ORIGINAL ARTICLE

Effect of Lentiviral shRNA of Nogo Receptor on Rat Cortex Neuron Axon Outgrowth

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ABSTRACT: ***Background and Aims:*** Axon growth is crucial for injured neural tissue to recover; however it is difficult to achieve in general. Axon outgrowth is inhibited by the activation of the Nogo receptor (NgR) by one of three different ligands. The present study aimed to suppress the inhibitory effect of the three inhibitory proteins to facilitate axon outgrowth. ***Methods:*** A lentiviral vector, siNgR199 (that has the capacity to interfere with the gene of NgR expression), was constructed for suppressing the gene transcription of NgR. Rat cortex neurons and oligodendrocytes were prepared to observe the effect of siNgR199 on facilitating axon outgrowth. ***Results:*** After transfection, the lentiviral siRNA of NgR remained in target neurons for almost two weeks whereas the conventional siRNA of NgR remained in neurons less than five days. Lentivirus-mediated delivery of exogenous small interfering RNA (siNgR199) targeting NgR significantly reduced the expression of this receptor and promoted axon outgrowth. In contrast, provision of naked siRNA targeting NgR (NgRsiRNA) showed less inhibitory effect on NgR protein expression and did not affect axon outgrowth. ***Conclusions:*** Lentiviral siRNA of NgR effectively suppresses the expression of NgR in cultured neurons that facilitates the axon outgrowth. The data implicate that lentiviral siRNA of NgR has therapeutic potential in facilitating the recovery of injured neural tissue.

RÉSUMÉ: Effet de l'ARNsh lentiviral du récepteur Nogo sur la régénération axonale de neurones du cortex chez le rat. ***Contexte et objectif :*** La croissance axonale est cruciale pour la guérison du tissu nerveux lésé. Cependant, elle est difficile à réaliser. La régénération axonale est inhibée par l'activation du récepteur Nogo (NgR) par l'un de trois ligands différents. Le but de cette étude était de supprimer l'effet inhibiteur des trois protéines inhibitrices pour faciliter la régénération axonale. ***Méthodes :*** Un vecteur lentiviral, siNgR199 (qui peut interférer avec l'expression du gène NgR), a été construit pour supprimer la transcription du gène NgR. Des neurones corticaux et des oligodendrocytes de rat ont été préparés pour observer l'effet de siNgR199 sur la régénération axonale. ***Résultats :*** Après transfection, l'ARNsi lentiviral de NgR est demeuré dans les neurones cibles pendant près de deux semaines alors que l'ARNsi conventionnel de NgR est demeuré dans les neurones moins de cinq jours. La livraison médiée par le lentivirus de petits ARNsi (siNgR199) exogènes interférents ciblant NgR a diminué significativement l'expression de ce récepteur et favorisé la régénération axonale. Par contre, l'ARNsi nu ciblant NgR a eu moins d'effet inhibiteur sur l'expression de la protéine NgR et n'a pas influencé la régénération axonale. ***Conclusions :*** L'ARNsi lentiviral de NgR supprime efficacement l'expression de NgR dans des neurones en culture, facilitant la régénération axonale. Ces données sont compatibles avec un effet thérapeutique potentiel de l'ARNsi lentiviral de NgR pour faciliter la récupération de tissus nerveux lésés.

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After neural injury, the axon regeneration is very difficult to achieve based on the mechanism that three inhibitory proteins, NogoA (also known as reticulon 4), MAG (myelin-associated glycoprotein) and OMgp (oligodendrocyte myelin glycoprotein), exist in the central nervous system that inhibit the neurite outgrowth[1-3]. These three inhibitory proteins share a common receptor, the Nogo receptor (NgR).[4] Nogo receptor (including NgR1, NgR2 and NgR3; the present study worked on NgR1) is a neuronal specific receptor with high effect and affinity by binding NogoA, MAG and OMgp mediating the inhibition of axon regeneration and induction of cone collapse[5].

It is proposed that suppressing Nogo receptor protein can suppress Nogo-A, MAG and OMgp's inhibitory effect on axon outgrowth[4]. In research of myelin-associated inhibitors in the central nervous system, the anti-NogoA antibody IN-1[6], myelin[7] or associated cDNA vaccine[8] and a Nogo A-derived peptide, NEP1-40[9], have been delivered to rats with spinal cord injury to facilitate the axon outgrowth. Various achievements in

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functional recovery and axonal outgrowth were reported[4,6,9]. However, IN-1 only inhibits Nogo-A and myelin[6]; associated cDNA vaccine[8] can not pass the blood brain barrier efficiently. In addition, other myelin inhibitors' function can not be antagonized in this method[5]. Although NEP1-40 can reduce Nogo-A, MAG and OMgp's inhibitory effect, the effect is transient[4,6,9]. Therefore, a long-lasting remedy that can inhibit these three inhibitory proteins is optimal for the axon outgrowth study.

