

lncRNA NEAT1-let 7b-P21 axis mediates the proliferation of neural stem cells *cultured in vitro* promoted by radial extracorporeal shock wave

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Abstract

In previous studies, we found radial extracorporeal shock wave (rESW), can promote the proliferation of neural stem cells (NSCs). Emerging evidence suggests that lncRNA NEAT1 can regulate NSCs proliferation. Whether lncRNA NEAT1 plays a role in the proliferation of NSC induced by shock waves is unclear. Cell Counting Kit-8 (CCK 8) method was used to detect the proliferation of NSCs, and the relative protein and mRNA expression of related genes of *Nestin*, *Cyclin D1* and *P21* were detected by Western Blot and Quantitative real-time PCR (RT-qPCR) respectively. Immunofluorescence staining was used to observe the changes in the number of BrdU/nestin positive cells. Overexpression of NEAT1 and let 7b in cells were used to explore whether rESW can rescue the decreased number of NSCs. We found that the optimal dose of R15 transmitter promoting NSCs proliferation is 1.5 bar, 500 pulse, 2 Hz. 1.2-1.5bar showed a dose-dependent effect on the proliferation of NSCs, but it was negatively correlated with the proliferation effect of NSC when it was more than 1.5bar. We revealed that let 7b-P21 axis was involved in regulating the inhibition of NSC proliferation which was activated by NEAT1 in NSCs. In addition, we demonstrated that rESW treatment resulted in the decrease of NEAT1 expression, which was accompanied by the improved biological function including proliferation. Our results confirm that low-intensity rESW (1.5bar, 500pulse, 2Hz) can promote the proliferation of NSCs through NEAT1-let 7b-P21 axis.

Introduction

Central nervous system (CNS) injuries are mainly caused by cerebrovascular accidents or mechanical contusions (for example, traumatic brain injury, TBI). Although the etiology is different, the common feature after injury is the loss of neurons (Arvidsson et al., 2002; Qian et al., 2021). It is believed that the nerve cells of the CNS of mammals lose their ability to regenerate shortly after birth, and the neuron loss caused by various reasons such as trauma and tumor can only be replaced by the proliferation of glial cells due to intracellular and extracellular factors that hinder neural regeneration (Russo & McGavern, 2016; He et al., 2020). This long hold concept has greatly restricted the development of treatment methods for neurological diseases. Currently, the available treatment options for CNS injury are limited, to palliative care (Romero-Ramírez et al., 2017). The discovery of neural stem cells (NSCs) provides new possibilities for the treatment of CNS injury. NSCs could differentiate into neurons and glial cells under specific conditions (Zhang & Jiao, 2015; Huang & Zhang, 2019). Adult NSCs in mammalian brains are mainly distributed in two areas—the subventricular zone (SVZ) of the anterior horn of the lateral ventricle and the subgranular layer (SG) of the dentate gyrs in the hippocampus (De Gioia et al., 2020). These cells could be used as source cells for regeneration by in situ induction with foreign factors.

Radical extracorporeal shock wave (rESW), as a kind of mechanical wave, has been widely used in rehabilitation medicine and orthopedics. Its therapeutic effects on bedsores, fractures, tendon injuries, aseptic inflammation and other diseases have been extensively confirmed by basic research and clinical trials (Hsiao et al., 2019; Liu et al., 2019; Kang et al., 2020; Kobayashi et al., 2020). Currently, rESW has achieved significant results in the field of regenerative medicine. In osteoarthritis (OA), rESW combined

with adipose-derived mesenchymal stem cells (ADMSCs) exhibited increased trabecular thickness and bone volume. The reduction of caspase-3 and platelet-derived growth factor (PDGF)-BB were also observed (Hsu et al., 2020). In a model of cultured fetal rat metatarsals, Sowmya R, etc. demonstrated that rESWT increased longitudinal bone growth by locally inducing chondrogenesis (Ramesh et al., 2020). Our previous studies have found that rESW can promote the proliferation and differentiation of NSCs, but the specific molecular mechanism needs to be further clarified (Zhang et al., 2017).

Recently, some studies have indicated that epigenetic modifications, such as DNA methylation, histone modification and non-coding RNA (ncRNA) are important factors affecting the development of the nervous system (Vieira et al., 2018). As a target gene of P53, lncRNA nuclear paraspeckle assembly transcript 1 (NEAT1) is a long non-coding RNA(lncRNA)essential for regulating the fate of stem cells (Mello et al., 2017; Zhang et al., 2019). Previous studies have shown that let 7b prolongs the cell cycle by inhibiting the expression of Cyclin D1—presumably due to increased expression of Pten, which is a negative regulator of Cyclin D1, thereby inhibiting the proliferation of NSCs(Zhao et al., 2010). At present, although some studies used dual luciferase reporter gene experiments have shown that NEAT1 and let 7b can interact with each other (Liu et al., 2019), whether the interaction between NEAT1 and let 7b regulates the proliferation of NSCs is not fully understood. Similarly, researchers have found that there is an interaction between let 7b and P21 in liver cancer cells (Hui et al., 2020), but they still cannot clearly explain how the two regulate the proliferation of NSCs. P21 is an important member of the Cyclin-dependent kinase inhibitor family. It regulates cell cycle, DNA replication and repair by inhibiting the activity of Cyclin-dependent kinases (Cyclin-dependent kinases CDKs) complex, and then regulating cell fate (Han et al., 2017; Patties et al., 2019).

Therefore, based on our previous experiment, the current explored whether the lncRNA NEAT1-let 7b-P21 axis mediates the promotion of NSCs proliferation by rESW.

Methods

Cell culture

NE-4C (Procell CL-0660) were provided by *Procell Life Science & Technology Co.,Ltd.* (Wuhan, China). Mouse NSCs were grown in Dulbecco's minimal essential medium with high glucose (DMEM), supplemented with 10% fetal bovine serum. Cells were cultured according to culture method guidelines and incubated at 37°C, 5% CO₂. NE-4C cells were dissociated with 0.05% trypsin-EDTA and resuspended in the incubator supplemented DMEM described above. Cells from passage levels 4–5 were used in the present study. The cell culture medium was refreshed every 1-2 days.

