

Establishment of Lipofection Protocol for Efficient miR-21 Transfection into Cortical Neurons *In Vitro*

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Dysregulated microRNAs in neurons could cause many nervous system diseases. The therapeutic manipulation of these pathogenic microRNAs necessitates novel, efficient delivery systems to facilitate microRNA modulators targeting neurons with minimal off-target effects. The study aimed to establish a lipofection protocol to upregulate expression levels of miR-21 in neurons under different conditions, including different serum-free medium, transfection conditions, and reagent concentration, by evaluating the expression levels of miR-21 and neuron injury. The expression levels of miR-21 were higher in neurons transfected by Neurobasal-A than by DMEM. Expression levels of miR-21 were already the highest at the ratio RNAiMAX:miR-21 = 3:5, but the increase of RNAiMAX's concentration had not caused the further upregulation of expression level of miR-21. Neuron injury was condition dependent and dose dependent after transfection. Compared to S-Neurobasal groups, neurons have a smaller injury in N-Neurobasal groups, and compared to ratios RNAiMAX:miR-21 = 4:5, 5:5, neuron injury was smaller at ratios of RNAiMAX:miR-21 = 1:5, 2:5, 3:5. Without the pretreatment of starvation *in vitro*, the lipofection protocol was that RNAiMAX/miR-21 agomir complexes were diluted in Neurobasal-A at the ratio of RNAiMAX:miR-21 = 3:5.

Introduction

DYSREGULATED MICRORNAs IN NEURONS could cause many central nervous system (CNS) diseases. The therapeutic manipulation of these pathogenic microRNAs necessitates novel, efficient delivery systems to facilitate microRNA modulators targeting neurons with minimal off-target effects. Neuronal injury occurs in different CNS diseases, including trauma (Wee *et al.*, 2015), tumor (Bingham *et al.*, 2011), and ischemic (Ji *et al.*, 2015) and neurodegenerative disorders (Li *et al.*, 2015), and leads to many functional deficits with serious impairments in patients (Reina *et al.*, 2013). The neurons display typical molecular and functional characteristics of mature neuronal cells *in vitro* and offer the opportunity to study various aspects of CNS diseases. The regulation of special gene is a crucial tool to identify the component necessary for a particular cellular process in many studies.

microRNAs play an important role in gene expression and bind to a special sequence of mRNA molecule to regulate the expression of mRNA (Choi *et al.*, 2014). In CNS diseases, miRNAs have been reported to regulate synaptic plasticity, neuroprotection, and neurodegeneration (Saugstad, 2010). microRNA-21 (miR-21) is one of the most studied miRNAs and has been found to play an important

role in most biological functions and diseases of CNS (Krichevsky and Gabriely, 2009). Growing evidence also suggests that miR-21 is detected in plasma sample in some diseases like glioma (Wang *et al.*, 2012). Meanwhile, miR-21 may be a novel therapeutic target in human cancer (Pan *et al.*, 2010) and miR-21 was also a therapeutic target for reversing drug resistance in cancer (Hong *et al.*, 2013). A thorough understanding of the physiological function of miR-21 is of great importance as it can promote the development of miR-21-based biomarkers and therapeutics. However, finding an efficient transfection method that delivers miRNAs into neurons is relatively difficult. Furthermore, when transfecting neurons with high efficiency, it is important that cell damage is avoided and the original function of neurons is maintained. Another crucial limitation of transfection neurons is that neurons are very sensitive to different transfection conditions, for example, transfection solvent, transfection reagent, or pretreatment of transfection, which all influence the transfection efficiency, neuron injury, or neuronal function.

Various methods, including chemical, physical, and biological methods, have been attempted to transfect neurons (Covello *et al.*, 2014). Physical methods, which include electrotransfection, biolistics, and injection, have been successfully used in segmentation cells but may be toxic for

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cells and usually have a high reagent and equipment cost and cell suspensions *in vitro* (Villemejeane and Mir, 2009), which are not appropriate for neurons. Biological methods, which include virus and plasmid transfection, have been applied to many hard-transfected cells (Du *et al.*, 2014), but the complexity and biosafety issues limited their widespread applications, which are not good methods for transfecting neurons. Chemical methods have widely been used to transfect, as they are relatively simple, cheap, and safe. There are many chemical methods, including calcium phosphate, liposomes, cationic lipids, cationic polymers, and cationic polysaccharides (Azzam and Domb, 2004), which are used to transfect a great number of cell lines and primary cells. In the traditional protocol, several steps are needed before transfection. Specially, the starvation pretreatment requires the cells to be cultured in only DMEM without any other substances, including fetal calf serum and antibiotics. However, the traditional protocol is also potentially harmful to cells, and transfection conditions need to be carefully optimized to improve efficiency and miRNA expression level in cells.

