the proliferation of NPCs via miR-106b and possibly by inhibiting the expression of p21.

Discussion

It has been shown that rTMS can induce plasticity in the brain (7,8) and can influence the gene expression profile of NPCs and cultured neural cells (27-29). As a clinical treatment, evidence from fMRI (7) and PET (8) analyses has demonstrated that rTMS alters prefrontal-hippocampal network dynamics in healthy volunteers and increases glucose metabolism in rats. It has also been found to modulate miRs *in vitro* (24). In the present study, it was observed that rTMS induced EdU-positive NPCs and upregulated the expression of miR-106b. Subsequently, miR-106b was either stably overexpressed or its siRNAs were transfected into NPCs, and it was confirmed that rTMS promoted the proliferation of NPCs through miR-106b and possibly by inhibiting the expression of kinase inhibitor p21. The data are presented in Fig. 2.

The protocols of rTMS are generally controversial in treatment of the nervous system (30). Stimulation frequency is the most important factor in terms of rTMS parameters. Low frequency rTMS is considered to have an inhibitory effect on the brain (26), whereas high frequency rTMS has excitatory effects (31). In animal experiments, a high frequency (>5 Hz) has been reported to promote neural plasticity and improve

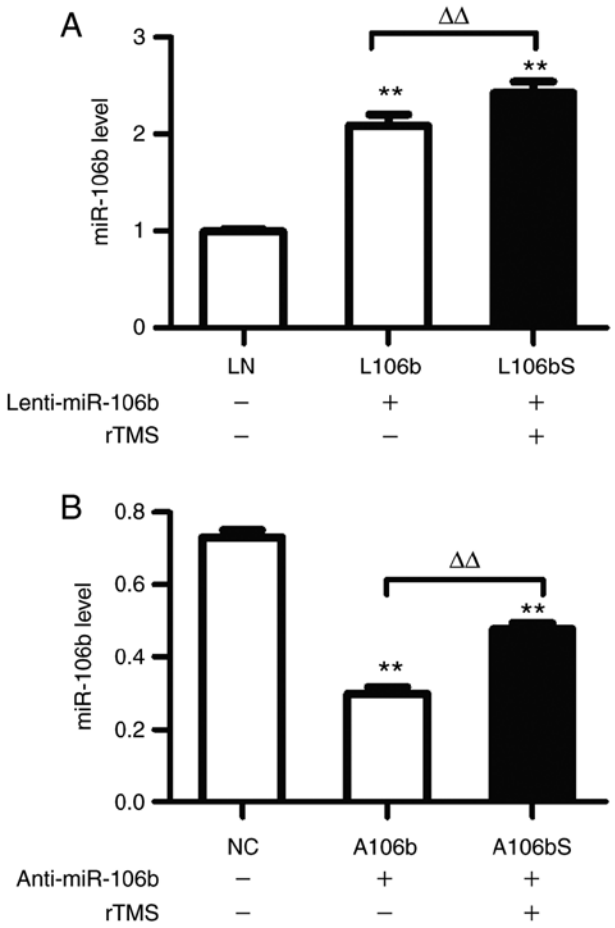

Figure 4. rTMS increases the expression of miR-106b in neural progenitor cells in miR-106b overexpression and inhibition. (A) Relative expression levels of miR-106b following the overexpression of miR-106b were assessed by RT-qPCR analysis. (B) Expression levels of miR-106b following inhibition of miR-106b were assessed by RT-qPCR analysis. Relative expression levels are expressed as the mean ± standard deviation. **P<0.01, vs. sham group, $^{\Delta\Delta}$P<0.01. miR, microRNA; rTMS, repetitive transcranial magnetic stimulation; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; NC, negative control + sham; L106b, Lenti-miR-106b + sham; L106bS, Lenti-miR-106b + rTMS; A106b, anti-miR-106b + sham; A106bS, anti-miR-106b + rTMS.

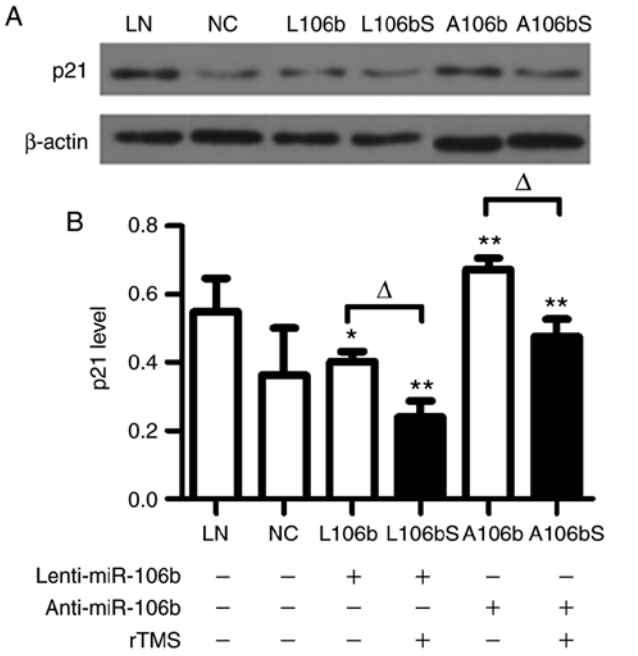

Figure 5. rTMS inhibits the protein expression of p21 in neural progenitor cells in miR-106b overexpression and inhibition. (A) Expression levels of p21 in each group were measured by western blot analysis. (B) Statistical graph of the protein expression levels of p21. Relative expression levels are expressed as the mean ± standard deviation. *P<0.05 and **P<0.01, vs. sham group; $^{\Delta}$P<0.05. miR, microRNA; rTMS, repetitive transcranial magnetic stimulation; LN, Lenti-null + sham; NC, negative control + sham; L106b, Lenti-miR-106b + sham; L106bS, Lenti-miR-106b + rTMS; A106b, anti-miR-106b + sham; A106bS, anti-miR-106b + rTMS.