Cell proliferation test

To investigate the possible impact of rESWT on the viability/proliferation of NE-4C cells, different doses of rESWT (from 1.0 bar to 1.6bar, 500 impulses, and 2 Hz) were used in the cultures. The proliferation of NE-4C cells was analyzed by Cell Counting Kit-8 (CCK 8, Sevenbio, Beijing, China) proliferation assay.

After being treated with rESWT, 5×10^3 cells were seeded into 96-well plates to grow for 12h, 24h, 36h, 48h, 60h, and 72 h. Then, 10 μ L of CCK 8 was added to each well, and culture plates were incubated at 37°C for 1 h. The absorbance was measured by photometry at 450 nm. We chose 1.5 bar, 500 impulses, 2 Hz as a therapeutic dose.

rESWT treatment

To evaluate the influence on cell proliferation *in vitro*, rESWT (R15 transmitter, STORZ MEDICAL AG, Switzerland) was applied to the cultures at a dose of 1.5 bar, 500 impulses, and 2 Hz, which maximized the therapeutic effects without significant reduction of the cell viability. NE-4C cells were dissociated with 0.05% trypsin-EDTA and resuspended in the tube supplemented DMEM described above. Transferred the cell suspension filled with the complete medium to a 2mL EP tube. After removing the air in the tube, then embedded the EP tube into a 50mL centrifuge tube filled with coupling agent. Coupling was used to minimize the loss of shock wave energy at the interface between the transmitter and tube. The transmitter of rESWT acted vertically on the upper part (Figure 1A-C). The control group was maintained under the same culture conditions, but without rESWT exposure.

Cell transfection

The mimic targeting let 7b and the negative control were obtained from *General Biol Co., Ltd.* (Anhui, China). The NEAT1 overexpression plasmid vector was constructed by *HanBio Co., Ltd.* (Shanghai, China). Transient transfection of mimics (100nM) and plasmid vector (10 nM) was performed using Lipofectamine 2000 (Thermo Fisher, CA, USA) according to the manufacturer's instructions. The empty vector and negative control of siRNA were also used as control. Transfected cells were harvested at 48 h after transfection for subsequent analysis and detection.

Experiment grouping

To explore whether NEAT1 mediates rESW regulation of NSCs proliferation, we divided the experiment groups into blank control group (Control), negative control group (Blank, plasmid, BP), overexpressing lncRNA NEAT1 (NEAT1) group, shock wave treatment group (rESW), shock wave + negative control group (rESW+BP), shock wave + overexpressing lncRNA NEAT1 group (rESW+NEAT1). Similarly, for let 7b, we divided the experiment groups into: blank control group [Control], negative control group (negative mimic, NM), shock wave treatment group (rESW), shock wave + negative control group (rESW+NM), shock wave + overexpression let 7b group (rESW+let 7b).

Quantitative real-time PCR

Total RNA was isolated from the NE-4C using TRIzol (TransGen Biotech, Beijing, China) according to the manufacturer's instructions. Then, 1 μ g of total RNA was used for reverse transcription using a One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech) according to manufacturer's instructions. Green qPCR SuperMix (TransGen Biotech) was used to qualify the expression levels of

Nestin, *Cyclin D1*, and *P21*. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β -actin were respectively used as endogenous controls (Sangong Biotech, Shanghai, China). Next, quantitative RT-PCR was performed using mmu-miR-let 7b-specific probes and specific gene primers (Sangong Biotech, Shanghai, China). All qRT-PCR reactions were performed using the Step ONE Plus RT-PCR System (Applied Biosystems, USA). The relative quantification $2^{-\Delta\Delta Ct}$ method was applied to calculate the gene expression values. Primers and probes used in this study are shown in Table 1.

Table 1 Primers and probes used in this study

Neat1-F	5'-GGCAGGTCTAGTTTGGGCAT-3'
Neat1-R	5'-CCTCATCCCTCCCAGTACCA-3'
let 7b-F	5'-GCGCGCTATACAACCTACTGC-3'
let 7b-R	5'-AGTGCAGGGTCCGAGGTATT -3'
Nestin-F	5'-CAACCACAGGAGTGGGAACT-3'
Nestin-R	5'-TCTGGCATTGACTGAGCAAC-3'
P21-F	5'-CCTGGTGATGTCCGACCTG-3'
P21-R	5'-CCATGAGCGCATCGCAATC-3'
Cyclin D1-F	5'-CAGACCTCTTAACCTTATAG-3'
Cyclin D1-R	5'-TTCCCAAGCACCTCATACTACCAGC- 3';

Western Blot

Total protein was extracted from cells using ice-cold radioimmunoprecipitation assay (RIPA) buffer (Sevenbio, Beijing, China) supplemented with protease inhibitors (10 mg/mL aprotinin, 10 mg/mL phenyl-methylsulfonyl fluoride [PMSF], and 50 mM sodium orthovanadate). The BCA protein assay kit (Beyotime Institute of Biotechnology) was used to determine the protein concentration of the supernatant. Equal amounts of protein samples (30 μ g) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrically transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore, Shanghai, China). Non-specific binding was blocked by incubation with 5% fat-free milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) at room temperature for two hours.

The membranes were subsequently incubated with primary antibodies as follows: *Nestin* (1:500; Proteintech, Chicago, IL, USA), *P21* (1:500; Proteintech), *Cyclin D1* (1:5000; Proteintech), and GAPDH (1:3000; Affinity, Cincinnati, OH, USA) at 4°C overnight. The membranes were washed and incubated with HRP-conjugated secondary antibodies (Sevenbio, Beijing, China), diluted at 1:3000 at room temperature for two hours. Immunoblots were visualized using an enhanced chemiluminescence kit (ECL; Sevenbio, Beijing, China) and detected by Bio-Rad Chemi-Doc (Bio-Rad, CA, USA) and then normalized to that of GAPDH or β -actin.

