African Journal of Pharmacy and Pharmacology Vol. 6(46), pp. 3215-3220, 15 December, 2012
Available online at http://www.academicjournals.org/AJPP
DOI: 10.5897/AJPP12.1183
ISSN 1996-0816 ©2012 Academic Journals

Full Length Research Paper

Small interfering RNA drug knockdown of EphrinB3 attenuates neuropathic pain after spinal cord injury in rats

Xiaodong Zhi[1], Mingyan Dong[1], Desui Yu[1], Yansong Wang[1], Da Huang[2] and Gang Lv[1]*

[1]Liaoning Medical University, Jinzhou 121001, P. R. China.
[2]CNPG Central Hospital, Jinzhou 121002, P. R. China.

Accepted 30 October, 2012

Erythropoietin-producing hepatoma-amplified sequence (Eph) receptor tyrosine kinases and their cell-surface-bound ligands, the ephrins, function as a unique signaling system triggered by cell to cell interaction and have been shown to mediate neurodevelopmental processes. However, the role of Eph in spinal cord injury (SCI) was unclear. Here, the lentiviral expressing vectors, pGCSIL-green fluorescent protein (GFP) vectors expressing an active small interfering RNA (siRNA) targeting EphB3 sequence were used to determine the effect of RNAi knockdown of EphB3 on function recovery of limb in adult rats by Basso-Beattie-Bresnahan (BBB) scores. Four weeks after intraparenchymal administration of the siRNA into the right lumbar, EphB3 mRNA and protein levels in siRNA group were significantly reduced ($P < 0.01$) in the spine when compared with the negative group animals. BBB locomotor scores were significantly increased ($P < 0.05$) in siRNA animals when compared with the control animals. These results indicate that vector-derived siRNAs can effectively produce spatial knockdown of EphB3 gene expression, and this knockdown selectively increased BBB scores of the rats. This preclinical study demonstrates the use of RNAi to target the expression of genes mediating SCI and the therapeutic potential of this approach.

Key word: Spinal cord injury, erythropoietin-producing hepatoma-amplified sequence (Eph) B3 receptors, quantitative reverse transcription polymerase chain reaction (qRT-PCR), EphB3, western-blot, Basso-Beattie-Bresnahan (BBB) scale.

INTRODUCTION

Spinal cord injury (SCI) is still a major clinical problem with permanent neurological deficits and secondary complications (Emine et al., 2012). It is characterized by a total or partial loss of motor and sensory functions due to the inability of neurons to regenerate. Inhibitory molecular cues of myelin origin, such as reticulon-4 (NOGO), myelin associated glycoprotein (MAG), oligodendrocyte myelin glycoprotein (OMgp) and erythropoietin-producing hepatoma-amplified sequence (Eph)/ephrins were the best known and most intensively studied neurite outgrowth inhibitors (Schnell and Schwab, 1990; McKerracher et al., 1994; Mukhopadyay et al., 1994; Wang et al., 2002; Irizarry-Ramírez et al., 2005; Silver and Miller, 2004). However, previous study showed that NOGO, MAG, and OMgp play minor role in neural regeneration after SCI (Liu et al., 2006). Therefore, it is important to understand the molecular role underlying Eph receptor in SCI to develop novel treatment strategies to improve better clinical outcomes for this disease.

The Eph receptors are a large family of receptor tyrosine kinases comprising eight EphA and six EphB receptors in humans. The distinction between EphA and B receptors is based on the similarity within each group

*Corresponding author. E-mail: ganglv514@yahoo.com.cn. Tel: +86-0416-467-3886. Fax: +86-0416-467-3528.

of the extracellular domain sequences and on the affinity for binding ephrin-A and -B ligands. Thus, EphA receptors bind to the ligands termed ephrin-A1, -A2, -A3, -A4, and -A5 which are anchored on cell membrane through glycosylphosphatidylinositol, whereas EphB receptors bind to the ligands termed ephrin-B1, -B2, and -B3, which are transmembrane molecules (Pasquale, 2010). This unique feature has been shown to play a critical role in establishing topologically organized neuronal connections in many regions of the developing nervous system (Du et al., 2007). Targeted delivery of imaging agents, such as chemotherapeutic drugs or siRNA which inhibit the expression of high levels of certain Eph/ephrin family members also offer medical promise for diagnosis or therapy.