RNA interference (RNAi) is a post transcriptional gene silencing process targeting homologous mRNA for degradation[10]. RNA interference targeted-genes still can be transcripted, but the mRNA can not be accumulated to function because it is degraded by transfected small interference RNA (siRNA)[10]. Since the effects of siRNA only last for a short time in target cells, transfection with designed vectors, such as plasmid and lentiviral vector, have been developed[11]. We speculated that using lentiviral vectors to carry siRNA of NgR into neurons can inhibit NgR for a relatively longer time that is sufficient for new axon outgrowth. We thus designed a lentiviral siRNA of NgR, the siNgR199, and transfected it into cultured neurons. The effect of siRgR199 was also compared to the effects of non-vector delivered siRNA. Indeed, transfection with siNgR199 dramatically increased the new axon outgrowth in cultured neurons.

Materials and Methods

Reagents and rats

siRNA of NgR and molecular related reagents, NativePure™ Lentiviral expression kit, NuPAGE gel, oligofectamine (Invitrogen, Shanghai, China). Antibody against Nogo receptor (sc-25659), Neuron-specific beta-Tubulin (sc-80011) (Santa Cruz Biotech, Santa Cruz, CA, USA). A2B5 antibody ((ab53521): abcam Inc (Cambridge, MA, USA). Isotype IgG, horseradish peroxidase conjugated second antibody (Sigma, Shanghai, China). Sprague Dawley rats were purchased from Shanghai Experimental Animal Institute. The animal experimental procedures were approved by the Animal Care Committee at the Second Military Medical University.

Construction of siNgR199

Following published procedures[12], siNgR199 was constructed into a short hairpin RNA construction as homologous NgR RNA succession, and cloned it into lentivirus vector using the E1bashir's criteria and the RNAi vector rule following published procedures[12]. The vector carried a built-in cGFP that was used as a tracer in the neurons. Then, we evaluated the recombinant by enzyme cutting and gene sequencing test. The results showed 100% fidelity in the sequence of siNgR199 (tcctgtggctgcactcaaa; NCBI: AF462390) as compared with the original sequence.

Western blotting

Levels of NgR in neurons were determined by Western blotting as refferenced in published procedures[12]. Briefly, protein was extracted from collected neurons and separated by pre-cast NuPAGE gel system and blotted onto a nitrocellulose membrane. The membrane was blotted with primary antibodies (or isotype IgG used as control) (1 μg/ml) and then incubated with horseradish peroxidase conjugated second antibody (1:1000). The enzymatic reaction was detected with enhanced chemiluminescent reagents and recorded with X-ray films.

Rat cortex neuron isolation

Neurons were isolated from two-day old Sprague-Dawley rats referred to a previous study[13]. Briefly, cultures of neurons were established from the cortex. The neurons were collected and treated with trypsin (0.25%) for ten min at 37 °C to remove the ectoblast. The cells were washed once with fresh media and then washed with fetal bovine serum (40%) to neutralize the effect of trypsin. After washing, neurons were filtered with a cell strainer and cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum, 50 U/ml penicillin–streptomycin, 2 mM l-glutamine, 25 mM glucose, 25 ng/ml nerve growth factor and 2 ng/ml glial-derived neurotrophic factor.

Oligodendrocyte preparation

Following our established protocol, two-day old rats were killed by cervical dislocation. The cortex was isolated and minced, followed by enzymatic digestion with 0.1% trypsin and 0.001% DNase, and centrifugation for five minutes (×600 g). The cell pellet was resuspended in culture medium (Dulbecco's modified Eagle's medium supplemented with 1% penicillin-streptomycin-fungizone and 10% foetal calf serum). After culture for 15-20 days, cortex-derived single cell suspension was subjected to magnetic cell sorting (MACS) to isolate oligodendrocytes (by A2B5 antibody). The purity of isolated cell population was over 95% as tested by flow cytometry.