Immunofluorescence staining

Add BrdU working solution one hour before cell fixation. Cells grown on coverslips were fixed with 4% paraformaldehyde, followed by treatment with 0.1 M glycine for 20 min at 25°C and 0.1% Triton X-100 for additional 5 min at 25°C to permeabilize. Cells were then incubated alternatively with the following primary antibodies: *Nestin* (1:500, Abcam, USA), *BrdU* (1:200, Abcam, USA), The primary antibodies were washed with PBS, and the cells incubated with goat anti-rat IgG-Dylight 594 (1:100 in PBS; A23440, Amylet Scientific, Wuhan, China) and goat anti-rabbit IgG-FITC (1:150; Abcam, USA) for 30 min at 25°C. The cell nuclei were counterstained with Hoechst 33342 (1:500; C0030, Solarbio, Beijing, China). Coverslips were finally mounted with Mowiol in PBS for observation. Images were taken with an Olympus BX51 microscope. Observations were made in 10 microscopic fields randomly taken from three different experiments.

Statistical analysis

Quantitative data were presented as mean \pm standard deviation (SD). GraphPad Prism 8 (GraphPad, La Jolla, CA, USA) software was used for statistical analysis. Student's t-test (two-tailed), one-way ANOVA or two-way ANOVA was employed to evaluate all statistical analyses. Differences were considered statistically significant when $P < 0.05$.

Results

1. rESW promotes the proliferation of NSCs

To clarify the optimal dose of rESW that affects NSCs proliferation, firstly, we used Cell Counting Kit-8 (CCK 8) to detect the effect of different rESW excitation pressure on NSCs. Compared with the control group, rESW at a dose of 1.5 bar, 500 points, and 2 Hz significantly increased the number of NSCs 72 hours after rESW treatment (Figure 2A-D). Between 1.2bar and 1.5bar, the proliferation-promoting effect of rESW on NSCs was positively correlated with the dose. Interestingly, when the dose was over 1.5 bar, the opposite effect was observed, and the Western Blot experiment confirmed the observation (Figure 2B, C). Therefore, the dose we used in this study was 1.5 bar, 500 points, 2 Hz. At the same time, at 72h, we used RT-qPCR to detect the expression of NSCs specific protein Nestin mRNA and used 5-Bromodeoxyuridine (BrdU) labeling method to detect cell proliferation. We observed that compared with the control group, the excitation pressure of 1.5 bar can significantly promote the proliferation of NSCs, showed by the number of BrdU+/nestin+ positive cells, which were increased significantly (Figure 2E, F).

2. rESW inhibits the expression of lncRNA NEAT1 and let 7b

In order to determine how rESW affects the expression of NEAT1 and let 7b, we used RT-qPCR to detect the expression of NEAT1 and let 7b within 72 hours after rESW treatment on NSC. It was found that rESW had the most significant inhibitory effect on the expression of NEAT1 at 48h after the treatment on NSC of rESW. Similarly, the expression of let 7b was the lowest at 48h after treatment (Figure 3A, B). The

expression of Nestin mRNA was highest at 48h after the treatment (Figure 3C). Interestingly, we used CCK 8 to detect the proliferation of NSC at 48h after the treatment of different excitation pressures of rESW and found that 1.5bar had the most obvious effect on the proliferation of NSC, there was a statistically significant difference between 1.5bar and 1.6bar. But there was no statistically significant difference between 1.5 bar and 1.4 bar (Figure 3D). Thence, we chose to complete our subsequent experiments at 48h after rESW treatment.

3. rESW mediates NSC proliferation through the lncRNA-let 7b axis

As the self-renewal ability of NSC decreases, let 7b expression increases. In a study with the proliferation of liver cancer cells, Liu Q et al. used the dual luciferase reporter gene experiment to show that there was an interaction between NEAT1 and let 7b(Liu et al., 2019). Therefore, we wanted to know whether the NEAT1-let 7b axis regulates NSC proliferation.

We overexpressed the NEAT1 in NSC by transfecting plasmid into the NSC (Figure 4A) and found that rESW mediates NSCs proliferation through the NEAT1 (Figure 4G). RT-qPCR and Western blot were used to detect the expression of Nestin mRNA and relative protein, and the results obtained were consistent with the results of CCK 8 (Figure 4B, E). Furthermore, by using BrdU to display NSCs proliferation, we found the number of BrdU+/Nestin+ positive cells were increased significantly, and the results were consistent with that of CCK 8 (Figure 5A).

At the same time, we overexpressed let 7b by transfecting let 7b mimics into the NSC (Figure 4C). We found that rESW mediates NSCs proliferation through the let 7b(Figure 4H). The results from RT-qPCR and Western blot were consistent with that of CCK8 (Figure 4D, F), so did that of BrdU (Figure 5B).

Interestingly, when we overexpressed NEAT1, the expression of let 7b was increased. But overexpression of let 7b did not change the expression of NEAT1 (Figure 4I-J).

4. let 7b regulates the proliferation of NSCs by promoting the expression of P21

As a member of the tumor suppressor gene family, P21 can regulate cell senescence, self-renewal, etc. Recently, it has been widely reported in research centered on NSCs proliferation. Hui L et al. have predicted and verified that let 7b and P21 can bind to each other in liver cells by using a dual luciferase reporter gene experiment (Hui et al., 2020). Therefore, we speculate that let 7b and P21 can interact with each other to affect NSCs proliferation.

We observed an increase in the expression of P21 by overexpression of let 7b in NSCs. At the same time, we detected the expression of Cyclin D1 and found that the expression of let 7b was negatively correlated with the expression of Cyclin D1. Moreover, rESW can rescue the increase in P21 expression and the decrease in Cyclin D1 expression caused by let 7b overexpression (Figure 6A-E).

Discussion

The recovery of the CNS is very slow after injury, and it is not easy to regenerate. The patient feels painful and causes great distress to their quality of life. After the cerebrovascular accident, even if the systemic thrombolytic treatment within 4.5 hours at the first time, 2/3 of the patients still have neurological dysfunction and disability (Azad et al., 2016). Therefore, promoting the repair of the central nervous system after injury is the current research focus on neurology and rehabilitation medicine. In recent years, because NSCs are able to promote repair after brain injury, increasing studies have focused on NSCs, providing new treatment ideas for repair after TBI. The increasing evidence indicates the existing of post-injury cell proliferation in SVZ (Jin et al., 2001; Parent et al., 2002) and SGZ (Yagita et al., 2001), where the NSCs originate. However, the time window for NSCs proliferation is narrow, and aging reduces SVZ cell proliferation and migration of neuroblasts after injury, even though neurogenesis is still observed (Moraga et al., 2015). As a non-invasive treatment, rESW has been proven to promote the proliferation of bone stem cells. In the current study with rESW, we found that low energy rESW (1.5bar, 2Hz, 500 points) not only does not cause cell damage, but also promotes the proliferation of NSCs, which brings new ideas to the limited repair of CNS after injury(Figure 2).