RNAi therapy for diseases of the central nervous system (CNS) may be delivered by chemically synthesized small interfering RNAs (siRNAs) or by viral vectors carrying genes that synthesize short hairpin RNAs (shRNAs). Therapeutic efficacy on neurological diseases has been demonstrated in animal models for neuropathic pain using directly delivered siRNAs (Dorn et al., 2004) and for spinal cerebellar ataxia 1 (SCA1) (Xia et al., 2004), Huntington disease (Harper et al., 2005) and amyotrophic lateral sclerosis, using viral vector-delivered siRNAs (Ralph et al., 2005; Raoul et al., 2005). However, spinal cord injury using siRNA treatment has not been reported. Therefore, we investigated the effects of intraparenchymal administration of recombinant lentiviral expressing vectors expressing an active EphB3 siRNA sequence into the spinal cord of adult rats on the expression levels of EphB3 mRNA and protein expression levels. We focused on the effects of EphB3 gene knockdown on the locomotion function recovery after SCI.

MATERIALS AND METHODS

siRNAs design and conduct

Three different siRNAs against EphB3 were designed as suggested by Elbashir et al. (2002). Sequences of the siRNAs used in this study are summarized in Table 1. An additional scrambled sequence was also designed as a negative control (NC) (Table 1). Replication deficient, self-inactivating lentiviral expressing vectors pGCSIL-GFP (Shanghai Gene Kaiji, China) were generated as follows. The cDNAs corresponding to the three siRNAs and NC were subcloned into the replication-deficient, self-inactivating lentiviral expression vector pGCSIL-RNAi-GFP (Shanghai Gene Chem, China). The resulting recombinant lentiviral vectors (LV) were designated as LV-siRNA 1, LV-siRNA 2, LV-siRNA 3 and LV-NC. To produce lentivirus, the 293T cells were transfected with 20 µg of eight pGCSIL-GFP-Eph receptor plasmid together with 15 µg of pHelper1.0 and 10 µg of pHelper2.0 packaging plasmids (Coleman et al., 2003). The culture medium was collected 48 h after transfection, concentrated by ultracentrifugation, aliquoted, and stored at -80°C until used. The titer of lentivirus was determined by hole-by-dilution titer assay (Deglon et al., 2000). Four days after a single exposure of 293T cells to the lentivirus, strong green fluorescence was shown in more than 90% of cells, indicating a high and stable transduction of the LV system. The final titer of pFU-shRNA1, pFU-shRNA-2, pFU-shRNA-3 and pFU-NC were 4×10^8, 5×10^8, 4×108 and 1×10^9 TU/ml, respectively.

Animal preparations

Fifty adult female Sprague-Dawley(SD) rats (200 to 240 g) were obtained from Liaoning Medical University Laboratory (Liaoning, China), and were kept at 24±1°C on a 12 h light-dark cycle and were given free access to laboratory chow and water. Following the International Association for the Study of Pain (IASP) guidelines for pain research in animals, all the animals studied were approved by the Animal Care and Use Committee at the Liaoning Medical University and were in accordance with the University's guidelines for the care and use of laboratory animals.