RNA interference (RNAi)

siNgR199 transfection was performed following reported procedures[12,14]. Briefly, 10^6 neurons were resuspended in 100 μl of infection cocktail, which contained lentiviral vectors (multiplicity of infection of 15) and left for two hours at 37°C in 5% CO_2. Transfections were performed in the presence of oligofectamine following the manufacturer's instruction. The efficiency of siRNA transfection was determined by flow cytometry (GFP was used as the marker) and Western blotting. The peak inhibitory effect was reached at 96 hours after transfection that lasted for another 96 hours and declined thereafter. The control siRNAs did not affect the target molecule expression. The transfection efficiency was over 95% and the inhibition was also over 95% and reproducible in all experiments.

Detection of GFP protein

GFP protein in lentiviral vector was detected by spectrophotometry. Optical density of protein extracts from transfected neurons was measured by a UV spectrophotometer (GENESYS 20, Thermo Scientific, ON, Canada) at 450 nm. Samples were measured in duplicate. The results were presented as OD value.

Statistical analysis

Data were expressed as mean ± standard deviation. A student t-test was used to compare the difference between means of two groups. Differences were considered significant if $p < 0.05$.

Results

Long-lasting effect of lentiviral siRNA of NgR

RNA interference has been shown to have a specific inhibitory effect on target genes that have been used extensively in basic research as well as some clinical studies. However, the weakness of conventional siRNA is its short effective lifespan that only lasts from several hours to one to three days. This shortcoming limits its application in some experiments that require longer time, such as axon regeneration. Following published procedures[12,14], we constructed lentiviral siRNA of NgR, the siNgR199. Sequence analysis showed the sequence of siNgR199 fully matched the designed sequence of NgR (data not shown). We transfected the primary culture neurons isolated from rat brain cortex with siNgR199 and conventional siRNA of NgR (NgRsiRNA). With the build-in cGFP as a tracer, we detected that the transfected gene reached its peak value at 48 hours after transfection that lasted up to 240 hours. However, although the NgRsiRNA also began its expression at 48 h, its extent was much less than that of siNgR199. Furthermore, it only lasted for 24 hours further and declined sharply (Figure 1). The results indicate that lentiviral siRNA of NgR is more efficient and its effective lifespan lasts much longer than NgRsiRNA.

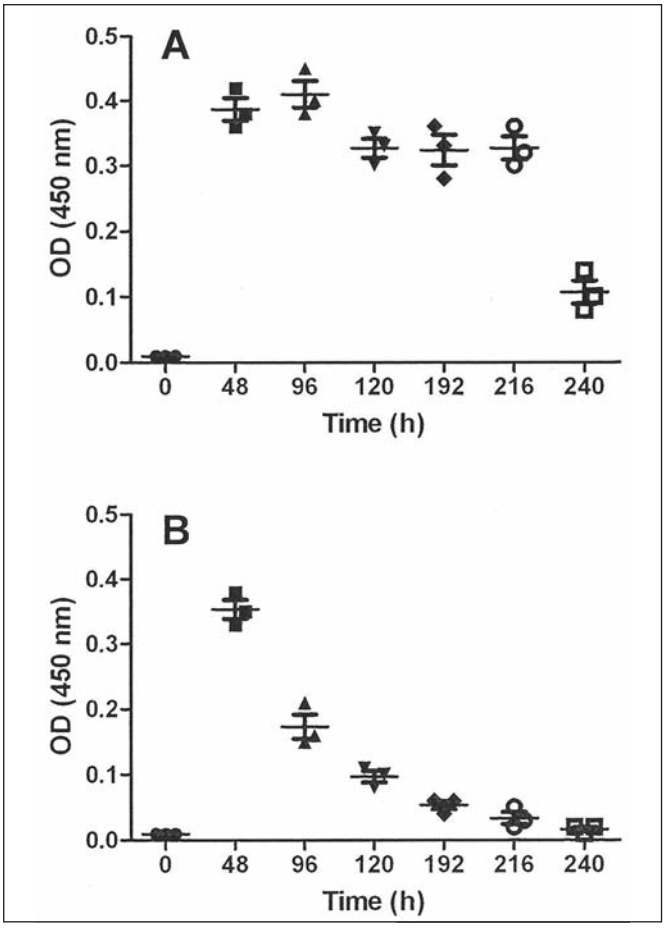

Figure 1: *siNgR199 suppresses NgR expression in target cells. Rat cortex neurons were cultured and transfected with siNgR199 or NgRsiRNA. Levels of cGFP (the tracer of siNgR199 and NgRsiRNA) in cellular extracts were determined by spectrophotometry at OD 450 nm. The scatter dot plots indicate the levels of cGFP in neurons at time points from 0 to 240 h. Each dot represents a single datum from one separate experiment.*