At present, the cognition of NEAT1 is mainly focused on its regulation of the progress of cancer cells, and little is known about whether it affects the development of the central nervous system. Interestingly, we found that rESW could promote the proliferation of NSCs inhibited by the expression of NEAT1. Our results were also in line with previous studies. Cui Y et al. found that the overexpression of NEAT1 in spinal cord neural progenitor cells (SC-NPCs) can cause SC-NPC to differentiate like neurons and inhibit apoptosis after spinal cord injury(Cui et al., 2019). On the contrary, the loss of NEAT1 will reduce the differentiation and migration of neurons. let-7 family continuously and dynamically express during neurogenesis with various functions (Yao et al., 2012). Our further exploration found that let 7b is the downstream target of NEAT1 to inhibit the proliferation of NSCs. rESW could regulate NSCs proliferation through inhibiting NEAT1-let 7b axis. Thus, our results also indicated that NEAT1 may have some connections in regulating cell behavior under pathological and physiological conditions.

miRNA is thought to regulate the proliferation of stem cells to ensure proper cell fate transition (Shi et al., 2008). As an important molecule that regulates the proliferation of NSCs, P21 can arrest the cell cycle and can also achieve the effect of inhibiting the proliferation of NSCs(Sun et al., 2010; Rackov et al., 2020). We therefore hypothesized that let 7b might mediate NSCs proliferation through P21. In our research, we proved that let 7b not only inhibits the expression of Cyclin D1, but also promotes the expression of P21, enriching the mechanism of let 7b inhibiting the proliferation of NSCs. In other words, our study identified P21 as a key target of let-7b in NSCs. Therefore, we concluded that let 7b can inhibit the proliferation of NSCs not only because it can inhibit the expression of Cyclin D1 to prolong the cell cycle, but partly because it can promote the expression of P21 to arrest the cell cycle. These studies, including ours, suggest that let-7b plays a role in NSC proliferation across a spectrum of developmental stages through targeting distinct key molecules. We can rescue the reduction of NSCs proliferation because of the overexpression of let 7b by low energy rESW.

However, our research currently has some limitations. How rESW, as an exogenous mechanical signal, was transformed into an endogenous chemical signal should be clearly verified. And the specific mechanism of excitement of cells caused by rESW should be explored. Meanwhile, the NSCs were cultured *in vitro*, which may not represent the *in vivo* status. Moreover, the mechanisms of *in vivo* experiments still need further exploration and validation.

There is no doubt that, however, rESW as a non-invasive and safe therapy can effectively activate NSCs compared to other treatments. It is certain that rESW as an innovative treatment for CNS injuries has far-reaching translational prospects. It continues to stimulate scientists and clinicians to explore specific mechanisms and provide a theoretical basis for future clinical applications. In addition, our results further verify the critical role of radial shockwave energy on biological effects, and the results provide a reference for clinical therapy.

Conclusion

In the present study, we demonstrated that radial shockwave is capable of enhancing the self-renewal capacity of NSC *in vitro*, and we screened out the optimized parameters in our experimental system. Moreover, we identified that the activation of NEAT1-let 7b-P21 axis contributed to this promising effect mediated by radial shockwave. (Figure 7).

Abbreviations

NSCs Neural stem cells

rESW radial extracorporeal shock wave

TBI traumatic brain injury

LncRNA Long non-coding RNA

Neat1 nuclear-enriched abundant transcript 1

q-PCR Quantitative real-time PCR

CCK 8 Cell Counting Kit-8

ANOVA Analysis of variance

Declarations

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

o Ethics approval and consent to participate:

Not Applicable

o Consent for publication:

All of the authors have given their consent for publication.

o Availability of data and materials:

Data sharing not applicable to this article as no datasets were generated or analysed during the current study

o Competing interests:

The authors declare that they have no competing interests.

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o Authors' contributions

KH and NK have substantial contributions to the conception and design; acquisition, analysis, and interpretation of the data; and manuscript writing. NK, JL and YWM contributed to the analysis and interpretation of the data and manuscript writing. KH and JL has substantial contributions to the cell culture. YWM, XTY and JL have substantial contributions to the conception and design, financial support, and analysis and interpretation of the data. All authors read and approved the final manuscript.

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o Footnotes

Kun Han and Nan Kang contributed equally to this work.