behavior in rats with depression (32,33) and in rats with focal cerebral ischemia (15), associated with plasticity genes, including brain-derived neurotrophic factor (BDNF) (33-35). In cell experiments, compared with low frequency (1 Hz) rTMS, high frequency (10 Hz) rTMS induced neuroprotective and anti-apoptotic effects in a cell model of hippocampal injury (36,37). In addition, high frequency (10 Hz) rTMS has been shown to induce neural plasticity in hippocampal slice cultures (31). In clinical experiments, high frequency rTMS is generally used for neuropathic pain (38,39), cognition and motor recovery in patients with Parkinson's disease and Alzheimer's disease (6), and leads to superior improvements over low frequency rTMS. The stimulation intensity is another important parameter; it decreases within the coil distance of 3.5 cm, and 60% of its intensity is maintained at a distance of 1 cm (40). Although transcranial magnetic stimulations should not be uniform on the suspended cell cultures in a dish due to the difference in distance, the electromagnetic field has been shown to be effective in inducing NPC proliferation (29). The results showed that the proliferation of NPCs was promoted by rTMS daily (1,000 stimuli) for 3 days at 10 Hz, with 1.75 T output.

The expression of miR-106b is high in the adult rat brain and influences thousands of target genes. One of these, minichromosome maintenance complex component 7, which is decreased in the brain of rats with Down's syndrome, suggests that miR-106b is closely associated with nerve generation (41). In addition, miR-106b influences the insulin/insulin-like growth factor-1-Forkhead box O pathway (19), which can promote NPC proliferation (42). Our previous study found that protein kinase inhibitor p21 as the target gene of miR-106b was another proliferative factor through regulating cyclins (24).

The molecular mechanism of p21 regulating the proliferation of NPCs remains to be fully elucidated. Cell cycle is regulated by cyclins, cyclin-dependent kinase (CDK) and CDKI (43). p21 as one of the CDKIs, is the direct target gene of miR-106b (20). It combines with CDK2, CDK4/6, cyclinA, cyclinD and cyclinE to arrest the cell cycle (44). miR-106b-medited p21 silencing can affect the cell cycle and promote the cells to exit the G1 stage and enter the S stage (44,45). In addition, p21 can be combined with enhancer SRY-box binding protein-2 (Sox2) regulatory region 2 (46), which is a Sox2 marker in NPCs (47). Low p21 increasing the expression of Sox2 can induce the proliferation of NPCs. Tailless (Txl) is an orphan nuclear receptor specifically expressed in NPCs and P21, as target gene of Txl, is crucial for the homeostasis of NPCs (48,49). In addition, Yoon *et al* (50) claimed that a

therapeutic effect of rTMS on subacute cerebral ischemia rat was associated with an anti-apoptotic effect. Liu *et al* (51) demonstrated that miR-106b modulated the anti-apoptotic effect through inhibiting p21. Decreasing apoptosis upregulates neuronal turnover, which is beneficial for neural plasticity (52). p21 is a protector preventing premature loss of the NSC population (53); when there is a lack of p21, cells have a higher proliferative activity. In the present study, it was found that rTMS decreased the expression of p21, which was consistent with the EdU-positive cells. These data are supported by an *in vivo* study (16), which showed that 14 days of chronic rTMS increased the number of BrdU-positive cells in the dentate gyrus of rats. The present study did not characterize cell differentiation of the cultured NPCs in the proliferation medium, which requires examination in future investigations.

There is an equilibrium system in place to balance the generation, proliferation or differentiation of cells in the brain, and the pool of stem cells can be depleted due to a weak proliferation rate (54-56). The results of the present study suggested that rTMS assisted in maintaining the equilibrium system by the appropriate continuous growth rate of NPCs in the brain. It is reported that, in the adult hippocampus, ~700 new neurons (annual turnover rate 1.75%) are exchanged every day, with a mild decline during aging (57). Treatment including regular physical activity has been suggested to resist aging due to promoting the proliferation of NPCs associated with increasing BDNF (58,59). Taken together, neurogenesis induced by rTMS may be another method to alleviate aging, which has application prospects in future healthcare and medical treatment.

According to the data, rTMS increases miR-106b and decreases p21 levels in NPCs *in vitro*, which is determined by overexpressing and downregulating miR-106b expression. The present study showed that rTMS-miR-106b was the main pathway influencing the action of NPCs. In conclusion, high frequency (10 Hz) rTMS promoted NPC proliferation via upregulating miR-106b, and possibly by inhibiting the expression of p21.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

HL and GL performed experiments; HL, GL, JW and CM wrote the manuscript; all authors contributed to manuscript preparation, discussed the results, analyzed data and commented on the manuscript; HL, YC and YY developed the concepts and designed the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All experimental procedures were approved by the ethics committee of the Wuhan Sports University (Wuhan, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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