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Ultrasound microbubbles combined with liposome-mediated pNogo-R shRNA delivery into neural stem cells★

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Abstract

In the present study, ultrasound-mediated microbubble destruction (UMMD) alone and combined with liposome technology was used as a novel nonviral technique to transfect a Nogo receptor (Nogo-R) shRNA plasmid (pNogo-R shRNA) into neural stem cells (NSCs). Using green fluorescent protein as a reporter gene, transfection efficiency of NSCs was significantly higher in the group transfected with UMMD combined with liposomes compared with that of the group transfected with UMMD or liposomes alone, and did not affect cell vitality. In addition, Nogo-R mRNA and protein expression was dramatically decreased in the UMMD combined with liposome-mediated group compared with that of other groups after 24 hours of transfection. The UMMD technique combined with liposomes is a noninvasive gene transfer method, which showed minimal effects on cell viability and effectively increased transfer of Nogo-R shRNA into NSCs.

Key Words: ultrasound microbubble; liposome; neural stem cell; gene transfection; Nogo receptor; neural regeneration

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INTRODUCTION

Neural stem cells (NSCs) can serve as a carrier for gene therapy of the central nervous system[1-3]. However, limited transfection efficiency has hindered the development of this promising therapeutic approach. Current gene transfection methods for stem cells are still restricted to virus-mediated, electroporation, microporation and liposome (Lip)-mediated[4-7], which are limited in application due to immunological reaction, high cell mortality and low efficiency. More recently, ultrasound microbubbles (UMs) have been reported to deliver genes into cells and tissues of interest and increase liposome-mediated exogenous gene delivery into eukaryotic cells *in vitro*[8]. The combination of these techniques overcame limitations and was developed into a new methodology for safe and efficient genetic modification. Ultrasound-mediated microbubble destruction (UMMD) combined with another vector to achieve high-efficiency may improve the field of genetically-modified NSCs[9].

UMMD forms transient nonlethal perforations in the cell membrane mainly by acoustic cavitation[10-13] and has been used in clinical applications[14-17]. Lips are also used as a nonviral vector for gene therapy[18], which has led to combination with UMs for a novel transfection model.

There are three known myelin-associated inhibitors including Nogo, myelin-associated glycoprotein and oligodendrocyte myelin glycoprotein. Nogo inhibits axonal regeneration after spinal cord injury. Nogo receptor (Nogo-R), a common receptor of the three inhibitors, is believed to be a point of convergence in signal transduction for myelin-associated inhibitory properties[19-22]. Consequently, Nogo-R gene expression has been inhibited using UM + Lip-mediated transfection to reduce Nogo-R protein expression. Previous studies have involved *in vitro* experiments, but very little data are available on UM + Lip-mediated transfection of NSCs. The present study delivered plasmid DNA into NSCs by combining UMMD with Lips to investigate the feasibility of decreasing Nogo-R protein expression.

RESULTS

Identification of NSCs

NSCs from the cerebrum of neonatal rats were cultured for 7 days and formed neurospheres (supplementary Figure 1 online), each of which consisted of several to hundreds of cells (Figure 1A). Passage three NSCs were characterized by

immunophenotyping nestin, a marker of undifferentiated NSCs (Figure 1B). In addition, numerous cells migrated out from neurospheres and pseudopodium-like apophysis stretched from neurosphere edges after plating onto poly-Lysine-coated coverslips in Dulbecco's modified Eagle's medium/Ham's nutrient mixture F-12 (DMEM/F12) medium supplemented with 10% fetal bovine serum for differentiation (supplementary Figure 2 online). Differentiated cells were positive for glial fibrillary acidic protein (GFAP) and neurofilament 200 (NF200; Figures 1C, D), indicating differentiation of neurons and astrocytes, respectively. These results suggested that the cultured NSCs maintained self-renewal and differentiation potential.

Transfection efficiency of NSCs under various conditions

Based on preliminary results from fluorescence microscopy after 48 hours of transfection, green fluorescent protein (GFP) expression, which indicated transfection efficiency, was observed in NSCs. Differences in the percentage of GFP-positive cells were then assessed. Transfection efficiency was 0% in the blank group and 9.79% in the negative control plasmid + UM group. Transfection efficiency was also low in pNogo-R shRNA + UM and pNogo-R shRNA + Lip groups at 9.92% and 10.89%, respectively. Transfection efficiency of the pNogo-R shRNA + Lip + UM group was significantly higher compared with that of other groups ($P < 0.05$; Figure 2).

