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Repetitive Magnetic Stimulation Promotes Neural Stem Cells Proliferation by Upregulating MiR-106b *In Vitro*[*]

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Summary: Neural stem cells (NSCs) proliferation can be influenced by repetitive transcranial magnetic stimulation (rTMS) *in vivo* via microRNA-106b-25 cluster, but the underlying mechanisms are poorly understood. This study investigated the involvement of microRNA-106b-25 cluster in the proliferation of NSCs after repetitive magnetic stimulation (rMS) *in vitro*. NSCs were stimulated by rMS (200/400/600/800/1000 pulses per day, with 10 Hz frequency and 50% maximum machine output) over a 3-day period. NSCs proliferation was detected by using ki-67 and EdU staining. Ki-67, p21, p57, cyclinD1, cyclinE, cyclinA, cdk2, cdk4 proteins and miR-106b, miR-93, miR-25 mRNAs were detected by Western blotting and qRT-PCR, respectively. The results showed that rMS could promote NSCs proliferation in a dose-dependent manner. The proportions of ki-67+ and Edu+ cells in 1000 pulses group were 20.65% and 4.00%, respectively, significantly higher than those in control group (9.25%, 2.05%). The expression levels of miR-106b and miR-93 were significantly upregulated in 600–1000 pulses groups compared with control group (P<0.05 or 0.01 for all). The expression levels of p21 protein were decreased significantly in 800/1000 pulses groups, and those of cyclinD1, cyclinA, cyclinE, cdk2 and cdk4 were obviously increased after rMS as compared with control group (P<0.05 or 0.01 for all). In conclusion, our findings suggested that rMS enhances the NSCs proliferation *in vitro* in a dose-dependent manner and miR-106b/p21/cdks/cyclins pathway was involved in the process.
Key words: repetitive magnetic stimulation; neural stem cells; EdU; ki67; microRNA-106b; cyclin-dependent kinase; cyclin-dependent kinase inhibitor

As a non-invasive brain stimulation (NIBS) technique, repetitive transcranial magnetic stimulation (rTMS) is considered to be safe and effective[1]. rTMS induces electric currents in the brain repeatedly so as to influence the excitability of the cortex, which has been proved by positron emission tomography (PET)[2] and functional magnetic resonance imaging (fMRI)[3] in many clinic tests. The therapeutic effect of rTMS was found to involve neural plasticity[4] and hippocampal neurogenesis in rats[5]. Ten Hz rTMS was demonstrated to promote the proliferation of neural stem cells (NSCs) in rat subventricular zone in our previous study[6]. However, the underlying mechanism is unclear.

By recognizing the 3' untranslated region (3'UTR)[7–9], microRNAs (miRNAs), short (approximately 22 nucleotides) non-coding RNAs, can silence the target mRNAs. A number of miRNAs can determine the cell fate, especially neurogenesis, neural differentiation and neural plasticity in the center nerve system[10–12]. MiR106b-25 cluster, which includes miR-106b, miR-25 and miR-93 with the similar sequence and targets, is strongly linked with NSCs functions[13–15]. Guo *et al* showed that miR-106b-25 cluster in the brain of rat with focal cerebral ischemia could increase EdU-positive NSCs after high frequency rTMS[6].

In contrast to numerous studies *in vivo*, there were only few studies on rTMS *in vitro*. In the present study, we used NSCs from neonatal rats to confirm that repetitive magnetic stimulation (rMS) could promote NSCs proliferation by regulating miR106b-25 cluster. We measured the expression of miR-106b, miR-25, miR-93 mRNAs and p21, p57, cyclinD1, cyclinA, cyclinE, cdk2 and cdk4 proteins after rMS, and found that miR-106b was the primary factor that affected the proliferation of NSCs. Because the stimulus intensity is an important parameter related to the rTMS effect[1], we also studied the relationship between the pulses and NSCs proliferation. Our findings indicated that rMS enhances the NSCs proliferation *in vitro* in a pulse dose-dependent manner. All of these results suggest that rMS at 10 Hz or higher can promote the proliferation of NSCs *in vitro*, and miR-106b/p21/cdks/cyclins pathway is involved in the process.