Suppressive effect of siNgR199 on NgR expression in cortex neurons

Nogo receptor is the common receptor of the three axon regeneration inhibitory molecules[4]. Thus, theoretically, inhibition of NgR can facilitate the axon regeneration. Based on published data[12,14], we designed this long lifespan siNgR199 with the expectation to inhibit expression of NgR for a relatively long time to allow the new axons to grow. We examined the expression of NgR protein in cultured neurons. The data showed that transfection with siNgR199 effectively suppressed the expression of NgR in cultured neurons. The suppressive peak appeared in eight and nine days after transfection; and then, the expression of NgR in cultured neurons gradually returned back

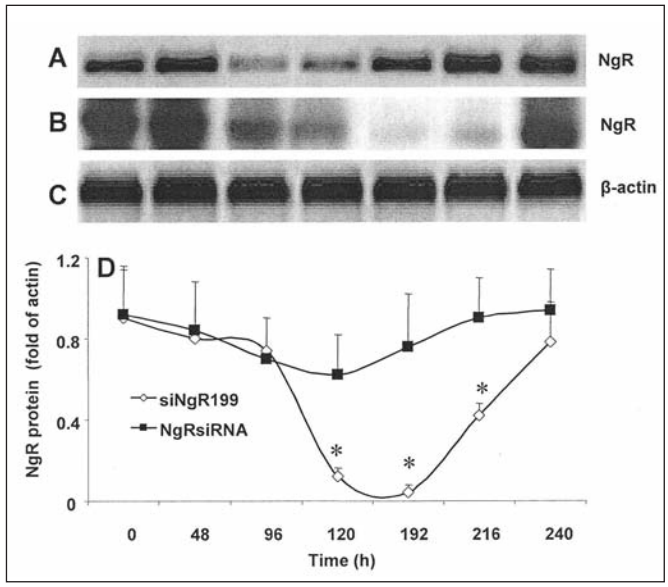

Figure 2: *RNAi suppresses expression of NgR in neurons. Rat cortex neurons were isolated and cultured in the presence of siNgR199 or NgRsiRNA for 0-240 h. Expression of NgR in neurons was examined by Western blotting. A-C, Western blotting gels show protein of NgR in NgRsiRNA group (A), siNgR199 group (B), and beta-actin protein (C). D, the line graph indicates the densitometry analysis for the Western blots. Data were normalized into fold of beta-actin and presented as mean ± SD. *, $p<0.05$, compared with NgRsiRNA group. Data were from three separate experiments. Scramble siRNA did not show any inhibitory effect on NgR protein expression (data not shown).*

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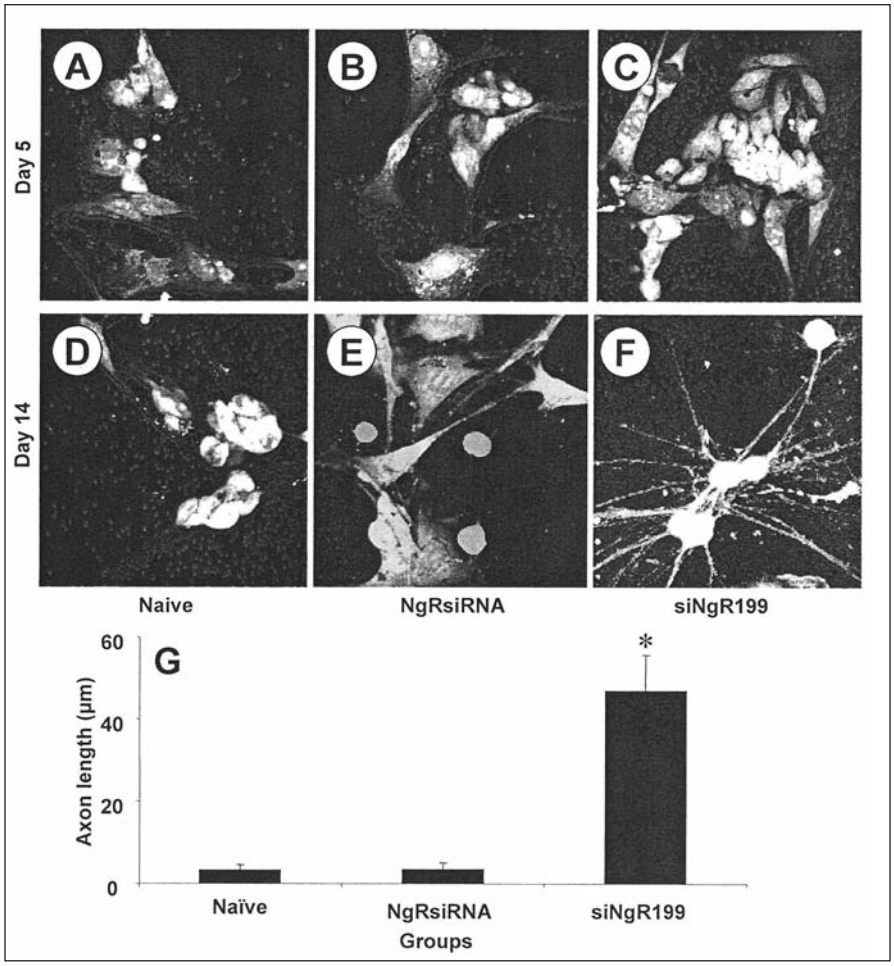