Spinal cord injury and virus injection

SD rats were anesthetized with a cocktail of 40 mg/kg Ketamine, 4 mg/kg Xylazine, and 0.9 mg/kg Acepromazine administered by intraperitoneal injection. A dorsal incision was made to expose T10 vertebra and a laminectomy was performed, leaving the spinal segment exposed. After exposure of the T10 segment by laminectomy, animals received a moderate contusion using the New York University (NYU) impactor that provides a contusion of 12.5 g/cm as previously described (Gruner, 1992; Miranda et al., 1999; Irizarry-Ramírez et al., 2005). Fifty SD rats of SCI were randomly divided into LV-siRNA 1, LV-siRNA 2, LV-siRNA 3, LV-NC and PBS group (n=10 in each group). Virus (5 µl) was administered 3 days after SCI, which was flushed by using 10 µl of PBS. Then, each group rats were administered to deliver with PBS, LV-NC, or LV-siRNA 1, LV-siRNA 2, LV-siRNA 3 plus PBS in a total volume of 15 µl, respectively. The T10-11 lumbar segment of the spinal cord was removed four week after administration, respectively. The protein and mRNA expression of EphB3 receptor in the spinal cord was measured by western blot analysis and quantitative reverse transcription polymerase chain reaction (qRT-PCR), respectively.

Real-time quantitative PCR

Total RNA was extracted using total RNA isolation reagent (Invitrogen, CA, USA) and 2 µg of RNA was reverse-transcribed in a 10 µl reaction using random primers and Transcriptor First Strand Synthesis Kit (Takara, Japan), both in accordance with the manufacturer's instructions. Amplification mixture (20 µl) contained 4 µl of cDNA, 5 µl of primers and 11 µl of Ex Taq SYBER Premix (Takara, Japan). The amplification was performed at 95°C for 2 min, followed by 40 cycles of 95°C for 30 s, 60°C for 1 min. All real time-PCR were performed in triplicate to ensure quantitative accuracy. PCR was performed on ABI 7500HT instrument and data were analyzed based on the $2^{-\Delta\Delta CT}$ method with normalization software. Primers utilized for the RT-PCR were as follows: EphB3, sense prime: 5′- ACTCAGCCTGGAGCCTGTCTAC-3′ and anti-sense prime: 5′- CGATCTGAGGGTAAAGCACGTA -3′; GAPDH, sense prime: 5′-TGG AGA AAC CTG CCA AGT ATG A-3′ and anti-sense prime, 5′-TGG AAG AAT GGG AGT TGC TGT-3′.

Western blot analysis

Lumbar spinal enlargements (L10–11) were removed and homogenized in sodium dodecyl sulfate (SDS) sample buffer containing a protease inhibitor cocktail (Sigma, USA). Protein samples were separated on an 8% SDS-polyacrylamide gel and transferred onto NC membrane (Millipore, USA). After being blocked in Tris-buffered saline (TBS) containing 5% skim milk and

Table 1. siRNA sequences used in the present study.

siRNA1: cggcaGAGGGTGGTTACGTGCTTTCTCGAGAAAGCACGTAACCACCCTCtgTTTTTg
siRNA2: CcgggaCCGGCTAGATCTACTTTGTCTCGAGACAAAGTAGATCTAGCCGGtcTTTTTg
siRNA3: CcgggaTCCCACCACGATTACTACACTCGAGTGTAGTAATCGTGGTGGGAtcTTTTTg
Negative:CcggTTCTCCGAACGTGTCACGTTTCAAGAGAACGTGACACGTTCGGAGAATTTTTg

0.1% Tween 20 for 2 h at room temperature, membranes were incubated overnight at 4°C in the primary rabbit monoclonal anti-EphB3 (1:500, Takara, Japan). Membranes were then washed and incubated with secondary antibody (1:2000, Santa Cruz Biotechnology, CA, USA) for 1 h. After extensively washing, the protein bands were visualized by an enhanced chemiluminescence assay (Millipore, German) following the manufacturer's instructions.

Analysis of locomotion function

Rats' spontaneous open-field locomotion was evaluated using the 22-point (0 to 21) Basso-Beattie-Bresnahan rating scale (BBB) (Basso et al., 1995, 1996). Briefly, the animals were allowed to acclimatize to the open-field environment during several sessions before testing. Subsequently, two double-blinded and trained observers assessed the locomotive function, joint movement, paw placement and rotation, coordination, and tail and trunk position and stability for 4 min. In this scale, a completely paralyzed rat scores 0, a rat with increasing joint movements but without weight support scores between 1 and 8, a rat with abnormal locomotion but with weight-supported steps (plantar or dorsal) and graded coordination patterns scores between 9 and 20, and a normal (and sham) rat scores 21.