1 MATERIALS AND METHODS

1.1 NSCs Culture

NSCs were harvested from the bilateral hippocampus of neonatal 3-day-old SD rats provided by the Experimental Animal Center, Huazhong University of

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Science and Technology (Wuhan, China). After mechanical isolation, NSCs were cultured in DMEM/F12 (Gibco, USA) supplemented with 1×B27 supplement minus vitamin A (Invitrogen, USA), 20 ng/mL recombinant human bFGF (Pepro Tech, USA) and 20 ng/mL recombinant human EGF (Pepro Tech, USA) at 5% CO_2 in a 37°C incubator at a density of 1×10^5/mL. The media were replaced every 2–3 days and the cells were passaged every 5 days when the neurospheres' diameter reached 100–200 μm. To obtain the single-cell suspension, we used 1×TrypLE™ express enzyme (Gibco, USA) to dissociate cells at 5% CO_2 in a 37°C incubator for 3 min, then pipetted cells mechanically with a 1-mL pipet for almost 30 times. Cells from original tissue were regarded as passage 1 cells.

1.2 rMS Exposure

rMS was administered by a customized magnetic stimulator with a 90-mm figure-of-eight coil (Yirui De, CCY-Ⅰ, Wuhan, China). Passage 2 NSCs in dishes and flasks were put on the cross of the "8" coil, where the magnetic field was strongest (fig. 1). The rMS parameters were as follows: the frequency, 10 Hz; the intensity, 50% of the device's maximum power (peak value 3.5T); the interval time of the stimulation, 10 s. A series of rMS (200/400/600/800/1000 pulses per day) were administered once a day for 3 consecutive days. For control group, the NSCs in dishes and flasks were also put on the cross of the "8" coil but subjected to no stimulation.

1.3 Immunofluorescence Assay

Nestin and SOX_2 staining was used to identify the hippocampus-derived NSCs. Besides the microscopy analysis, the proliferation of cells was assessed by ki-67 and 5-ethynyl-2-deoxyuridine (EdU) staining. After 1-h exposure to rTMS, neurospheres were plated on 48-well plate coverslips coated with poly-L-lysine (0.1 mg/mL) (Sigma, USA) at 5% CO_2 in a 37°C incubator for at least 30 min. Then NSCs were fixed with absolute methyl alcohol for 20 min for ki-67/nestin/SOX_2 immunofluorescence before 200 μL solution A (1:1000) of EdU (Ruibo Company, China) was added into each well for incubation at 5% CO_2 in a 37°C incubator for 4 h. Briefly, each coverslip was covered with 20 μL mouse anti-nestin (1:100; BD, USA) and goat anti-SOX_2 (1:100; Santa Cruz Biotechnology, USA) antibody at 4°C overnight for NSCs immunostaining. FITC-anti-ki67 (1:50; eBioscience, USA) was added to cells for staining directly. The next day, nestin- and SOX_2-staining cells were incubated with secondary FITC-labeled rabbit anti-mouse IgG (1:400; Jakson ImmunoResearch, USA) and Cy3-labeled rabbit anti-goat IgG (1:100; EarthOx, USA) for 2 h in the dark. DAPI was added for nuclei staining for 15min.

Wells were rinsed by PBS (5 min×3), 0.5% TritonX-100 PBS (10 min) and PBS (5 min×1) for preparing for EdU staining. 1× Apollo® (500 μL) containing solution B 25 μL, solution C 5 μL, solution D 1.5 μL and solution E 5 mg was applied and incubated for 30 min at room temperature in the dark. Then cells were washed by 0.5% TritonX-100 PBS (5 min×3) and absolute methyl alcohol (5 min×1) respectively. 1× Hoechst 33342 staining was used for nuclei staining for 30 min. Apollo® solution excitation wavelength was 550 nm, emission wavelength 565 nm, Hoechst 33342 solution excitation wavelength 350 nm, and emission wavelength 461 nm. The time of exposure was below 30 ms[16].

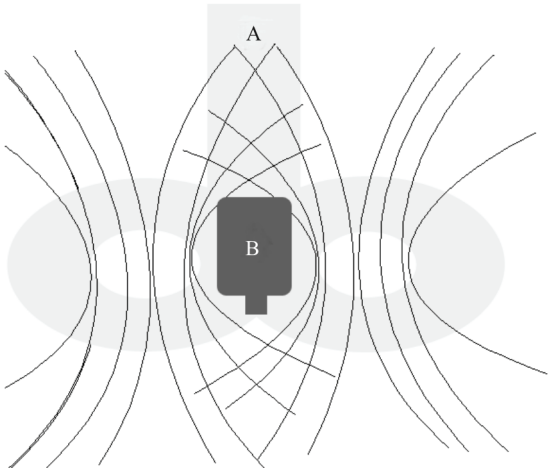

Fig. 1 The position of the NSCs culture flask and the stimulation parameters
A: the flask put on the cross of "8" coil; B: rMS stimulator. The cambered line means the magnetic field.