Cationic lipofection, a reagent of chemical methods, is the simplest and most readily available way for RNA oligo delivery. Lipofectamine RNAiMAX (RNAiMAX), a new cationic lipofection reagent, has shown higher efficiency in delivering miRNA into many stem cells and cell lines (Zhao *et al.*, 2008; Malamas *et al.*, 2013), although its transfection efficiency is still unclear in most primary cells, especially neurons. Here, we sought to establish a lipofection protocol to upregulate the expression level of miR-21 in neurons under different transfection conditions, including different transfection solvents (DMEM or Neurobasal), pretreatments of transfection (with starvation or not), and different reagent ratios, while maintaining viability and functional abilities of neurons. The lipofection protocol will become the basic research transfection method of miRNAs in neurons *in vitro*, which will provide insight into developing a novel therapeutic approach of miR-21 or other miRNA gene therapy for CNS diseases.

Materials and Methods

Neuron culture

Primary cortex fragments from the postnatal Wistar rats (<24 h), which were purchased from Experimental Animal Laboratories of the Academy of Military Medical Science and complied with NIH guidelines, were dissected and dissociated into single cells. The cell was suspended in DMEM with 10% fetal bovine serum and plated on six-well plates coated with poly-L-lysine (Sigma-Aldrich) at a density of 5×10^5 per well. After 2 h, neurons were cultured in the Neurobasal-A medium with 2% B27 and 1% glutamine (2 mL) (Gibco) for 7 days at 37°C in 95% air and 5% CO₂ atmosphere.

The transfection efficiency of miR-21 and lipofection protocol

Labeling of the miR-21 agomir with a 5'-FAM oligonucleotide (FAM-miR-21; GenePharma) was done to confirm whether miR-21 oligomers could be transfected into neuronal cells or not. Immunofluorescence staining for

microtubule-associated protein 2 (MAP-2) and transfection for FAM-miR-21 were performed. The neurons were cultured for 7 days, and translation was performed with RNAiMAX according to the traditional protocol with the minimum concentration transfection reagent (0.5 μL of RNAiMAX), which was used to test the transfection efficiency of miR-21. Dilute 0.5 μL RNAiMAX reagent and 2.5 μL of FAM-miR-21 agomir (20 μM) in DMEM, and the overall solution reached to 500 μL and was incubated for 20 min. Transfection solution was added to six-well plates and maintained up to 6 h. Then, the cells reacted with mouse anti-MAP-2 (1:100; BOSTER) as a neuron marker for 12 h at 4°C and reacted with TRITC-(tetra-methyl-5,6-isothiocyanate)-labeled phalloidin (1:100; BOSTER) for 1 h. DAPI was used for DNA staining. The number of MAP-2-positive cells, FAM-positive cells, and the total number of cortical neurons were counted under 200× magnification.

Neurons were cultured for 7 days and randomly divided into five groups: starvation+DMEM (S-DMEM), unstarvation+DMEM (N-DMEM), starvation+Neurobasal-A (S-Neurobasal), unstarvation+Neurobasal-A (N-Neurobasal), and the sham group (Sham). As described previously, miRNA oligomers (sequences listed in Table 1) were diluted to a final concentration of 20 mM. miRNA oligomers (2.5 μL) were then combined with RNAiMAX (0.5, 1, 1.5, 2, 2.5 μL) (Invitrogen) in an RNase-free polymerase chain reaction (PCR) tube and incubated for 20 min at room temperature, with the ratio RNAiMAX (μL):miR-21 (μL) = 1:5, 2:5, 3:5, 4:5, 5:5. The overall transfection solution reached to 500 μL (N-DMEM/Neurobasal-A) and 250 μL (S-DMEM/Neurobasal-A). Before transfection, neurons plated on six-well plates were treated with 250 μL of DMEM or Neurobasal-A for 1 h in S-DMEM/S-Neurobasal-A groups for starvation pretreatment but not in N-DMEM/N-Neurobasal-A groups. Transfection solution was added to six-well plates and maintained up to 6 h, and then the neurons were cultured in the Neurobasal-A medium with 2% B27 and 1% glutamine (2 mL). Neurons were further cultured for 48 h before analysis (Li *et al.*, 2009). Cells of the sham group underwent the same transfection procedure without RNAiMAX. The antibiotics, such as penicillin or streptomycin, were not used in the