Effect of pNogo-R shRNA + Lip + UM transfection on NSC viability

To evaluate viability, cells were stained with trypan blue and quantified by microscopy. The percentage of live cells was 95.25 ± 0.87% in the blank group, 84.43 ± 0.67% in the negative control plasmid + UM group, 85.06 ± 0.49% in the pNogo-R shRNA + UM group, 91.35 ± 0.17% in the pNogo-R shRNA + Lip group and 80.25 ± 0.35% in the pNogo-R shRNA + Lip + UM group. There were no significant differences in cell viability (> 80%) among groups.

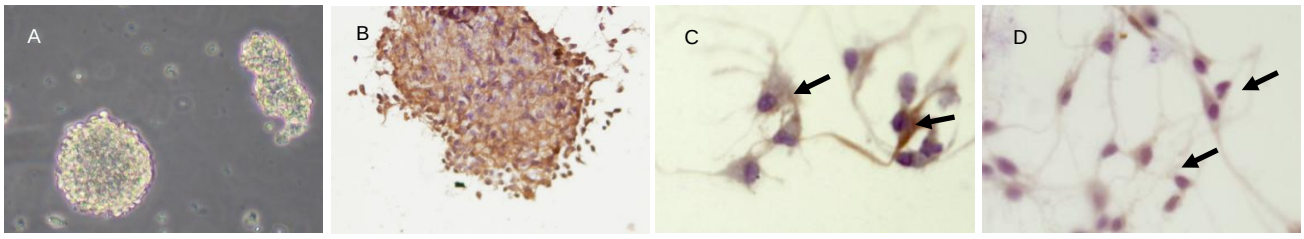

Figure 1 Isolation and culture of neural stem cells.

(A) Neural spheres cultured for 1 week (inverted microscopy, × 200).

(B) Nestin-positive (brown) neurospheres (immunohistochemical staining, × 200).

(C) Glial fibrillary acidic protein-positive (brown, arrows) astrocytes (immunohistochemical staining, × 400).

(D) Neurofilament 200-positive (brown, arrows) neurons (immunohistochemical staining, × 400).

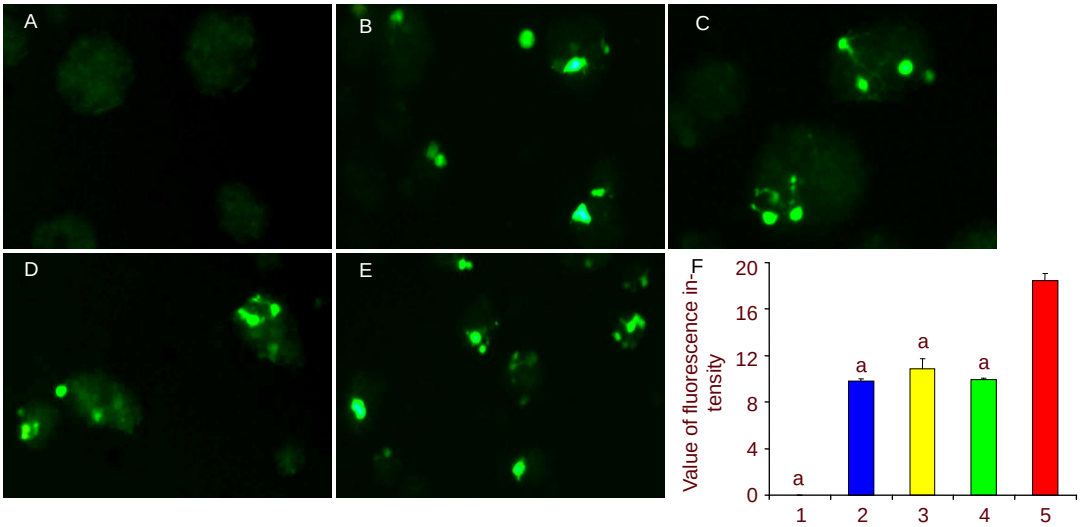

Figure 2 Transfection efficiency of neural stem cells. UM: Ultrasound microbubble; Lip: liposome; Nogo-R: Nogo receptor.

(A) Blank, (B) negative control + UM, (C) pNogo-R shRNA + UM, (D) pNogo-R shRNA + Lip, (E) pNogo-R shRNA + Lip + UM. Green fluorescent protein (pGPU6/GFP/Neo-Nogo-R-shRNA) expression in neural stem cells was determined by fluorescence microscopy (× 200).

(F) Relative fluorescence intensity of groups (1: Blank; 2: negative control + UM; 3: pNogo-R shRNA + UM; 4: pNogo-R shRNA + Lip; and 5: pNogo-R shRNA + Lip + UM). $^aP < 0.05$, *vs*. pNogo-R shRNA + Lip + UM group. Data were expressed as mean ± SEM.