Immunofluorescence images were observed and recorded under the Olympus B×51 microscope. Five microscopic fields were randomly selected and the cells counted using image processing software (Image J, USA) for quantification.

1.4 Quantitative Real-time PCR (qRT-PCR)

According to the manufacturer's instructions, total RNA was extracted from stimulated passage 2 neurospheres. RNA was quantified by using a spectrophotometer. To quantify the expression of miR-106b, miR-25 and miR-93, reverse transcribed RNA was carried out by using TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) at 70°C for 5 min, 42°C for 60 min, and 95°C for 5 min. Each 20 μL qPCR system contained 100 μmol/L forward (F) primer, 100 μmol/L reverse (R) primer, 10 μL SYBR Green Supermix/Flourescein qPCR Master Mix (2×) (TaKaRa, Japan) and 4 μL cDNA (10×). The program was 1 cycle of 50°C for 2 min; 95°C for 10 min; 40 cycles of 95°C for 30 s, and 60°C for 30 s. Samples were run in triplicate and miRNA expressions were analyzed by $2^{-\Delta\Delta CT}$ method[17]. The primers are listed in the table 1.

1.5 Western Blotting

The crude lysates of whole cells were extracted by using RIPA buffer, and then they were transferred to prechilled eppendorf tubes and centrifuged at 12 000 g for 10 min at 4°C. The protein concentrations were quantified by using the Protein Assay Kit for bicinchoninic acid (Pierce, UK) according to the manufacturer's protocol. Proteins (50 μg) were resolved on a 15% SDS PAGE, transferred onto PVDF membranes and probed sequentially with antibodies. Antibodies were listed as follows: ki-67 (1:500, eBioscience, USA), p21 (1:200, Santa Cruz, USA), p57 (1:200, Santa Cruz, USA), cyclinA (1:400, Abcam, USA), cyclinD1 (1:1000, Cell Signaling Technology, USA), cyclinE (1:200, Santa Cruz, USA), cdk2 (1:200, Santa Cruz, USA) and cdk4 (1:200, Santa Cruz, USA). The expression levels of protein were analyzed by Odyssey IR imaging system (LI-COR Biosciences, USA).

Table 1 Primer sequences of miR-106b, miR-93 and miR-25

Primers	Loop	Forward	Reverse
U6		5'-CGCTTCGGCAGCACAT-ATAC-3'	5'-AAATATGGAACGCT-TCACGA-3'
rno-miR-106b	5'-GTCGTATCCAGTGCAGGGTCCGAGGT-ATTCGCACTGGAACGACATCTG-3'	5'-TGCGCTAAAGTGCTGA-CAGTG-3'	5'-CTCAAGTGTCGTGG-AGTCGGCAA-3'
rno-miR-25-3p	5'-GTCGTATCCAGTGCAGGGTCCGAGGT-ATTCGCACTGGATACGACTCAGA-3'	5'-TGCGCCATTGCACTTG-TCTCGG-3'	5'-CTCAAGTGTCGTGG-AGTCGGCAA-3'
rno-miR-93-5p	5'-GTCGTATCCAGTGCAGGGTCCGAGGT-ATTCGCACTGGATACGACCTACC-3'	5'-TGCGCCAAAGTGCTGT-TCGTGCA-3'	5'-CTCAAGTGTCGTGG-AGTCGGCAA-3'

1.6 Data Analysis

Results are expressed as $\bar{x}\pm s_{\bar{x}}$ and the data were analyzed by one-way ANOVA followed by a subsequent sidark post-hoc test via the statistical software SPSS (version 17.0; SPSS, USA). Statistical significance was set at $P<0.05$.

2 RESULTS

2.1 Identification of NSCs

Light microscopy revealed smooth and shiny passage 2 neurospheres, indicating a good activity of the neurospheres (fig. 2A). Nestin and SOX_2, the common markers of fetal and adult NSCs[18], were found to be highly expressed in neurospheres (fig. 2B–2E).