TABLE 1. THE SEQUENCES OF miRNA OLIGOMERS AND PRIMERS USED FOR QUANTITATIVE REAL-TIME RT-PCR

miRNA oligomers	Sequences
miR-21 agomir	5'-UAGCUUAUCAGACUGAUGUUGA-3'
Agomir negative control	5'-UUCUCCGAACGUGUCACGUTT-3'
RT primers	
miR-21 U6	5'-AATGGTTGTTCTCCACACTCTC-3' 5'-GGAACGCTTCACGAATTTG-3'
PCR primers	
miR-21-F	5'-ACGTTGTGTAGCTTATCAGACTG-3'
miR-21-R	5'-AATGGTTGTTCTCCACACTCTC-3'
U6-F	5'-ATTGGAACGATACAGAGAAGATT-3'
U6-R	5'-GGAACGCTTCACGAATTTG-3'

RT-PCR, reverse transcription polymerase chain reaction.

traditional or lipofection protocol. There was no serum used in this transfection protocol.

Isolation of total RNA and quantitative real-time PCR

After 48 h of transfection, the total RNA from neurons subjected to transfection protocols was extracted with the TRIzol reagent (Invitrogen) following the manufacturer's instructions. To get the mature miR-21, the total RNA and the Hairpin-it™ miRNAs RT-PCR Quantitation Kit/U6 snRNA RT-PCR Normalization Kit (GenePharma) were used in reverse transcription following the manufacturer's protocol. U6 small nuclear RNA (snRNA) was used as a housekeeping gene. Cycling conditions were 16°C, 30 min; 42°C, 30 min; 85°C, 10 min; and 4°C, keeping. Then, quantitative real-time (qRT)-PCR was performed with the CFX Connect™ Real-Time System (Bio-Rad Laboratories). Furthermore, the cycling conditions used were as follows: 95°C, 3 min; 40 cycles of 95°C, 12 s and 62°C, 40 s. Expression of miR-21 was calculated through a $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001). Primers for amplification of miR-21 and U6 are listed in Table 1.

Western blots

After 48 h of transfection, the total protein was extracted from neurons in S/N-Neurobasal groups transfected with miR-21 agomir for immunoblotting analysis. The protein concentrations were detected with the Pierce BCA Protein Assay Kit. Equal amounts of protein were separated by SDS-PAGE and transferred onto the PVDF membrane. Then the blots were blocked by 5% nonfat milk dissolved in TBST for 2 h and incubated overnight at 4°C in the following primary antibodies: PTEN, PDCD4, and GAPDH. Subsequently, the blots were incubated with the second antibody HRP goat anti-rabbit.

TUNEL and MTT assay

To eliminate the biological effect of miR-21, miR-21-negative control (miR-21NC) was transfected into neurons according to the transfection protocol. To detect the cortical neurons and neuronal apoptosis, double immunofluorescence staining for MAP-2 and TUNEL were performed after 48 h of transfection. MAP-2 immunostaining was performed using anti-MAP-2 for 12 h and then reacted with TRITC-(tetra-methyl-5, 6-isothiocyanate)-labeled phalloidin for 1 h. Subsequently, cortical neurons were incubated with TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) reacting mixture from the TUNEL Apoptosis Detection Kit (Roche) for 2 h. Finally, DAPI (4,6-diamidino-2-phenylindole) was used as a counterstain. The number of MAP-2-positive cells, TUNEL-positive cells, and the total number of neurons were counted under 200× magnification.

The cytotoxicity of the RNAiMAX was quantified by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide] assay. Briefly, 15 μ L of MTT (5 mg/mL) dissolved in the cell culture medium were added to each well and incubated for 4 h at 37°C after 48 h of transfection. After this period, the remaining MTT solution was removed, 150 μ L of DMSO were added to each well to dissolve the formazan crystals, and then the 96-well plate was vibrated for 10 min.

The absorbance was detected using a microplate reader at 490 nm.