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Figures

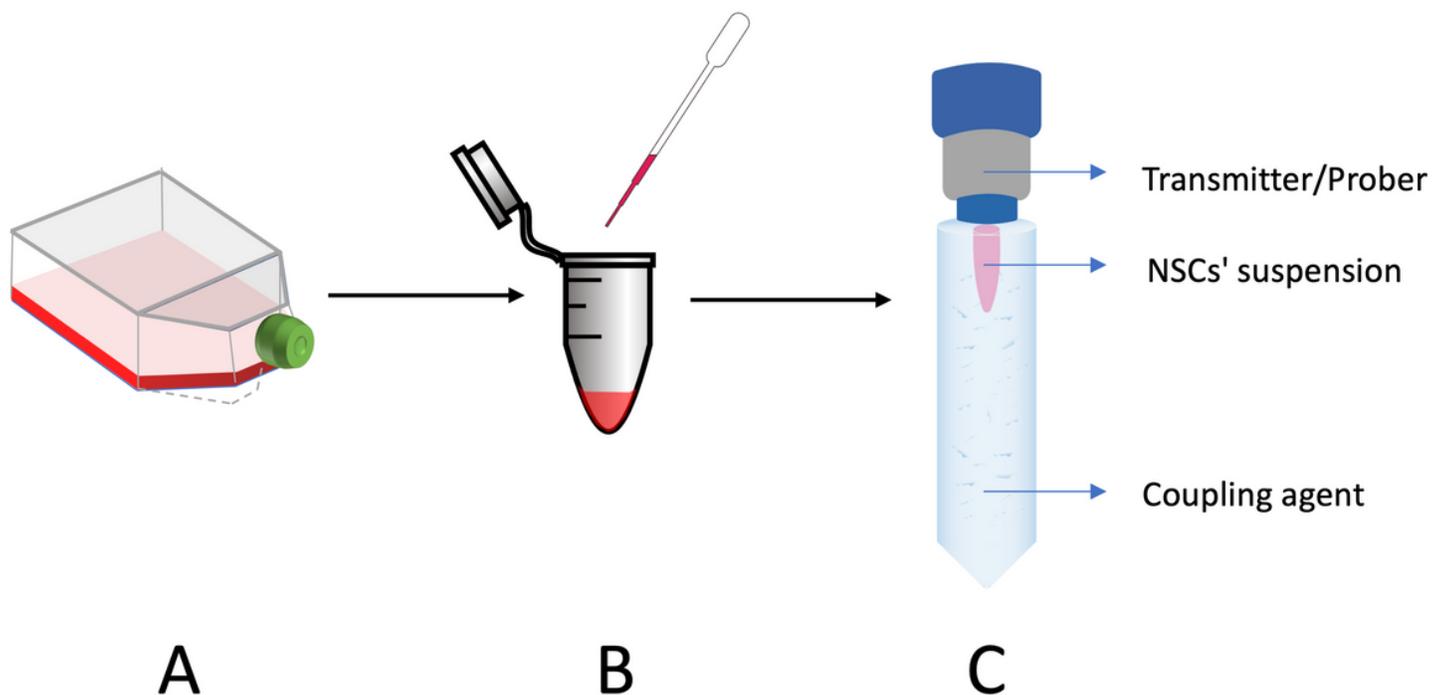


Figure 1

Representative images showing the protocols. (A) NE-4C cells are dissociated with 0.05% trypsin-EDTA and resuspended in the tube supplemented DMEM. (B) The cell suspension filled with the complete medium was transferred to a 2mL EP tube. (C) After the air is removed in the tube, the EP tube is embedded into a 50mL centrifuge tube filled with coupling agent. The transmitter of rESWT acts vertically on the upper part.

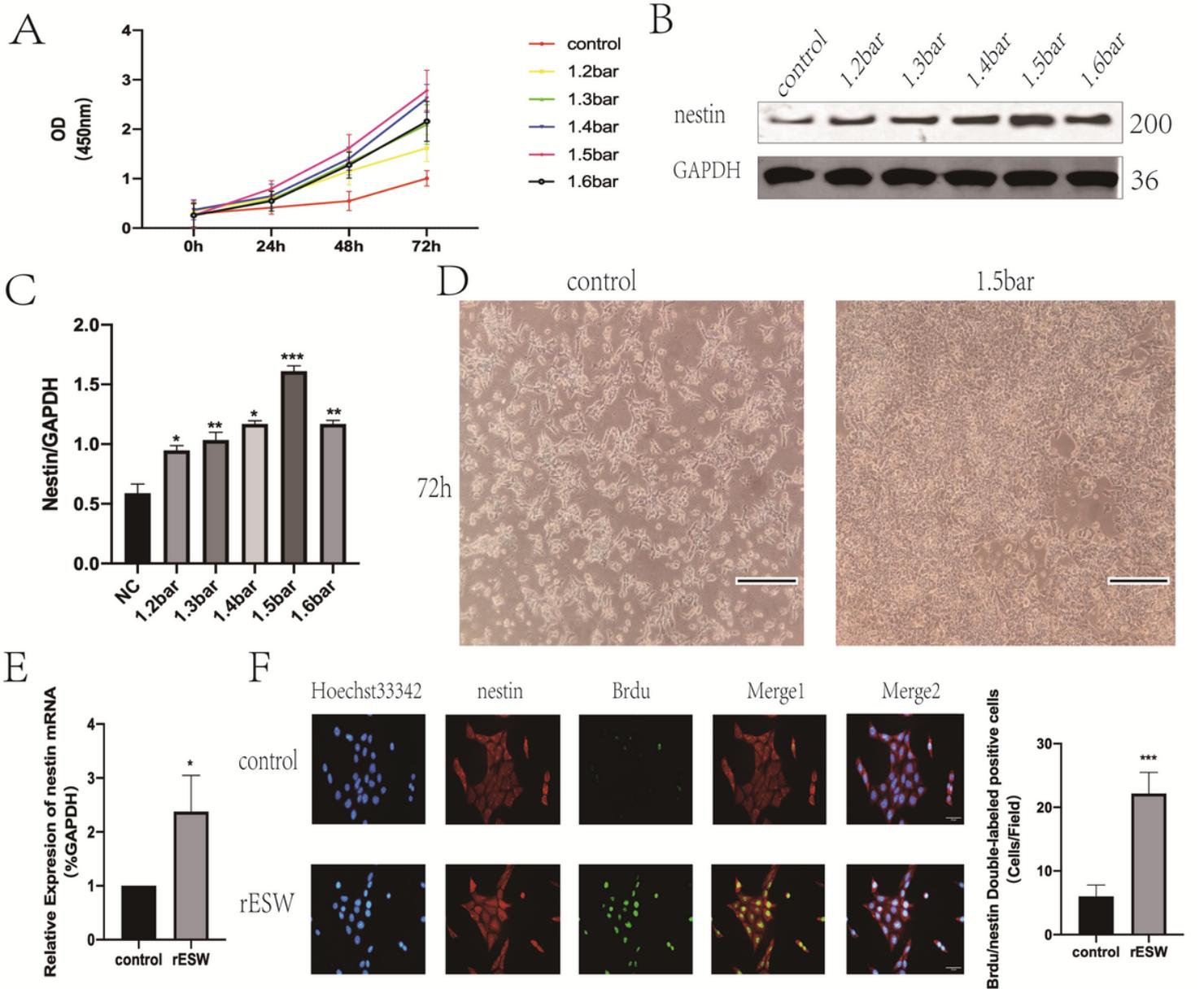


Figure 2

(A) CCK8 results showed the optimal excitation pressure of rESW to promote the proliferation of NSCs ($p < 0.05$ by two-way ANOVA). (B)–(C) The chart GAPDH of Western Blot demonstrates Nestin expression 72h after rESW treatment. The data shown in (C) were analyzed by one-way ANOVA* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (D) The microscopic pictures show the number of NSCs at 72h in the 1.5bar treatment group is significantly more than that in the control group (Bar=50 μ m) (E) The chart graph of RT-qPCR results demonstrates the Nestin mRNA expression 72h after 1.5bar rESW treatment. (F) The immunostaining of NSCs demonstrates the proliferation changes 72h after rESW treatment, with cell proliferation marker BrdU and NSCs marker Nestin. Bar=20 μ m. * $p < 0.05$, *** $p < 0.001$ by Student's t-test.