Statistical analysis

All data are expressed as mean ± standard error of mean (SEM). Statistical analysis between two samples was performed using Student t test. Statistical comparison of more than two groups was performed using one-way analysis of variance (ANOVA) followed by a Tukey post hoc test. The significance of any differences in behavioral data of different experimental groups was assessed using two-way ANOVA. $P<0.05$ was considered statistically significant.

RESULTS

LV transduction of shEphB3 into neurons *in vivo*

LV-shEphB3 injection induced strong fluorescence in a large numbers of cell bodies (Figure 1). There was no green fluorescence in the PBS group. Fluorescence appeared primarily in the cytoplasm, indicating a successful infection. On the other hand, no cell death or significant morphology change was shown had little toxic effect on cells.

Down-regulation of EphB3 mRNA expression by siRNA

Real time-PCR was performed 28 days after injection to evaluate the level of EphB3 mRNA expressions. Our results showed that no significant inhibition in EphB3 mRNA expression was found *in vivo* treated either by LV-NC. In contrast, LV-siRNA-1, LV-siRNA-2 and LV-siRNA-3, treatment induced 85.8 79.6 and 90.4% reduction, respectively, in EphB3 mRNA ($P<0.01$, Figure 2), which indicated that most EphB3 mRNA were degraded by EphB3-siRNA in rats *vivo*.

Down-regulation of EphB3 protein expression by siRNA

The effect of EphB3 siRNA treatment on protein expression was assessed by western blotting analysis. As shown in Figure 3, there was no significant inhibition in EphB3 protein expression *in vivo* treated by LV-NC ($P>0.05$), while the band density decreased dramatically in the LV-siRNA-1, LV-siRNA-2 and LV-siRNA-3 as compared with the LV-NC ($P<0.01$). These results demonstrated that siRNA targeting EphB3 significantly silenced EphB3 protein expression in rats *in vivo* ($P<0.01$).

Effects of siRNA on hindlimb recovery during SCI

The BBB locomotor grading scale was used to assess the effects of EphB3-siRNA on the locomotive behavior of injured rats. Two-way ANOVA followed by Bonferroni post-hoc testing after employing this approach demonstrated very significant differences in locomotor behavior between LV-siRNA-1, LV-siRNA-2 and LV-siRNA-3 and the LV-NC ($P<0.01$) groups (Table 2). Our data transformation and analysis revealed improved BBB scores in the LV-siRNA-1, LV-siRNA-2 and LV-siRNA-3 compared with the LV-NC ($P<0.01$) group, which showed that EphB3-siRNA can increase BBB scores in some extent.

DISCUSSION

SCI triggers the re-expression of inhibitory molecules present in early stages of development, contributing to prevention of axonal regeneration. Eph receptor and Eph, was the best known and most intensively studied neurite outgrowth inhibitory molecules (Irizarry-Ramírez et al., 2005; Silver and Miller, 2004). The roles of the Eph/ephrin

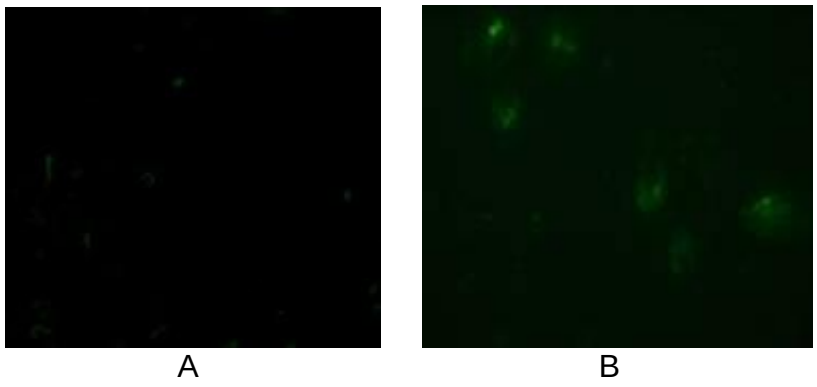

Figure 1. Fluorescence microscopy images of neurons after infection with LV-shEphB3 in spinal cord cells of rats (20 X). Untransfected group are shown as a negative control. No green fluorescence is seen in the control. A: Untransfected LV-shEphB3; B: Transfected LV-shEphB3.