Effect of pNogo-R shRNA + Lip + UM transfection on Nogo-R protein expression in NSCs

As shown in Figure 3, no significant difference was found in the gray values of Nogo-R between blank and negative control plasmid + UM groups ($P = 0.357$), while band density was decreased in pNogo-R shRNA + UM, pNogo-R shRNA + Lip and pNogo-R shRNA + Lip + UM groups compared with that of blank and negative control groups ($P < 0.05$). Moreover, the pNogo-R shRNA + Lip + UM group (Figure 3E) showed a decrease of Nogo-R expression compared with that of pNogo-R shRNA+UM (Figure 3C) and pNogo-R shRNA+Lip groups (Figure 3D; $P < 0.01$). These findings suggested that the synergistic effect of Lips combined with UMs enhanced inhibition of Nogo-R protein expression in NSCs after transfection.

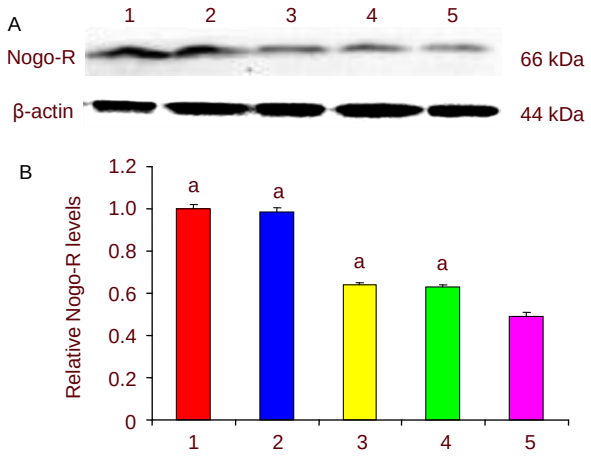

Figure 3 Nogo-receptor (Nogo-R) protein expression in neural stem cells after transfection (western blot). UM: Ultrasound microbubble; Lip: liposome.

(A) Nogo-R protein band measured by western blot. 1: Blank; 2: negative control + UM; 3: pNogo-R shRNA + UM; 4: pNogo-R shRNA + Lip; 5: pNogo-R shRNA + Lip + UM.

(B) Quantification of Nogo-R protein expression. The expression level in the blank group was set as 1. [a]$P < 0.05$, *vs*. pNogo-R shRNA + Lip + UM group. The data were expressed as mean ± SEM of gray value ratio of target protein/β-actin in experimental group/blank group

Efffect of pNogo-R shRNA + Lip + UM transfection on Nogo-R mRNA expression in NSCs

As shown in Figure 4, RT-PCR analysis supported the western blot data.

The data were expressed as mean ± SD of gray value ratio of target protein/β-action in experimental group/ blank group.

Nogo-R mRNA expression decreased in pNogo-R shRNA + UM, pNogo-R shRNA + Lip and pNogo-R shRNA + Lip + UM groups compared with that of blank and negative control groups. In particular, Nogo-R mRNA expression in the pNogo-R shRNA + Lip + UM group showed lower levels compared with those of other groups ($P < 0.05$; data not shown). However, there was no significant difference between pNogo-R shRNA + UM and pNogo-R shRNA + Lip groups.

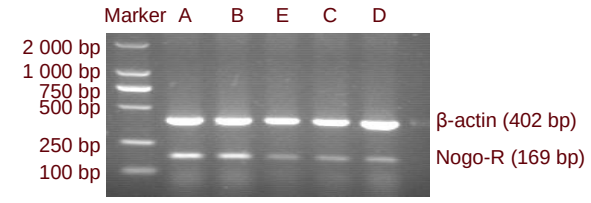

Figure 4 Nogo-receptor (Nogo-R) mRNA expression in neural stem cells after transfection (reversetranscription-PCR). UM: Ultrasound microbubble; Lip: liposome.

A–E: Blank, negative control, pNogo-R shRNA + UM, pNogo-R shRNA + Lip, and pNogo-R shRNA + Lip + UM, respectively.

DISCUSSION

UMs have been recently proposed as a potential nonviral vector for gene delivery[23], which is based on acoustic cavitation that forms transient reversible pores, thereby allowing extracellular macromolecules to instantaneously enter cells. UMMD possesses several properties such as transient reversible delivery, site-specific delivery and nonviral gene delivery[24-26], providing a highly promising approach for gene delivery *in vitro*. After a Lip-plasmid mixture attaches to gas-filled microbubbles that interact with receptors on the cell membrane, the energy created by ultrasound is believed to form transient access to the cell membrane for delivery of macromolecules containing drugs and/or genes into cells and tissues. Additionally, energy release from microbubble bursting may contribute to cell transfection[27]. Thus, UMs may be a helpful tool to enhance transfection efficiency of Lip-plasmid complexes by altering cell membrane permeability for a short time compared with that of the use of one method alone. Moreover, the simple transfection process is not cytotoxic.