Figure 3: *siNgR199 facilitates axon regeneration. Rat cortex neurons were cultured with oligodendrocytes for two weeks in the presence of NgRsiRNA, or siNgR199. The neurons are in round shape; oligodendrocytes are irregular in shape; axons are in line shape. Control group using medium alone. G, bars indicate the length of axon in cultured neurons on Day 14. Data were presented as mean ± SD from 50 neurons of each group. *, p<0.05, compared with naïve group. Some neurons were treated with scramble siRNA; axon outgrowth was similar to naive group (data not shown).*

to baseline (Figure 2). Conventional siRNA of NgR also showed suppressive effect on the expression of NgR on cultured neurons on Day 4 after transfection. However, the suppressive effect on NgR expression was only maintained for one day. The results indicate that conventional NgR RNAi does suppress the expression of NgR, but only for a short time. The effect of NgRsiRNA on axon regeneration may be limited since it is only transient. siNgR199 has much better effect on suppressing the NgR expression and the inhibitory effect sustains for much longer time than NgRsiRNA does. The control siRNA did not show any effect on NgR expression (data not shown).

Effect of siNgR199 facilitates axon outgrowth

After injury, the axon outgrowth is usually refractory in the central nervous system[15]. The three inhibitory proteins are responsible for this phenomenon[1,2]. In this study, we aimed to avoid the inhibitory effect from these inhibitory proteins by effectively suppressing the expression of NgR on the neurons; such a remedy is expected to facilitate the axon regeneration. To test the hypothesis, we generated neurons and oligodendrocytes from rat cortex and observed axon growth for two weeks. As depicted in Figure 3, the axon outgrowth of the naïve group could be observed on Day 5, but grew slowly and almost stopped growing after Day 10. Transfection with NgRsiRNA showed facilitating effect. The axon outgrowth appeared on Day 4, which lasted only two days and fell to the same rate as naïve control group. Transfected with siNgR199 showed the best effect on axon outgrowth. The new axons appeared on Day 4 and grew fast that lasted until Day 12, and then declined gradually. Another group of neurons were treated with an irrelevant protein ovalbumin, which did not show any facilitating effect on axon outgrowth (data not shown). The results indicate transfection

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with siNgR199 has a useful effect on facilitating neuron axon growth; its effect is much better than NgRsiRNA, the conventional siRNA.

Discussion

Although the mechanism to prevent injured nerve from regeneration, in which the three inhibitory molecules to inhibit axon outgrowth, remains to be fully understood[1-3], various methods have been tried to facilitate the growth of new axons from the injured neurons, or neural tissue, some encouraging data have been reported although the *in vivo* studies need to be further executed[4,6,9]. In line with these pioneer studies[1-6,9], the present study successfully constructed a recombinant targeting NgR-specific siNgR199 with Lentiviral vector. The recombinant can effectively suppress NgR expression in rat' cortical neurons, facilitating the outgrowth of axons in the presence of oligodendricytes. The results indicate that lentivirus carrying siRNA may have therapeutic potential for the treatment of spinal injury.