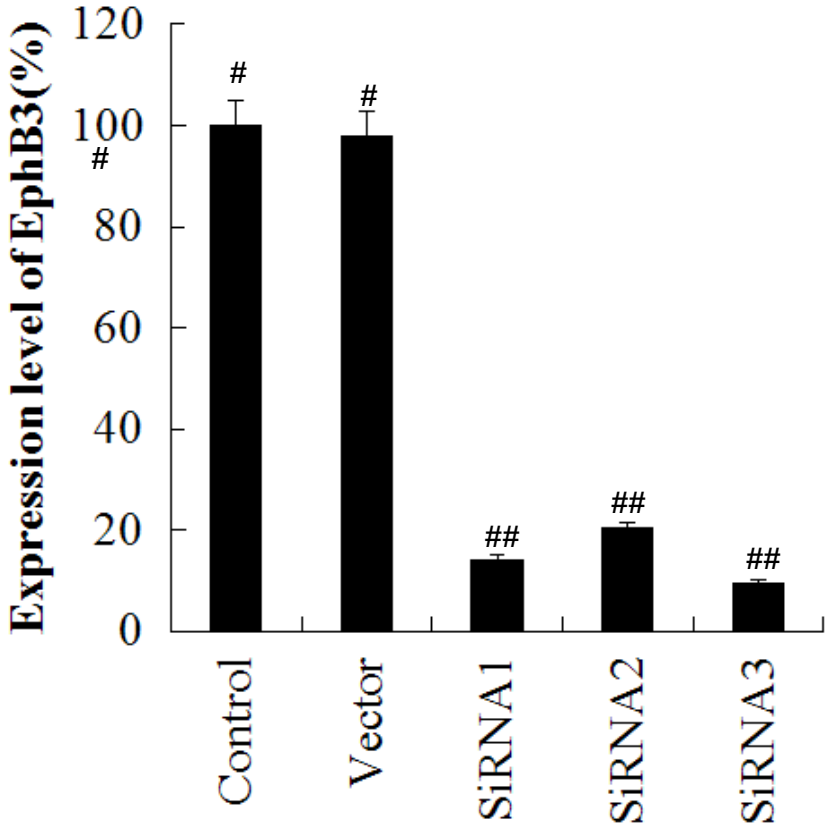

Figure 2. siRNA inhibited the EphB3 mRNA expression in spine tissue. The images are representative results from three independent experiments. Different marks represent the significant difference at $P<0.05$.

system in stem cell proliferation and differentiation could also be exploited in regenerative medicine (Genander and Frisen, 2010). Furthermore, interfering with the Eph system in the nervous system may also be useful to treat diseases where excessive extracellular levels of the neurotransmitter glutamate cause hyperexcitability or toxicity (Carmona et al., 2009). Therefore, in this study, RNA interference strategies was used to reduce the expression of EphB3 in rats *vivo*, and to evaluate the role of EphB3 in hindlimb recovery, the results showed that