Counting and statistics analysis

The immunostaining was analyzed with the ImageJ software. The percentage of positive cells was calculated as positive neurons/total neurons \times 100%. Data are expressed as mean \pm standard deviation (SD). Statistical analysis was performed with SPSS 19.0. Comparisons between any two groups of data were done using the single-factorial analysis of variance (ANOVA). When *p*-values were less than 0.05, statistical significance was considered.

Results

Transfection efficiency and the expression level of miR-21 in neurons

To manifest that miR-21 oligomers could be transfected into cortical neurons through RNAiMAX by the traditional protocol, we detected the transfection efficiency of FAM-miR-21 oligomers in cortical neurons. We found some intense FAM-green staining in the cytoplasm of cortical neurons, and data analyzed with the ImageJ software showed that the positive cells were 38.7% (Fig. 1A), which illustrated that the cationic lipofection reagent, RNAiMAX, could transfect miR-21 agomir into cortical neurons by the traditional protocol, but the transfection efficiency of miR-21 was not at a high level.

To investigate the different results of lipofection protocol and traditional protocol, we detected the miR-21 expression level in cortical neurons using qRT-PCR. Although both solvents were able to mediate transfection, the expression levels of miR-21 were obviously different, and compared to S/N-DMEM groups, the expression levels of miR-21 were higher at almost all ratios in S/N-Neurobasal groups (Fig. 1B). However, the miR-21 expression level in S-DMEM groups was not different compared to N-DMEM groups and it was similar in S-Neurobasal groups and in N-Neurobasal groups (Fig. 1B). Interestingly, compared to the sham group, the highest expression levels of miR-21 was obtained at the ratio RNAiMAX:miR-21=3:5 and the increase of RNAiMAX's concentration had not caused the further upregulation of expression levels of miR-21 at ratios RNAiMAX:miR-21=4:5, 5:5 (Fig 1B). The results showed that the transfection solvent Neurobasal-A was better used to transfect miR-21 agomir into neurons than DMEM and the starvation pre-treatment of transfection had no effect on the expression levels of miR-21. The above results also indicated that the expression levels of miR-21 in neurons had reached the highest level at the ratios RNAiMAX:miR-21=3:5, 4:5, 5:5 in S/N-Neurobasal groups *in vitro*.

Protein levels of PTEN and PDCD4

To further confirm the function of miR-21, we tested the protein levels of PTEN (phosphatase and tensin homolog deleted on chromosome ten) and PDCD4 (programmed cell death protein 4), which were the target genes of miR-21 (Junker *et al.*, 2015; Xu *et al.*, 2015). The data showed that miR-21 suppressed the protein levels of PTEN and PDCD4, and the protein levels of PTEN and PDCD4 decreased apparently at ratios RNAiMAX:miR-21=3:5, 4:5, 5:5