It is known that the three inhibitory molecules, myelin-associated glycoprotein, oligodendrocyte glycoprotein, and Nogo in the central nervous system myelin act as inhibitors on axon growth[1-3]. All three molecules have a common receptor, the NgR, on the neurons[4]. Ligation of NgR by any of the three inhibitory proteins would inhibit axon growth[4]. Therefore, to inhibit the NgR can be an effective way to promote axon growth. Although siRNA can be used to knock down specific genes to silence target proteins, the short lifespan of transfected siRNA limits its application. In the present study, we constructed a lentiviral vector carrying siRNA with advanced technique. The results demonstrate that this technique overcomes the shortcomings of conventional siRNA by markedly raising the lifespan of transfected siRNA in target cells, which enables the target neurons to be unresponsive to the three inhibitory proteins. The target neurons thus have the potential to grow out new axons.

The advantage of RNAi is that it can target specific genes to suppress its function but does not affect its gene transcription[16]. Therefore, this technique is specifically suitable for the recovery of neural injury. In fact, the three inhibitory proteins are not "bad" proteins; they have certain physiological functions. They are important in the process of myelinization of nerves in the central nervous system[17]. It is speculated to serve as a necessary "adhesion molecule" to provide structural integrity to the myelin sheet[18]. Therefore, they are necessary in maintaining neural physiological function and repairing injured nerves. Based on this information, it would be better not destroy the genes of these proteins to get rid of their inhibitory effect on axon outgrowth in managing neural injury, but employ other remedies. The present study provides a useful approach by which the receptor of the three inhibitory proteins can be silenced for a short time. Our results indicate that transfection with siNgR199 can effectively suppress the expression of NgR for almost two weeks, which provides an opportunity for the injured neurons to outgrow new axons. Indeed, we observed that neurons transfected with siNgR199 grew out substantial axons over a two week period.

The effect of transfection with siNgR199 only lasted for about two weeks. The mechanism may be because the neurons eliminate this "foreign device" from the cells. The fact indicates that siNgR199 is also temporary; it only stays in target cells longer as compared with conventional siRNA transfection. This feature is actually what we expected because we only need a certain time period in which the neurons do not respond to the inhibitory proteins in order to allow new axons to grow. On the other hand, we also need these three proteins to seal the newly generated axons. Then the NgR may become useful in neural myelinization to complete the neural repair[19]. Further *in vivo* studies are needed to study the role of siNgR199 in neural repairing.

The study on elucidating the role of NgR on preventing neural repairing was commenced more than ten years ago. Conflicting results were reported. Kim et al indicate that Nogo-66 receptor prevents raphespinal and rubrospinal axon regeneration and limits functional recovery from spinal cord injury[20]; previous studies employed siRNA of NgR and found significant effect to facilitate the dorsal root ganglion cell neurite outgrowth[21-23]. Our results are in line with these pioneer studies by confirming the effect of siRNA of NgR on neurite outgrowth; furthermore, our study had a previous un-described finding that construction of siRNA of NgR with lentiviral vector could greatly extend the inhibition of NgR expression in neurons and demonstrated that this extension of time period was crucial for neurite outgrowth.

On the other hand, in a previous study, Chivatakarn et al found that blocking NgR1 expression in primary cortical neurons does not result in enhanced neurite outgrowth[24]. The same authors also showed that soluble myelin inhibitors, when applied acutely, induce growth cone collapse that is significantly attenuated in neurons where NgR1 has been silenced. They propose that NgR1 has a circumscribed role in regulating cytoskeletal dynamics after acute exposure to soluble myelin inhibitors but is not required for these ligands to mediate their growth-inhibitory properties in chronic outgrowth experiments[24]. Administration of a function-blocking NgR1 ectodomain resulted in sprouting of corticospinal and raphespinal fibres, which correlated with improved spinal cord electrical conduction and improved locomotion[25]. Some of the robust regeneration following spinal cord injury has been observed in animals treated with the NgR1 antagonistic peptide NEP1-40 or siRNA of NgR.[9,22,23] However, the results could not be reproduced by some recent studies[9,24]. The difference between these studies and the present data may be because we used lentiviral vector to carry siRNA of NgR that has the advantage in which the effect of siRNA of NgR lasts for much longer time as compared to using conventional siRNA.

Conclusion

In summary, the present study reports a lentiviral siRNA of NgR, the siNgR199, was constructed. As compared with conventional siRNA, siNgR199 had longer lifespan in dwelling in target neurons; had stronger inhibitory effect on the expression of NgR, which benefited for new axon outgrowth. The siNgR199 may have therapeutic potential for the treatment of neural injury.

Acknowledgement

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