2.2 Proliferation of NSCs *In Vitro*

The percentage of ki-67+ cells was significantly increased in 800 and 1000 pulses groups ($P<0.01$ for both; fig. 3, and the expression levels of ki-67 protein were also significantly increased in 600, 800 and 1000 pulses groups as compared with control group ($P<0.01$ for all; fig. 4).

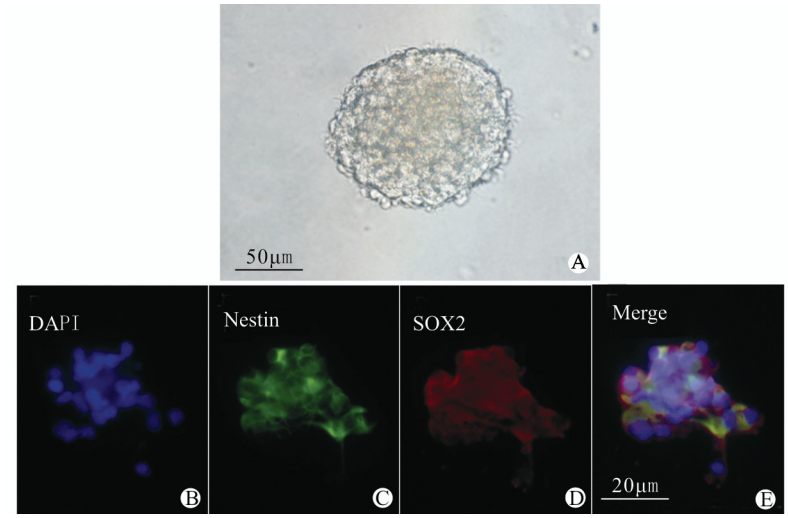

Fig. 2 Immunocharacterization of NSCs

NSCs are cultured and identified by nestin and SOX_2 fluorescent staining. A: Neurospheres were observed under the light microscope. Bar=50 μm. B: DAPI expressing nuclei (blue). Bar=20 μm. C: nestin (green) in neurosphere. Bar=20 μm. D: SOX_2 (red) in neurosphere. Bar=20 μm. E: merged picture. Bar=20 μm.

We evaluated NSCs proliferation by EdU staining, which has recently been considered to be an alternative of BrdU staining[19]. The results showed that EdU+ cells were significantly increased in 600, 800 and 1000 pulses groups as compared with control group ($P<0.01$ for all; fig. 5). The percentage of ki-67+ and Edu+ cells in 1000 pulses group was 20.65% and 4.00%, respectively, significantly higher than that in control group (9.25%, 2.05%).

2.3 Expression of miRNA-106b Cluster in NSCs Stimulated by rMS

The expression levels of miR-106b and miR-93 were significantly increased in 600 pulses stimulation group ($P<0.05$ for all; fig. 6) and in 800/1000 pulses groups (all $P<0.01$; fig. 6) as compared with control group. However, the expression levels of miR-25 were significantly reduced in 400/600/800/1000 pulses groups (all $P<0.01$, fig. 6).

2.4 Expression of p21 and p57 in NSCs Treated by rMS

The expression levels of p21 protein were decreased significantly in 800/1000 pulses groups ($P<0.01$ for all; fig. 7), while p57 protein expression levels were increased significantly in 600 pulses group ($P<0.05$; fig. 7) and in 800/1000 pulses groups as compared with control group ($P<0.01$ for all; fig. 7).