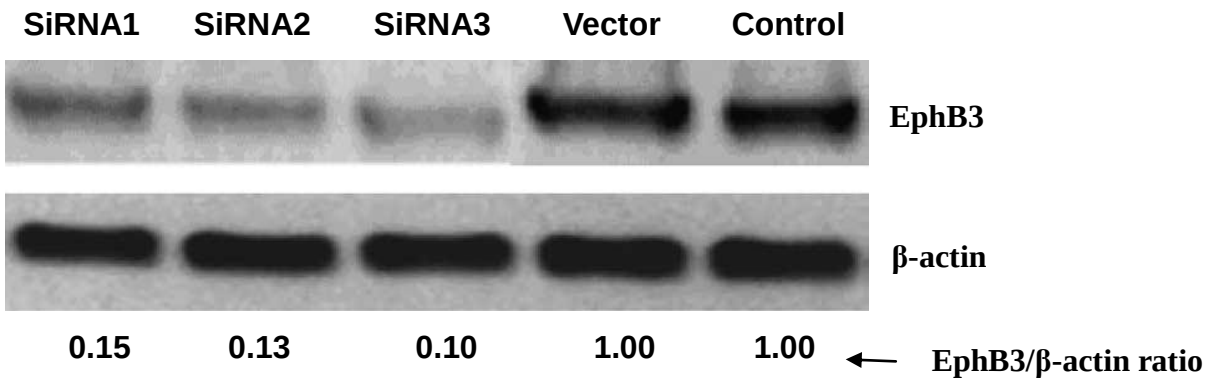

Figure 3. Effect of siRNA on EphB3 protein expression in spine tissue. The expression of β-actin was used as an internal control.

Table 2. BBB score in limb of rats 4 weeks after SiRNA treatment. Different marks represent the significant difference at $P < 0.05$.

Group	BBB scores
SiRNA1	$15.17 \pm 0.84^{\Delta}$
SiRNA2	$15.68 \pm 0.67^{\Delta}$
SiRNA3	$15.48 \pm 0.90^{\Delta}$
Vector	$12.64 \pm 0.96^{\#}$
Control	$11.98 \pm 0.69^{\#}$

down-regulation of EphB3 increased BBB scores and help hindlimb recovery. These results further demonstrated that inhibition of EphB3 expression *in vivo* can help health in SCI.

Key properties of an ideal gene delivery tool are the fact that it should be safe (including low toxicity), stable, cell type specific, and could be a marker (Song and Yang, 2010). Viral vector-mediated RNAi has already been shown to be effective in inhibiting gene expression in a number of diseases (Couto and High, 2010). Viral-mediated gene transfer is currently believed to be the most efficient system for delivering therapeutic proteins *in vivo* (Adriaansen et al., 2006; Vervoordeldonk et al., 2008). LV also have a relatively limited host-inflammatory response and potential to yield sustained (in theory life-long) gene silencing (Manjunath et al., 2009). In our study, we observed high levels of green fluorescent protein (GFP) in spinal cord cells, suggesting the successful infection of the recombinant LV *in vivo*. These studies also showed a significant down-regulation of EphB3 and *in vivo* after transfecting LV-shEphB3. When the LV-siRNA was used *in vivo* in the SCI model in rats after administration, BBB score was increased. Furthermore, the general behavior of rats, such as weight, intake of food and water and reactive ability of the rats were observed after LV-shRNA administration during the whole study. These results suggest a potential clinical use of shRNA expression vectors as a gene therapy approach to SCI.

Conclusively, the EphB3 knockdown does not only result in a decrease of the mRNA and protein expression, but also help to increase BBB scores. Management and LV delivery strategy could be a suitable approach for future studies on gene functions of EphB3. This pre-clinical study demonstrates the use of RNAi to target the expression of genes mediating pain and the therapeutic potential of this approach.

ACKNOWLEDGEMENTS

This work was supported by Science and Technology Research and Innovation Team funded by Liaoning provincial (LT2010062) and the National Natural Science Foundation of Liaoning provincial (201202142).

REFERENCES

Adriaansen J, Vervoordeldonk MJ, Tak PP (2006). Gene therapy as a therapeutic approach for the treatment of rheumatoid arthritis: innovative vectors and therapeutic.Opin. Cell. Biol. 22:611-616.

Basso DM, Beattie MS, Bresnahan JC (1995). A sensitive and reliable locomotor rating scale for open field testing in rats. J. Neurotrauma 12:1-21.

Basso DM, Beattie MS, Bresnahan JC (1996). Graded histological and locomotor outcomes after spinal cord contusion using the NYU weight-drop device versus transection. Exp. Neurol. 139:244-256.

Carmona MA, Murai KK, Wang L, Roberts AJ, Pasquale EB (2009). Glial ephrin-A3 regulates mhippocampal dendritic spine morphology and glutamate transport. Proc. Natl. Acad. Sci. USA. 106:12524-12529.