Among current nonviral methods for delivery of exogenous genes into cells and tissues, gas-filled microbubbles have been applied to various organs such as the kidney, retina[28] and saphenous vein[29]. We expanded on previous studies, which delivered exogenous genes by only one method, and further investigated whether UMMD combined with Lips can enhance transfection efficiency to improve NSCs for use in therapeutic strategies. The Nogo-R gene was selected as a target gene to investigate the synergistic effect of UMMD and Lips on increasing transfection efficiency. Optimized ultrasound parameters (frequency, 300 kHz; power, 1.0 W/cm^2; total time, 30 seconds) were based on a previous study[30]. Fluorescence microscopy showed a significantly higher number of GFP-positive cells in the UM + Lip-mediated group compared with that of other groups. However, similar apoptotic rates were observed in the pNogo-R shRNA + UM group compared with that

of the pNogo-R shRNA+Lip group. Trypan blue staining showed no significant difference in cell viability among groups. Furthermore, Nogo-R expression at mRNA and protein levels showed no significant change in both pNogo-R shRNA + UM and pNogo-R shRNA + Lip groups after 48 hours of transfection. However, Nogo-R expression levels in the pNogo-R shRNA + Lip + UM group was significantly decreased, suggesting that either UM or Lip inhibited Nogo-R gene expression. Moreover, the effect of combining UMs and Lips was more significant.

In the present study, the combination of UMs and Lips was used for gene transfection of NSCs. Despite the potential of combinatorial strategies for transfection of NSCs, safety issues remain and must be solved to harness the full potential of stem cells. Further studies are needed to reduce adverse reactions to gene therapy and improve transfection efficiency. In conclusion, the combination of UMs and Lips is a valuable transfection method for efficient gene delivery into NSCs.

MATERIALS AND METHODS

Design

An *in vitro*, cytobiological observation.

Time and setting

Experiments were performed at the Molecular Medicine and Cancer Research Laboratory of Chongqing Medical University, China between June 2009 and July 2010.

Materials

A total of 60 neonatal Sprague-Dawley rats born within 24 hours, of clean grade, were provided by the Laboratory Animal Center of Chongqing Medical University (No. SCXK (Yu) 2007-0001) and housed in a light, humidity (50%) and temperature-controlled (21–27°C) room. Animal experiments were performed in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, published by the Ministry of Science and Technology of China[31].

Methods

NSC isolation and culture

The cerebrum was sterilely obtained from neonatal rats (supplementary Figure 3 online), and tissue samples were triturated in cold sterile D-Hank's (HyClone, Logan, USA), then incubated in 1.25 g/L trypsin and 0.2 g/L EDTA for 30 minutes at 37°C. Samples were homogenized to a single cell suspension by a fine-polished pipette. Cells were centrifuged and washed three times with D-Hank's and resuspended in 5 mL DMEM/F12 serum-free culture medium (HyClone) containing 0.02% B27 (Gibco, Los Angeles, USA), 20 μg/L epidermal growth factor (Peprotech, Rocky Hill, USA) and 20 μg/L basic fibroblast growth factor (Peprotech), transferred to 50 cm^2 culturing flasks at 5 × 10^5 cells/L and incubated in 5% CO_2 at 37°C. Culture medium was exchanged every 3 days, and cells were passaged every 7 days. Passage three NSCs were identified by immunophenotyping of nestin, and their differentiation ability was identified by NF200 and GFAP expression[32-34].

NSC transfection with Lips and UMs

Passage three NSCs were randomly assigned into five groups according to transfection conditions: blank, negative control + UM (negative plasmid (GenePharma, Shanghai, China) + ultrasonic irradiation), pNogo-R shRNA+UM (Nogo-R shRNA plasmid (GenePharma) + ultrasonic irradiation), pNogo-R shRNA + Lip (lip/shRNA plasmid, Nogo-R shRNA plasmid: lipofectamine 2000 (Invitrogen, CA, USA) ratios of 1: 3), and pNogo-R shRNA + Lip + UM (Nogo-R shRNA plasmid + lip + ultrasonic irradiation) groups.

Preparation of DNA-loaded microbubbles

The lower liquid phase containing microbubbles was provided by the Institute of Ultrasound Imaging of Chongqing Medical University. The microbubble density was 3 × 10^8/mL. pNogo-R shRNA (50 μg) was gently added to 100 μL microbubble suspension and incubated at 4°C for 30 minutes.