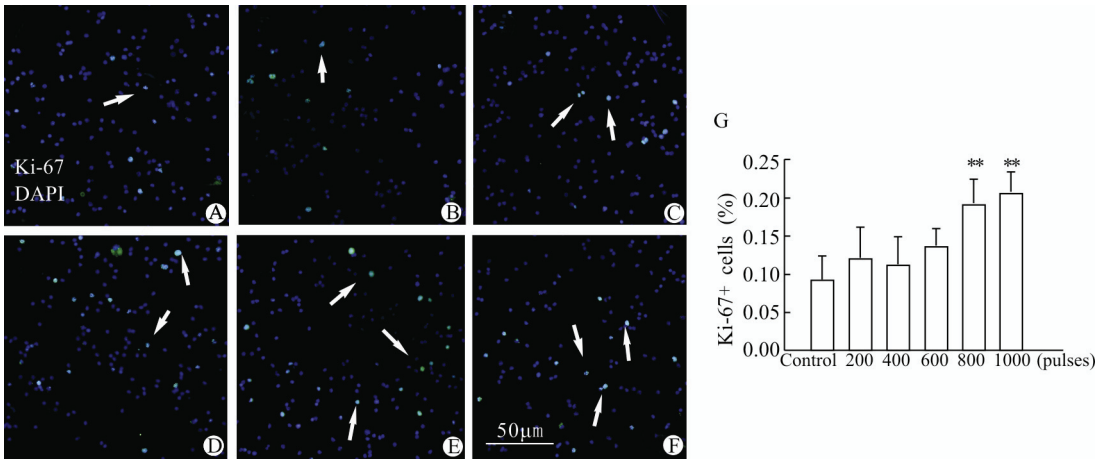

Fig. 3 Ki-67 staining showing the effect of rMS on the proliferation of NSCs *in vitro*
A: control group; B–F: 200, 400, 600, 800 and 1000 pulses groups, respectively. Ki-67 fluorescence (green) was expressed in nuclei, and all nuclei were counterstained using DAPI (blue). The arrows indicate ki-67+ cells. Bar=50 μm. G: the statistical graph. $^{**}P$<0.01 *vs*. control group

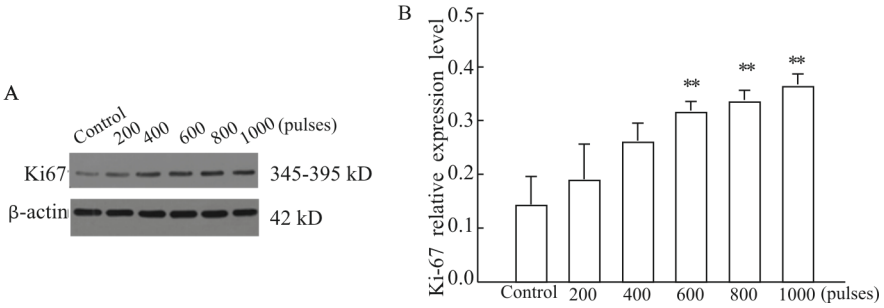

Fig. 4 Ki-67 protein expression in NSCs after rMS
A: expression changes of ki-67 in different groups; B: the statistical graph. $^{**}P$<0.01 *vs*. control group

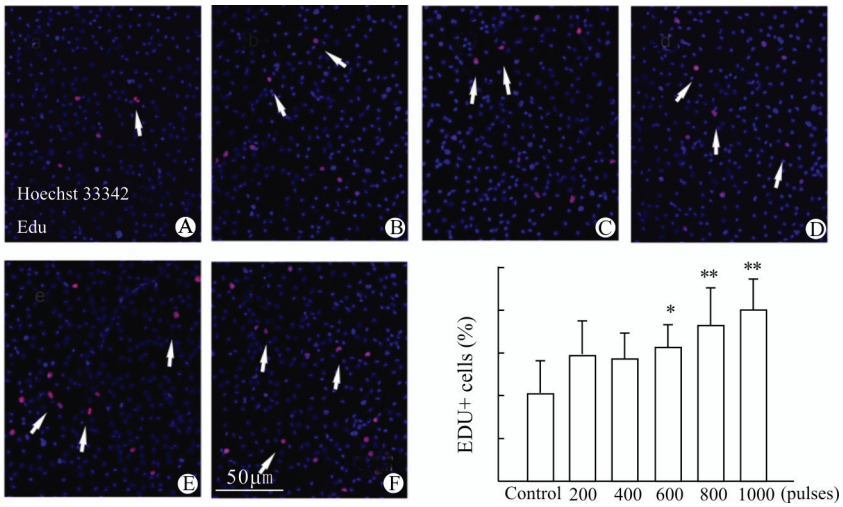

Fig. 5 EdU staining showing the effect of rMS on the proliferation of NSCs *in vitro*
A: control group; B–F: 200, 400, 600, 800 and 1000 pulses groups, respectively. Proliferative nuclei were counterstained by EdU (red), and nuclei were counterstained using Hoechst 33342 (blue). The arrows indicate Edu+ cells. Bar=50 μm. G: the statistical graph. $^{*}P$<0.05, $^{**}P$<0.01 *vs*. control group