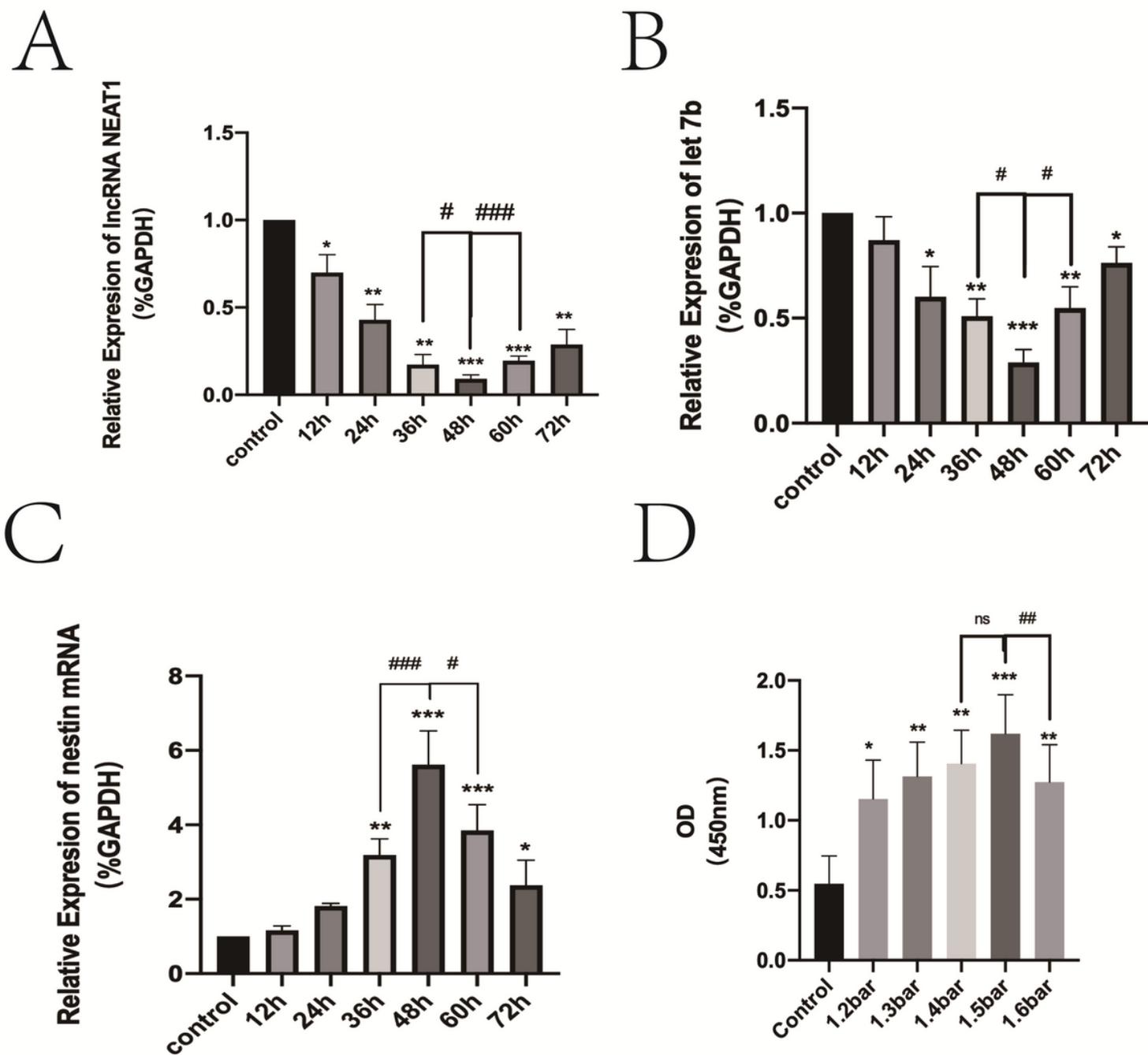


Figure 3

Relative expression of NEAT1(A), let 7b (B) and nestin (C) within 72 hours after rESW treatment in NSCs. (D) The effect of different excitation pressures on the proliferation of NSCs at 48h after rESW treatment. The data are shown as the mean \pm SD * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