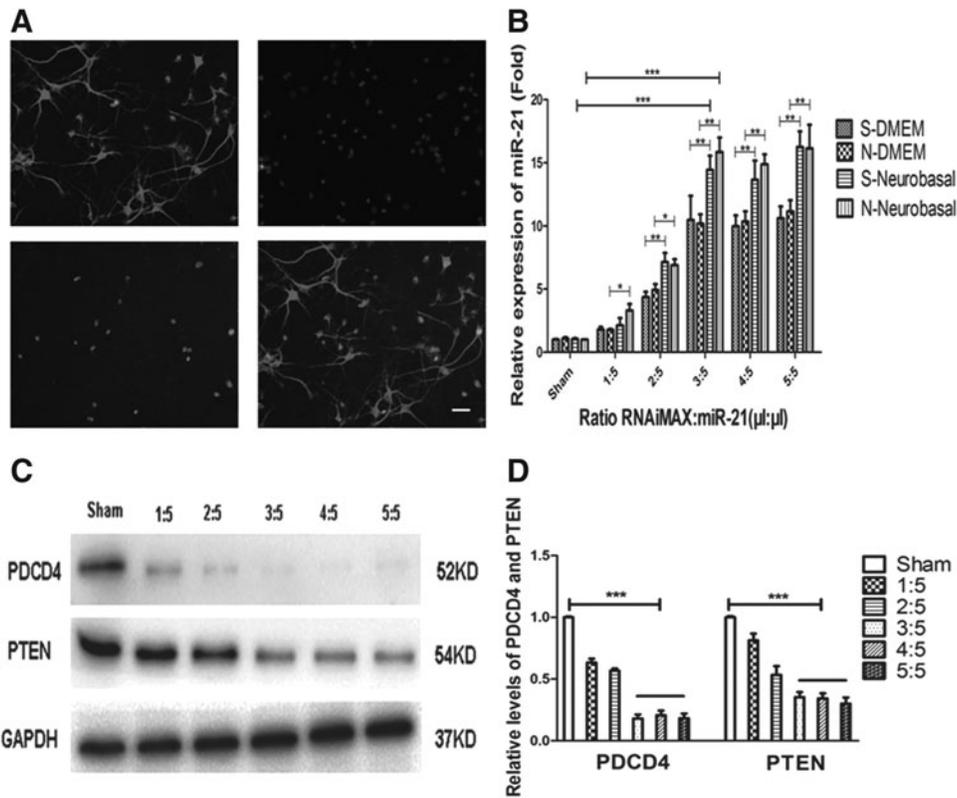


FIG. 1. The transfection efficiency and expression level of miR-21 in cortical neurons. **(A)** Cortical neurons labeled MAP-2, DAPI, and FAM-miR-21 agomir and merged. The transfection efficiency of miR-21 oligomers was FAM-miR-21/DAPI. Scale bar: 100 μ m. **(B)** The expression levels of miR-21 in cortical neurons. Compared to S/N-DMEM groups, the expression levels of miR-21 were higher in neurons in S/N-Neurobasal groups at ratios RNAiMAX:miR-21 = 1:5, 2:5 (* p < 0.05) and RNAiMAX:miR-21 = 3:5, 4:5, 5:5 (** p < 0.01). Compared to sham, the expression levels of miR-21 were highest at ratios RNAiMAX:miR-21 = 3:5, 4:5, 5:5 in S/N-Neurobasal (*** p < 0.001) and the expression levels of miR-21 were not different at ratios RNAiMAX:miR-21 = 3:5, 4:5, 5:5 (p > 0.05). **(C, D)** Protein levels of PTEN and PDCD4. The protein levels of PTEN and PDCD4 decreased remarkably at ratios RNAiMAX:miR-21 = 3:5, 4:5, 5:5 (*** p < 0.001). MAP-2, microtubule-associated protein 2.

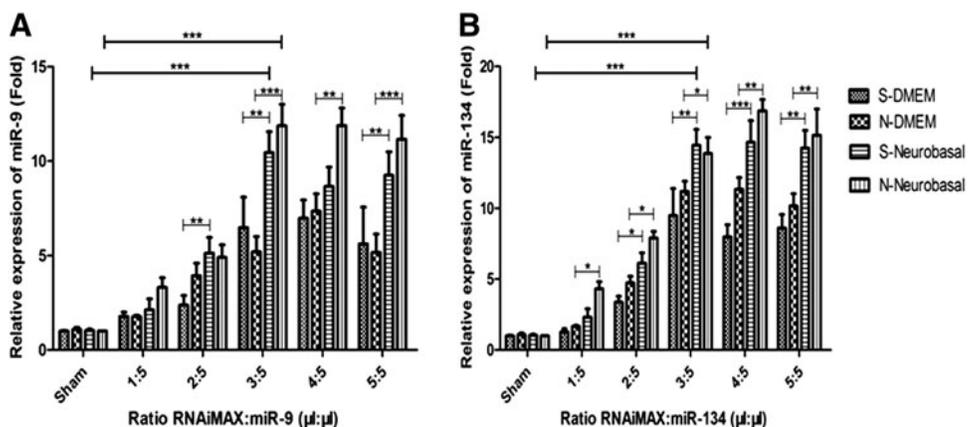
(Fig. 1C, D), which indicated that miR-21 had higher expression levels at ratios RNAiMAX:miR-21 = 3:5, 4:5, 5:5.