2.5 Regulatory Effects of Cyclin-dependent Kinase Inhibitor p21 on Cyclin-dependent Kinases

The expression levels of cyclinA protein were significantly enhanced in 600/800/1000 pulses groups (P<0.05 or P<0.01; fig. 7) as compared with control group. Those of cyclinD1 protein were significantly increased in 800/1000 pulses groups (P<0.01 for all); cyclinE protein expression levels were much higher in 200/400/600/800/1000 pulses groups (P<0.05 or P<0.01); cdk2 protein expression levels were obviously increased in 400/600/800/1000 pulses groups (P<0.05 or P<0.01), and cdk4 protein expression levels were also markedly

increased in 1000 pulses group (P<0.05).

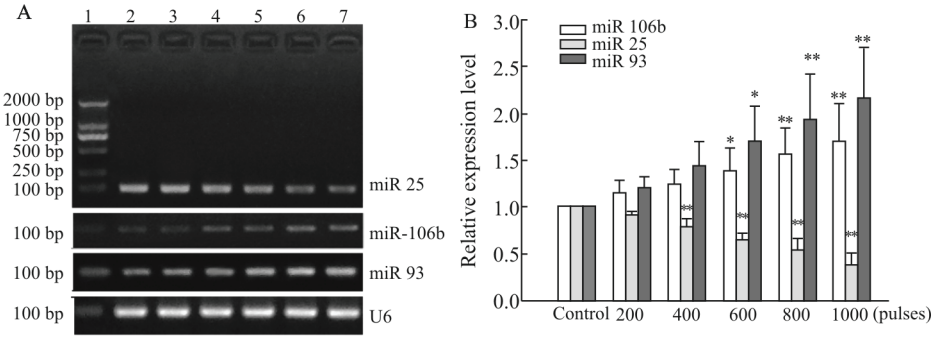

Fig. 6 Effect of rMS on the expression of the miR-106b family members

A: The expression of miR-106b cluster members were assessed by qRT-PCR in different groups; 1: DL2000, 2: control group, 3–7: 200–1000 pulses groups; B: the statistical graph. $^{*}P$<0.05, $^{**}P$<0.01

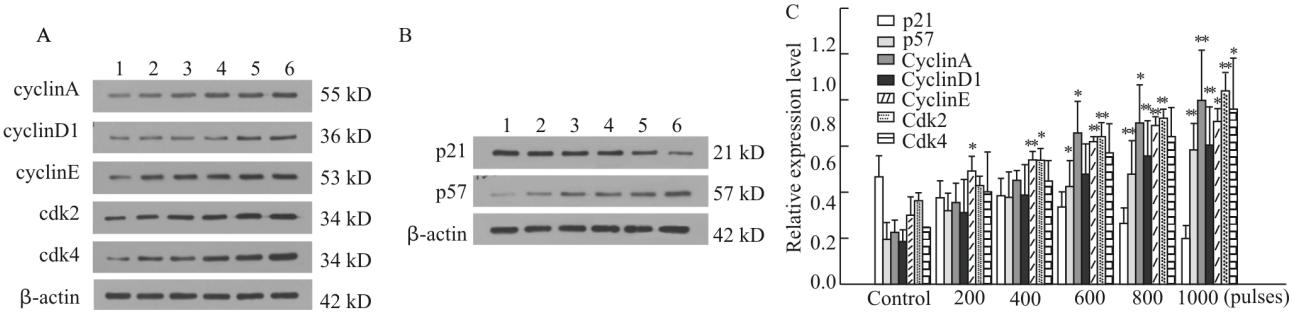

Fig. 7 p21-regulated potential signaling pathways

A: cyclin-dependent kinase protein expressions in different groups; 1: control group; 2–6: 200–1000 pulses groups; B: p21 and p57 protein expressions in different groups; C: the statistical graph. $^{*}P$<0.05, $^{**}P$<0.01 *vs*. control group

3 DISCUSSION

3.1 Proliferation of NSCs and rMS Pluses

Ki-67 is a marker of cells at each stage of cell cycle except G_0 phase[20]. As an alternative to BrdU, EdU is recently used to examine the proliferation of cells in S phase[16]. In our present study, ki-67 and EdU immunofluorescence showed that the percentage of ki-67+ and EdU+ cells was increased by 2.23 and 1.95 folds, respectively, in 1000 pluses group as compared with control group, suggesting that rMS promoted NSCs proliferation *in vitro*.