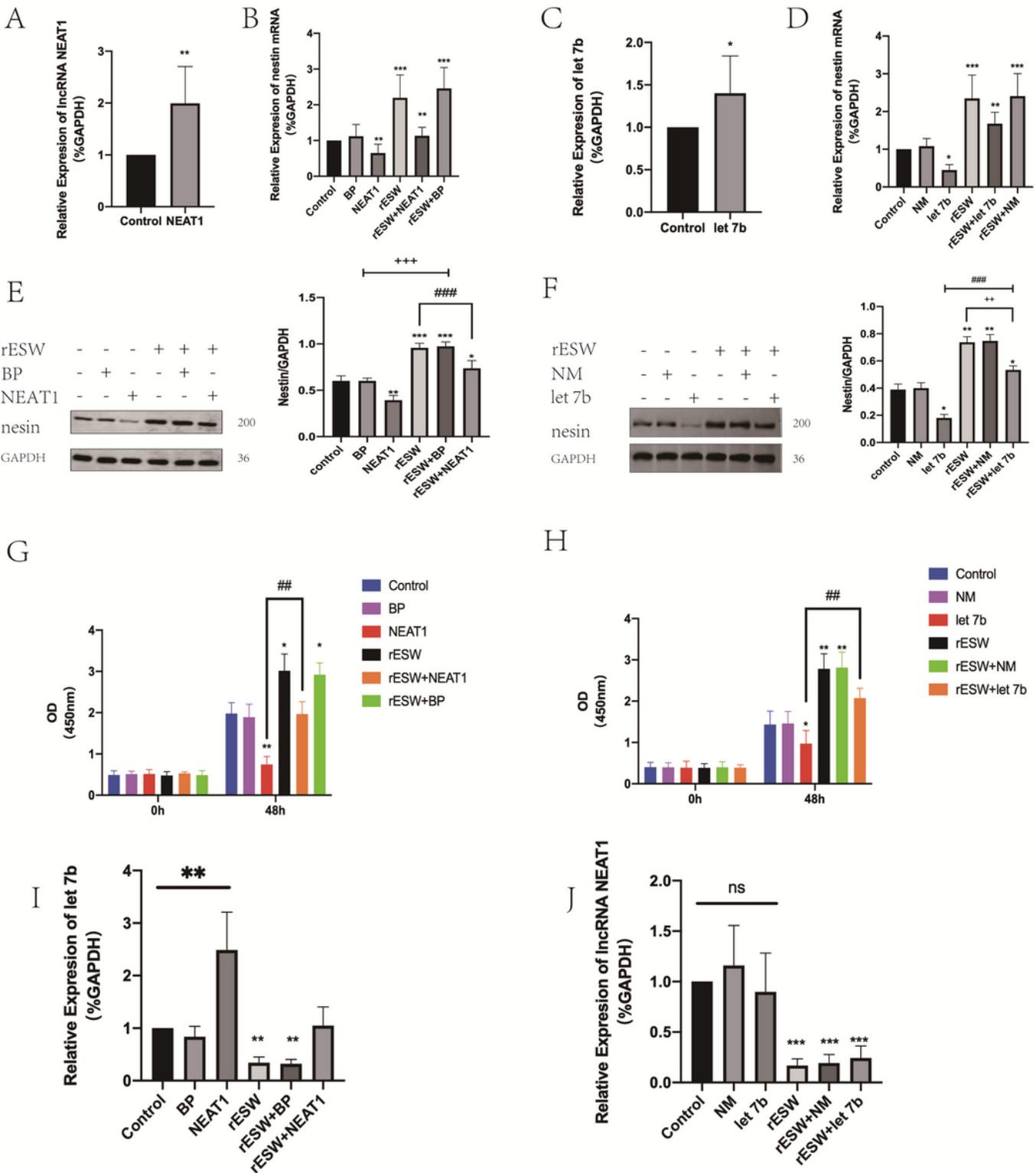


Figure 4

(A) The RT-qPCR results demonstrate the transfection efficiency of NEAT1 overexpression in NSCs. (C) The RT-qPCR results demonstrate the transfection efficiency of let 7b overexpression. Results of RT-qPCR and Western Blot show that NEAT1 overexpression inhibits the expression of Nestin gene and protein. CCK 8 results show the effect of NEAT1 overexpression on NSCs proliferation. Treatment with rESW rescues the reduction. Similar effects are observed in NSCs overexpressing let 7b. (I-J) The

results demonstrate the effect of NEAT1 and let 7b overexpression on each other. The data are shown as the mean \pm SD * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

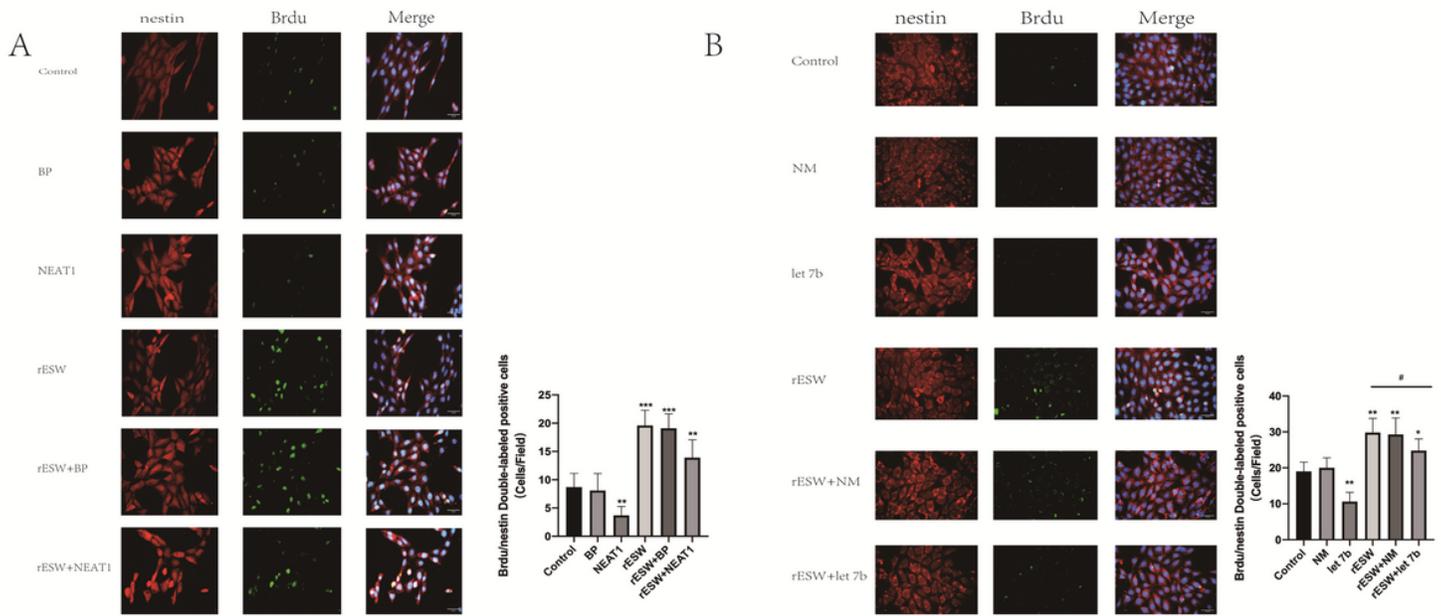


Figure 5

(A-B) The immunostaining results demonstrate the inhibitory effect of NEAT1 or let 7b overexpression on proliferation of NSCs, which is reversed by rESW treatment. Bar=20 μ m. The data are shown as the mean \pm SD * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