The expression levels of miR-9 and miR-134 in neurons

To see whether other miRNAs have similar expression levels in cortical neurons after transfection by lipofection protocol, we

tested the expression levels of miR-9 and miR-134. The results showed that miR-9 and miR-134 expression levels were similar to miR-21 expression levels in cortical neurons (Fig. 2A, B). The miR-9 and miR-134 expression levels in S/N-Neurobasal groups were higher than in S/N-DMEM groups; compared to the sham group, the expression levels of miR-9 and miR-134 also achieved the highest at the ratio RNAiMAX:miR-21 = 3:5; and the increase of RNAiMAX's concentration had not caused the

FIG. 2. The expression levels of miR-9 **(A)** and miR-134 **(B)** in cortical neurons. The expression levels of miR-9 and miR-134 were similar to miR-21 in cortical neurons. Compared to S/N-DMEM groups, the expression levels of miR-9 and miR-134 were higher in S/N-Neurobasal groups at different ratios of RNAiMAX:miR-21 and not different at ratios RNAiMAX:miR-21 = 3:5, 4:5, 5:5 (p > 0.05) in cortical neurons. * p < 0.05, ** p < 0.01, *** p < 0.001.



further upregulation of expression levels of miR-9 and miR-134 at the ratios RNAiMAX:miR-21 = 4:5, 5:5 (Fig. 2A, B). These were all similar to miR-21 expression levels in cortical neurons. The data indicated that lipofection protocol could transfect miRNAs into cortical neurons.

The neuron injury after transfection

We found that neuron injury was condition dependent and dose dependent after transfection (Fig. 3A–C). Compared with the sham group, the TUNEL-positive cells appeared as transfected RNAiMAX/miR-21NC complexes and were apparently increased at ratios RNAiMAX:miR-21NC = 4:5, 5:5 (Fig. 3A, B), and the cellular viability was also dra-

matically decreased at ratios RNAiMAX:miR-21NC = 4:5, 5:5 (Fig. 3C), which suggested that the RNAiMAX caused cortical neuron injury and the increase of RNAiMAX's concentration caused more neuron injury. Meanwhile, compared with N-Neurobasal groups, the percentage of TUNEL-positive cells was higher in the S-Neurobasal group (Fig. 3B), and the cellular viability was dramatically decreased in the S-Neurobasal group (Fig. 3C). These results indicated that cortical neurons in the N-Neurobasal group had a smaller injury than those in the S-Neurobasal group after transfection. The results above demonstrated that RNAiMAX caused injury of neurons, and compared to ratios RNAiMAX:miR-21NC = 4:5, 5:5, neuron injury was smaller at ratios RNAiMAX:miR-21NC = 1:5, 2:5, 3:5.