It has been reported that rTMS has therapeutic effects on depression, because it can reverse the decrease of NSCs[21] and enhance neurogenesis[22]. Neurogenesis is believed to be a potential therapeutic method for center nerve system diseases based on regulation of endogenous and exogenous factors[23–25]. A variety of cells are reported to be influenced by rTMS, including neurons[26], gails[27], NSCs[28], p12 and HL-60 cells[29]. However, the mechanism of neurogenesis by rMS remains elusive.

Our data demonstrated that rMS could promote the proliferation of NSCs in a dose-dependent manner. rMS protocol consists of stimulation frequency, amplitude duration and coil shape, which have been reported to be important in the rMS treatment[1]. As the frequency of 10 Hz was shown to have an exciting effect on rat brain[30], we chose 10 Hz in the present study. Additionally, the effect of rTMS depends on the number of stimuli *in vivo*[31]. We investigated the relationship between the number of pluses and proliferation of NSCs *in vitro* in the current study and observed that the highest pulses had the best proliferation effect as *in vivo*.

3.2 Relationship between MicroRNA Cluster miR-106b-25 and NSCs Proliferation after rMS

The miR-106b cluster members were found to be correlated with cancer[32–33], NSCs proliferation[13] and neovascularization[34]. They are located in the 13th intronic region of the Mcm7 oncogene and they can control DNA synthesis and cells entry into S phase[35]. In the present study, the result showed that the expression levels of miR-106b and miR-93 were significantly increased and those of miR-25 were reduced with the number of pluses increasing. This indicates that rMS can drive the proliferation of NSCs by regulating miR-106b cluster.

Our study showed that miR-25 has an opposite trend to miR-106b and miR-93 after rMS *in vitro*. It is known that miR106b-25 belongs to intronic microRNAs[36]. Given that intronic microRNAs can regulate host genes by synergistic or antagonistic effects[37], miR-25 may silence the promoter of host gene to keep the equilibrium of miR106b-25 cluster. The proliferative potential of NSCs is finite *in vivo*[38]. The miR-25 down-expression after rMS may be a contributor to this limitation. miR-25 may play a complicated role in the proliferation of NSCs after rMS, and further work will be

needed to find out how miR-25 is influenced by rMS in rat NSCs.

3.3 Potential Signaling Pathways Regulated by p21

MicroRNAs affect the proliferation of cells through four common mechanisms[39]. Targeting cell cycle regulators is a very important one[40]. To predict all conserved targets of rat miR-106 cluster members, TargetScan (www.targetscan.org) and KEGG database were used in this study. We found that the cell cycle progression regulator p21 was a direct target of miR-106b, and p57 was a target of miR-25[40–42].

Overexpressing the entire miR-106b-25 clusters in adult NSCs could promote cells proliferation[13]. This is related to the expression of cyclin-dependent kinase inhibitors such as p21, which can control G_1-to-S cell cycle transition[40]. P21 that represses the downstream protein of cyclinD1-cdk4/6, cyclin A-cdk2 and cyclin E-cdk2 complexes regulates cells maintenance by negatively prohibiting cell progression through G_1 phase[43–45]. Marques-Torrejon *et al* showed that p21 could also repress the expression of Sox2 gene[46], which is highly expressed in NSCs[47]. Lange *et al* reported that overexpression of cdk4/cyclinD1 could shorten the G_1 phase of progenitors and increase the expansion of progenitor[48], while loss of cdk2 and cdk4 could reduce the length of S-phase and be prone to neuronal differentiation[49]. Moreover, as a repressor, p21 can bind to E2F1, c-Myc or STAT3 to control cells fate[50]. Our data indicated that rMS suppressed the expression of p21 in NSCs and up-regulated the expressions of its downstream protein (cyclinD1, cyclinA, cyclinE, cdk2 and cdk4) to promote the proliferation of NSCs.

In conclusion, we demonstrated that rMS can promote the proliferation of NSCs in a dose-dependent manner *in vitro*. miR-106b/p21/cdks/cyclins pathway is involved in the proliferation of NSCs after rMS by modulating cell cycle progression.

Conflict of Interest Statement

The authors declare no conflict of interest in this study.

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