Gene transfection

Transfection was performed using a CGZZ ultrasound gene transfection instrument, designed by the Institute of Ultrasonic Engineering in Medicine, Chongqing Medical University, and GFP was used as a transfection marker. pNogo-R shRNA target sequences were determined by a pre-test[35]: 5'-CCG AAT CTC TTA CGT GCC A-3'. Cells (5 × 10^4) were plated in a 24 well culture plate. A coupling agent (Yousheng, Shanghai, China) was placed on the ultrasonic transducer. The ultrasound launch parameters were 0.3 MHz, mechanical index was 1.0 W/cm^2, and irradiation time was 30 seconds. After ultrasonic irradiation, 24 well plates were placed in a 5% CO_2 cell incubator. After 4 hours incubation at 37°C, the compound solution was replaced with 500 μL serum-free medium. GFP reporter gene expression was analyzed after 48 hours. All transfection experiments were performed in triplicate.

Trypan blue staining of transfected cells

After 48 hours of gene transfection, cells were harvested, washed once in PBS, and centrifuged at 1 000 r/min for 5 minutes. The cell suspension was then stained with 0.4% trypan blue and examined under a microscope (Olympus, Tokyo, Japan). Cell survival rate (%) = blue stained cells / total cells × 100%.

Western blot analysis of Nogo-R protein expression in NSCs

Nogo-R protein expression was measured by western blotting at 2 days after transfection. Collected cells (2 × 10^5/mL) were homogenized in cell lysis buffer on ice for 30 minutes, and centrifuged at 12 000 r/min for 5 minutes at 4°C. The suspension was boiled in loading buffer for 5 minutes at 95°C. Total protein was separated in a 10% sodium dodecyl sulfate-polyacrylamide gel (Bio-Rad, Hercules, CA, USA) and transferred to a polyvinylidene difluoride membrane. The membranes were incubated with blocking solution (5% milk powder in Tris-buffered saline with Tween-20) for

1 hour. Immobilized proteins were incubated with a rabbit anti-Nogo-R polyclonal antibody (1: 300; Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C, followed by a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1: 1 000; Zhongshan Goldenbridge Biotech Co., Beijing, China) for 2 hours. Membranes were developed using an enhanced chemiluminescent reagent and the gray value was scanned using an Odyssey Infrared Imaging System (Bio-Rad). Experiments were performed in triplicate. The ratio of gray values for Nogo-R and β-actin were analyzed in triplicate.

RT-PCR analysis of Nogo-R mRNA expression in NSCs

Total RNA was extracted with Trizol reagent according to the manufacturer's instructions (Takara, Tokyo, Japan). RNA content and purity were detected by ultraviolet spectrophotometry (GE Healthcare, USA), and analyzed on a 1.5% agarose gel[36]. RNA samples were reverse transcribed using an RT reagent kit (Takara), and used for PCR under standard conditions with Taq DNA polymerase. Primer sequences are as follows:

Gene	Sequence (5' –3')	Length of PCR product (bp)
Nogo-R	Forward: TGC AGG CAC TTC CCG ACA ACA C Reverse: AAG GCA TGT GGG TGC ACA CGA G	169
β-actin	Forward:TCG GCA ATG AGC GGT TCC GAT G Reverse: ACG CAG CTC AGT AAC AGT CCG C	402

PCR conditions consisted of denaturation for 2 minutes at 94°C, followed by 30 cycles of amplification (15 seconds at 94°C, 30 seconds at 56°C and 1 minute at 72°C), and then 5 minutes at 72°C. The β-actin housekeeping gene was used for normalization. PCR products were analyzed on a 1.5% agarose gel, and visualized in an ImageMaster VDS (Bio-Rad) image analyzer.

Statistical analysis

Data were expressed as mean ± SEM and were analyzed using SPSS 13.0 statistical software (SPSS, Chicago, IL, USA). Differences between groups were analyzed using one-way analysis of variance, and $P < 0.05$ was considered statistically significant.

Author contributions: Youde Cao was responsible for funds and supervised the study. Weixia Ye generated experimental data and prepared the manuscript. Xueping Huang provided technical support. Yangyang Sun, Hao Liu and Jin Jiang performed statistical analysis and provided data.

Conflict of interest: None declared.

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Ethical approval: This study received permission from the Animal Ethics Committee of Chongqing Medical University, China.

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Supplementary information: Supplementary data associated with this article can be found, in the online version, by visiting www.nrronline.org, and entering Vol. 7, No. 1, 2012 after selecting the "NRR Current Issue" button on the page.

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