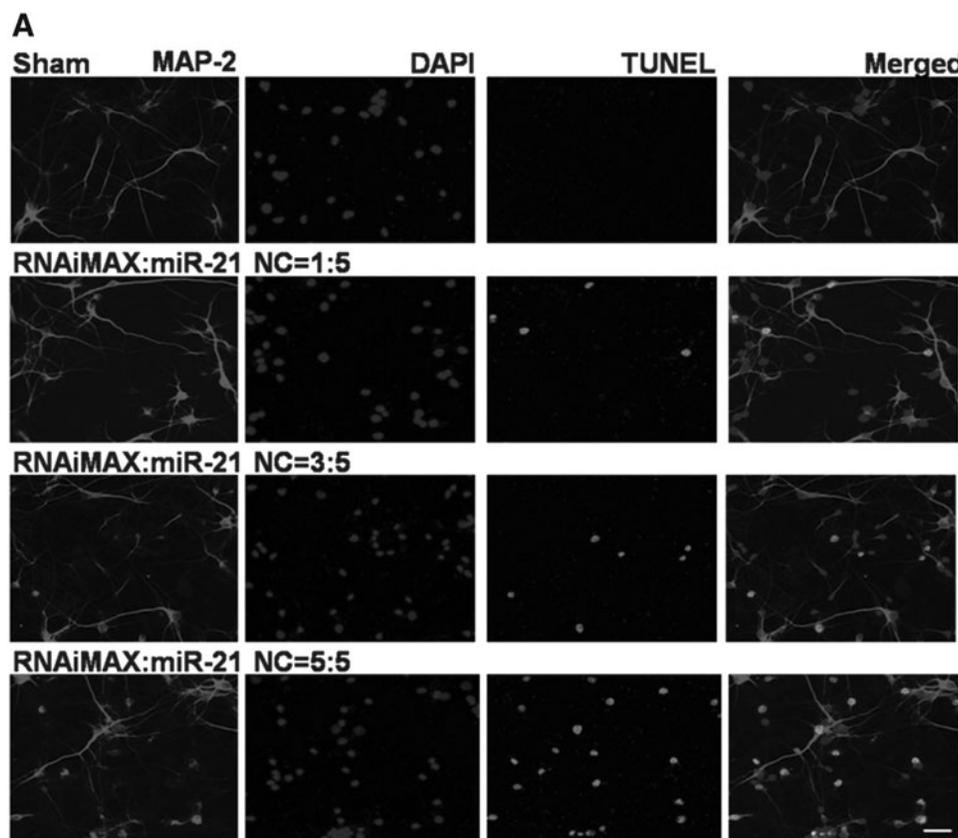
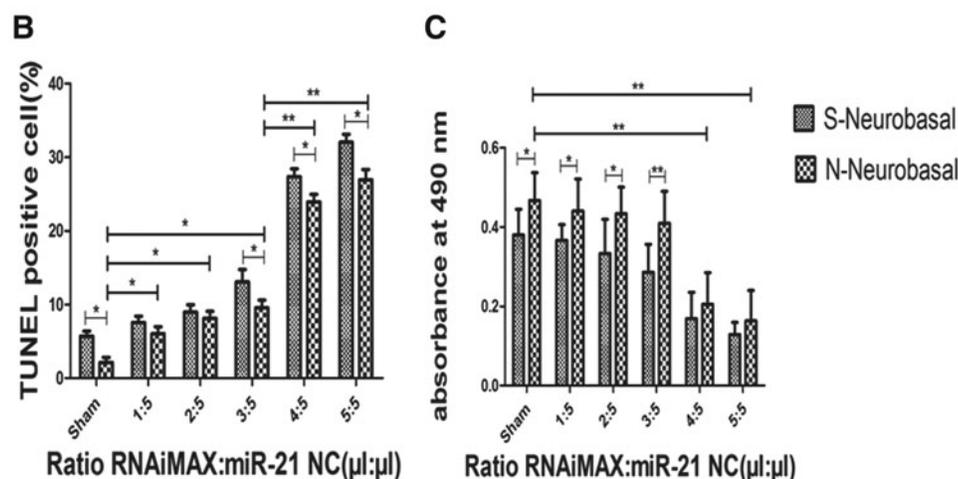


FIG. 3. The apoptosis and cellular viability of cortical neurons. Cortical neurons transfected with RNAiMAX/miR-21NC complexes labeled for MAP-2, DAPI, and TUNEL and merged. Compared with RNAiMAX:miR-21NC = 1:5, 3:5, the number of apoptotic cortical neurons increased at the ratio RNAiMAX:miR-21NC = 5:5 (A). Scale bar: 100 μ m. The percentage of TUNEL-positive cell: compared with S-Neurobasal groups, the percentage was lower in N-Neurobasal (* p < 0.05), and compared with ratio RNAiMAX:miR-21NC = 3:5, the percentage increased remarkably at ratios RNAiMAX:miR-21NC = 4:5, 5:5 (** p < 0.01) in N-Neurobasal groups (B). MTT for the cellular viability in cortical neurons: compared to the cortical neurons in S-Neurobasal groups, the cellular viability was higher in N-Neurobasal groups (* p < 0.05), and compared to the sham group, the cellular viability decreased dramatically at ratios RNAiMAX:miR-21 = 4:5, 5:5 in N-Neurobasal groups (** p < 0.01) (C).



Discussion

The goal of this study was to produce a parallel comparison of the traditional and lipofection protocol, including transfection conditions, solvents, and reagent concentrations, to establish the lipofection protocol for delivering active, noncoding miRNAs into cortical neurons. Some transfection reagents are currently used to introduce miRNAs into cells, but poor transfection efficiency and low expression level of miRNAs are a common problem in neurons. The cationic lipofection has been shown to perform well in many passage cells, such as osteoclasts (Liu *et al.*, 2015), endothelial cells (Rohde *et al.*, 2015), and human-derived epithelial cells (Le Corre *et al.*, 2015), but the mature primary cells, like neurons, are still described as difficult-to-transfect (Morris and Labhasetwar, 2015). In some studies, the cationic lipofection reagents, like Lipofectamine 2000, were efficient in terms of delivery in primary cells (Chen *et al.*, 2014), but there are no special protocols to maintain the functionality of the internalized RNA oligos.