Coleman JE, Huentelman MJ, Kasparov S, Metcalfe (2003). Efficient large-scale production and concentration of HIV-1-based lentiviral vectors for use *in vivo*. Physiol. Genomics 12:221-228.

Couto LB, High KA (2010). Viral vector-mediated RNA interference. Curr. Opin. Pharmacol. 10:534-542.

Deglon N, Tseng JL, Aebischer P (2000). Self-inactivating lentiviral vectors with enhanced transgene expression as potential gene transfer system in Parkinson's disease. Hum. Gene Ther. 11:179-190.

Dorn G, Patel S, Wotherspoon G (2004). siRNA relieves chronic neuropathic pain. Nucl. Acids Res.32:e49.

Du J, Fu C, Sretavan DW (2007). Eph/ephrin signaling as a potential therapeutic target after central nervous system injury. Curr. Pharm.

Des. 13:2507-2518.
Emine AK, Bulent B, Sebnem K , Kamer K (2012). Neuroprotective effects of racemic ketamine and (S)-ketamine on spinal cord injury in rat. Injury (In press).
Genander M, Frisen J (2010). Ephrins and Eph receptors in stem cells and cancer. Curr. genes. Rheumatol. 45:656-668.
Harper SQ, Staber PD, He X (2005). RNA interference improves motor and neuropathological abnormalities in a Huntington's disease mouse model. Proc. Nat. Acad. Sci. 102:5820-5825.
Irizarry-Ramírez M, Willson CA, Cruz-Orengo L, Figueroa J (2005). Upregulation of EphA3 receptor after spinal cord injury. J. Neurotrauma 22(8):929-935.
Liu X, Hawkes E, Ishimaru T, Tran T (2006). EphB3: an endogenous mediator of adult axonal plasticity and regrowth after CNS injury. J. Neurosci. 26(12):3087-3101.
Manjunath N, Wu H, Subramanya S, Shankar P (2009). Lentiviral delivery of short hairpin RNAs. Adv. Drug Deliv. Rev. 61:32-45.
McKerracher L, David S, Jackson JL (1994). Identification of myelin-associated glycoprotein as a major myelinderived inhibitor of neurite growth. Neuron. 13(4):805-811.
Mukhopadyay G, Doherty P, Walsh FS (1994). A novel role for myelin-associated glycoprotein as an inhibitor of axonal regeneration. Neuron. 13(3):757-767.
Pasquale EB (2010). Eph receptors and ephrins in cancer: bidirectional signalling and beyond. Nat. Rev. Cancer 10:165-180.
Ralph GS, Radcliffe PA, Azzouz M (2005). Silencing mutant SOD1 using RNAi protects against neurodegeneration and extends survival in an ALS model. Nat. Med. 11:429-433.
Raoul C, Abbas-Terki T, Aebischer P(2005). Lentiviral-mediated silencing of SOD1 through RNA interference retards disease onset and progression in a mouse model of ALS. Nat. Med. 11:423-428.
Schnell L, Schwab ME (1990). Axonal regeneration in the rat spinal cord produced by an antibody against myelin-associated neurite growth inhibitors. Nature 343(6252):269-272.
Silver J, Miller JH (2004). Regeneration beyond the glial scar. Nat. Rev. Neurosci. 5:146-156.
Song H, Yang PC (2010). Construction of shRNA lentiviral vector. North Am. J. Med. Sci. 2:598-601
Vervoordeldonk MJ, Aalbers C, Tak PP (2008). Advances in local targeted gene therapy for arthritis: towards clinical reality. Future. Rheumatol. 3:307-309.
Wang KC, Kaprovica V, Kim JA (2002). Oligodendrocyte-myelin glycoprotein is a NOGO receptor ligand that inhibits neurite outgrowth. Nature 417(6892):941-944.
Xia H, Mao Q, Eliason S, Davidson BL (2004). RNAi suppresses polyglutamine-induced neurodegeneration in a model of spinocerebellar ataxia. Nat. Med. 10:816-820.