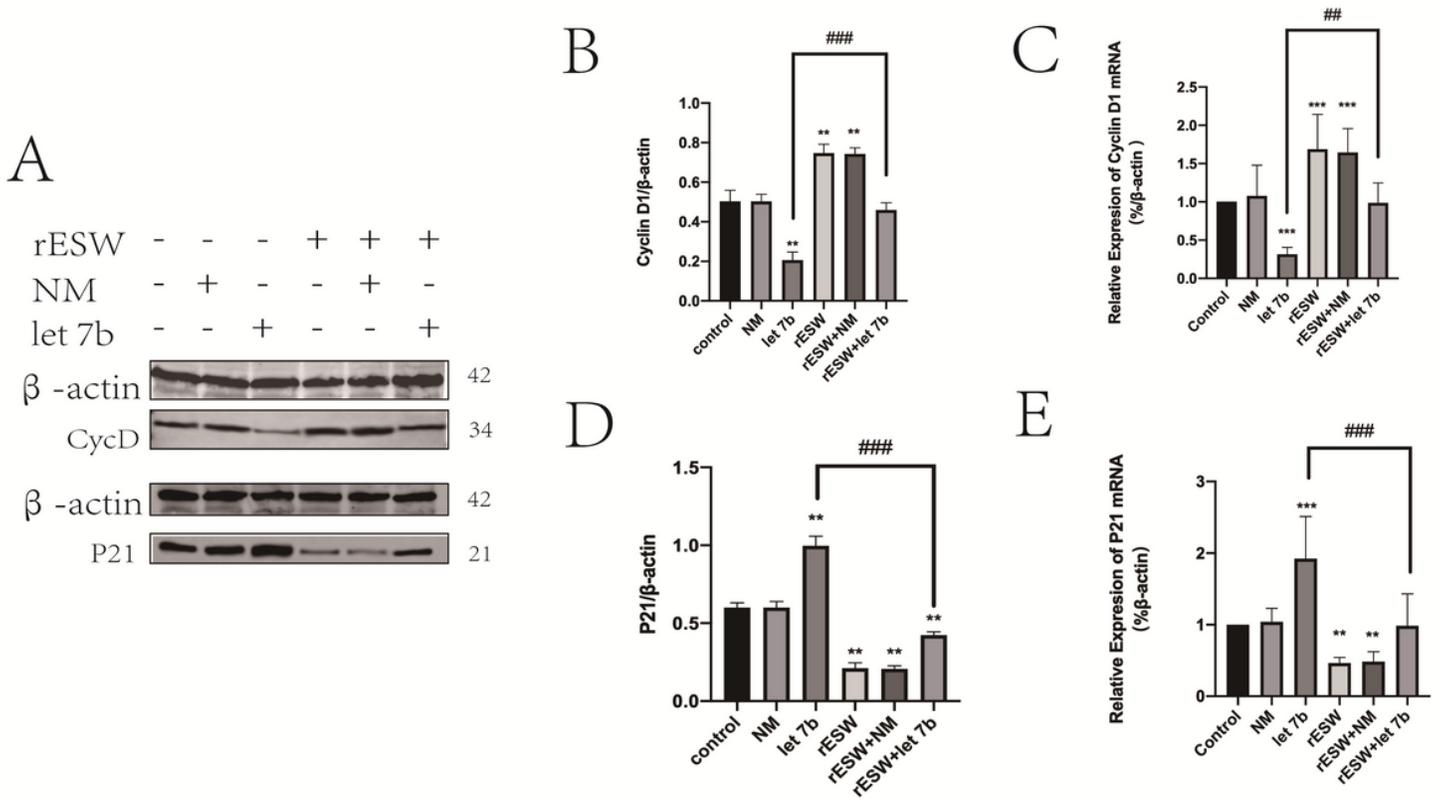


Figure 6

let 7b inhibits the expression of Cyclin D1 (A, B Western Blot (C, RT-qPCR), and promotes the expression of P21 (A, D Western Blot (E, RT-qPCR). Treatment with rESW rescues the reduction. The data are shown as the mean \pm SD * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

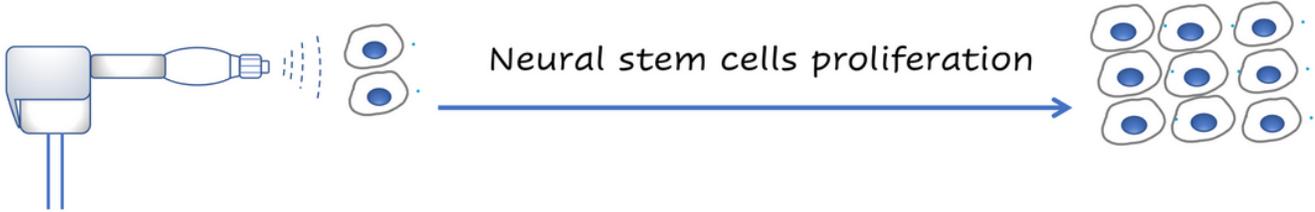
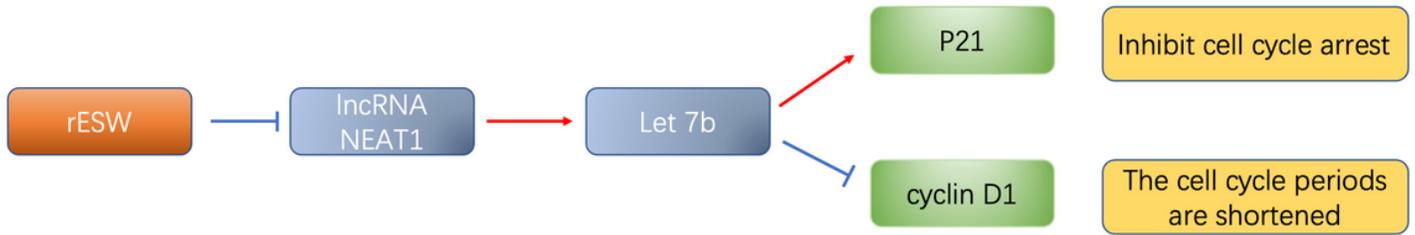


Figure 7

let 7b mediates the regulation of the expression of Cyclin D1 and P21 caused by IncRNA NEAT1 and then regulating the proliferation of NSCs. rESW can regulate NSCs proliferation through NEAT1-let 7b-P21 axis.