In this study, by detecting the expression levels of miR-21 and injury of neurons transfected with RNAiMAX/miR-21 complexes, we successfully established the lipofection protocol of miR-21 transfecting into cortical neurons. First, we examined whether the cultured cells were cortical cells or not. The MAP-2 is a cytoskeletal protein localized in the neuronal dendritic compartment, and it is considered a marker of structural integrity because it is involved in the morphological stabilization of dendritic processes (Di Stefano *et al.*, 2001). The cultured cells had a strong MAP-2 staining in dendrites. Meanwhile, in other primary cells, such as primary human aortic and coronary artery endothelial and vascular smooth muscle, RNAiMAX has been reported an effective gene vector (Nabzdyk *et al.*, 2012). RNAiMAX has been used in multigene transfection and is considered an effective tool for gene delivery. As a new cationic liposome agent, RNAiMAX has a higher transfection efficiency compared with several other transfecting agents such as Oligofectamine and Lipofectamine 2000 (Zhao *et al.*, 2008; Kamaci *et al.*, 2011). Then, we found that RNAiMAX was effective for delivering FAM-miR-21 agomir into cortical neurons.

Second, we investigate the expression levels of miR-21 in neurons transfected with RNAiMAX/miR-21 complexes. We chose two solvents to dilute RNAiMAX/miR-21 complexes, DMEM and Neurobasal-A. DMEM is a common medium used for culturing cells and transfection (Xu *et al.*, 2014). Neurobasal-A is a special medium used for culturing neurons (Liu *et al.*, 2013). Both are often used as transfection solvents. By analyzing the expression levels of miR-21, we found that the expression levels of miR-21 were remarkably higher in neurons transfected by Neurobasal-A than by DMEM. Meanwhile, with different ratios of RNAiMAX:miR-21, our qRT-PCR results showed that expression levels of miR-21 were already the highest at the ratio of RNAiMAX:miR-21 = 3:5 and the increase of RNAiMAX's concentration had not caused the further upregulation of expression levels of miR-21 at ratios RNAiMAX:miR-21 = 4:5, 5:5, which may be because the saturation of transfection is reached at RNAiMAX:miR-21 = 3:5 and the expression levels of miR-21 did not have obvious differences in neurons with pretreatments of transfection. Our research certified that the

expression levels of miR-21 in neurons were the highest under the conditions of RNAiMAX/miR-21 complexes diluted in Neurobasal-A and at ratios of RNAiMAX:miR-21 = 3:5, 4:5, 5:5.

Previous studies have reported that compared with untreated cells, viability of transfected cells decreased and depended on the concentration of RNAiMAX (Kamaci *et al.*, 2011). Also, some studies reported that cationic liposome agents have been shown to cause apoptosis in human lung epithelial cell (Kongkaneramt *et al.*, 2008). In other studies, the cell apoptosis can be induced by serum deprivation (Ferriere *et al.*, 2013). In our research, the results demonstrated that RNAiMAX caused injury of cortical neurons and neuron injury depended on the RNAiMAX concentration. Compared to ratios RNAiMAX:miR-21 = 4:5, 5:5, the injury of primary cortical neurons was smaller at the ratio of RNAiMAX:miR-21 = 3:5, which determined that the higher RNAiMAX concentration caused more cortical neuron injury. In contrast, the pretreatment, starvation of transfection, increased neuron injury. Compared to N-Neurobasal groups, the cortical neurons' viability decreased significantly in S-Neurobasal groups and the percentage of TUNEL-positive cells increased.

Conclusion

miR-21 agomir can be transfected into neurons by RNAiMAX. Without the pretreatment of starvation *in vitro*, the lipofection protocol was that RNAiMAX/miR-21 agomir complexes were diluted in Neurobasal-A at the ratio of RNAiMAX:miR-21 = 3:5. The starvation pretreatment led to less apoptosis of cortical neurons, and the better solvent is Neurobasal-A, and the ratio RNAiMAX:miR-21 = 3:5 is the best transfection reagent concentration in lipofection protocol.

Acknowledgments

This work was supported by the Tianjin Research Program of Application Foundation and Advanced Technology (No. 13JCYBJC23700), the National Clinical Key Subject Construction Project of NHFPC Fund, and the National Natural Science Fund (81471252).

Disclosure Statement

No competing financial interests exist.

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Received for publication January 7, 2015; received in revised form August 15, 2015; accepted August 18, 2015.