Int J Clin Exp Pathol 2015;8(6):7028-7033
www.ijcep.com /ISSN:1936-2625/IJCEP0008427

Original Article

Expression of exogenetic enhanced green fluorescent protein in rat endocranium through lentivirus infection

Qi Zhang*, Qiang Li*, Li Li, Zhaolong Zhang, Yina Wu, Yi Xu

*Department of Neurosurgery, Second Military Medical University, Changhai Hospital, Shanghai, China. *Equal contributors.*

Received March 25, 2015; Accepted May 20, 2015; Epub June 1, 2015; Published June 15, 2015

Abstract: The study aims to investigate whether exogenetic green fluorescent protein is able to express in the endocranium of rats, and to establish a method for further study in exogenetic gene knock-in or gene overexpression. Forty female Sprague Dawley (SD) rats were randomly divided into 4 groups with 10 in each: low and high dose groups, treated with 10% and 100% EGFP-lentivirus, respectively; negative control group, treated with virus enhancer; sham group, treated with normal saline. Seven days later, half rats' brain tissues were perfusion fixed and fresh brain tissues were obtained from the rest after euthanasia in each group. Immunohistochemical analysis, Western blotting and RT-PCR were respectively performed to detect the site where EGFP expressed and its levels. Immunohistochemical analysis demonstrated that EGFP was successfully expressed in brain tissue of those rats infected with EGFP-lentivirus. Both Western blotting and RT-PCR showed that EGFP was expressed after treatment with EGFP-lentivirus, and the expression level increased with the dosage of the vector. Exogenetic EGFP gene can express in brain tissue of the rat, which laid a solid foundation for future studies in exogenetic gene knock in or gene overexpression.

Keywords: Lentivirus, Infection, enhanced green fluorescent protein (EGFP), exogenetic gene

Introduction

The endocranium is the basal, inner part of the cranium, and it also refers to the outer layer of the dura mater in human anatomy [1]. A dural arteriovenous fistula (DAVF) is a vascular anomaly caused by an abnormal connection between a meningeal artery and a meningeal vein or dural venous sinus [2]. Many factors, including sinus thrombosis, head trauma, chronic central nervous system, hypercoagulable state, surgery, and hormonal influence can pre-dispose and initiate this disease. Although the reported incidence of intracranial DAVFs is approximately 10-15% of all intracranial vascular abnormalities, the true incidence may be much greater [3, 4]. Endovascular treatment is still, at present, one of the first line options for DAVF management [4].

Lentiviruses are a subclass of Retroviruses, which have a long incubation period [5]. They are able to deliver various viral RNA into the host DNA. But most of all, they have the unique ability among retroviruses to infect non-dividing cells as other Retroviruses can infect only dividing cells [5, 6]. So lentivirus infection is one of the most efficient methods for gene delivery, and they have been adapted as gene delivery vehicles for many years [7]. Generally speaking, DNA reverse-transcribed by the viral genome inserts into the genome at a random position, and such a unpredictable integration can lead to activation of oncogenes [8]. However, both *in vitro* and *in vivo* studies have proved that lentivirus infection has a less possibility to integrate in places that potentially cause cancer than gamma-retroviral vectors [9, 10]. What's more, lentivirus utilization to deliver gene therapy for treating HIV in clinical trials is not accompanied with any increase in mutagenic or oncologic events [8].

In this study, we focused our attention on the point whether lentivirus could carry exogenous and integrate into host genome and effectively express, hoping the positive results would be the basis for us to step forward to the further

study on the pathology and therapy of the pachymeningopathy.

Materials and methods

Experimental animals and grouping

Thirty female SD (Sprague Dawley) rats, which were purchased from Shanghai Laboratory Animal Center (Chinese Academy of Sciences, China) and weighed 210 ± 10 g, were randomly divided into 4 groups (n = 10): two experimental groups, including low dose and high dose groups, and a control group, namely sham group.

Experimental surgery

In experimental groups, the rats' superior sagittal sinus and the left transverse sinus were surgically blocked under anesthesia. After the right transverse sinus was isolated, different concentrations of EGFP-lentivirus were injected and the right transverse sinus was temporarily blocked for 30 min. While for sham group, we only blocked the rats' superior sagittal sinus and the left transverse sinus and then treated with saline.

Immunohistochemical analysis

The perfusion-fixed brain tissues were typically embedded with paraffin and then sliced into series of sections. After dewaxed, the paraffin sections were incubated with 3% H_2O_2 at room temperature for 10 min to inactivate the endogenous peroxidase. The sections were washed with distilled water and immerged in PBS for 5 min, after which (repeated twice), the samples were blocked with 5~10% normal goat serum (diluted in PBS) and incubated at room temperature for 10 min. The serum was discharged without washing and primary antibodies were added, which was followed by incubation at 37°C for 1~2 h. After the samples were washed with PBS for 5 min thrice, biotin-labeled secondary antibodies were dropwise added and incubated at 37°C for 10 to 30 min. Horseradish peroxidase-labeled streptavidin was subsequently added to the samples after they were washed with PBS again. The incubation at 37°C for 10~30 min and washing with PBS were repeated one more time. Color developing agent DAB (diaminobenzidine) was used for coloration for 3~15 min. Finally, the sections were sufficiently swashed with running water, counterstained, dehydrated, cleared (with xylene), mounted for the ultimate microscopic examination.

Western blotting

Upper tissues close to cortex in the brain were obtained from the fresh brain samples. The tissues were homogenized with appropriate amount of tissue lysis solution in an ice-bath until visible tissue block disappeared. The tissue homogenate was transferred to centrifuge tubes to prepare the lysate for 20 min. After centrifugation at a high speed, the supernatant was obtained and used for the following experiments. Before the analysis, total protein in the supernatant was quantified using BCA method. Same amount of total protein was added into loading buffer and it was brought to the same final volume with tissue lysate. The protein samples were separated with gel electrophoresis on a 10% polyacrylamide and subsequently transferred to nitrocellulose membrane. Following the block with skim milk, incubation with primary and secondary antibodies, electrochemiluminescence (ECL) was performed and scanned using an imaging system.

RNA extraction and RT-PCR

Upper tissues close to cortex in the brain were obtained and lysed in Trizol lysis buffer (Invitrogen SARL, Cergy Pontoise, France). The lysate was transferred to centrifuge tubes to obtain supernatant after centrifugation. Twenty percent volume of chloroform was added to the supernatant and evenly mixed with each other. After 3 min of standing, centrifugation was performed at 2~8°C under a force of 12,000 g for 15 min, and three layers were shown in the end. The upper layer (water phase) was transferred to an RNase-free centrifuge tube. Subsequently, 50% volume of isopropanol was evenly mixed in the solution and kept still for 10 min. The above mentioned centrifugation was repeated again to precipitate RNA. The RNA was washed with 75% ethyl alcohol, and the solution was vortex-mixed, following which, centrifugation under 7,500 g at 2~8°C was performed for 5 min. The residual ethyl alcohol was withdrawn, and the precipitate was dissolved in RNase-free water after it was dried in the air. The concentration and purity of the RNA were detected by measure the absorptions at 260 nm and 280 nm.

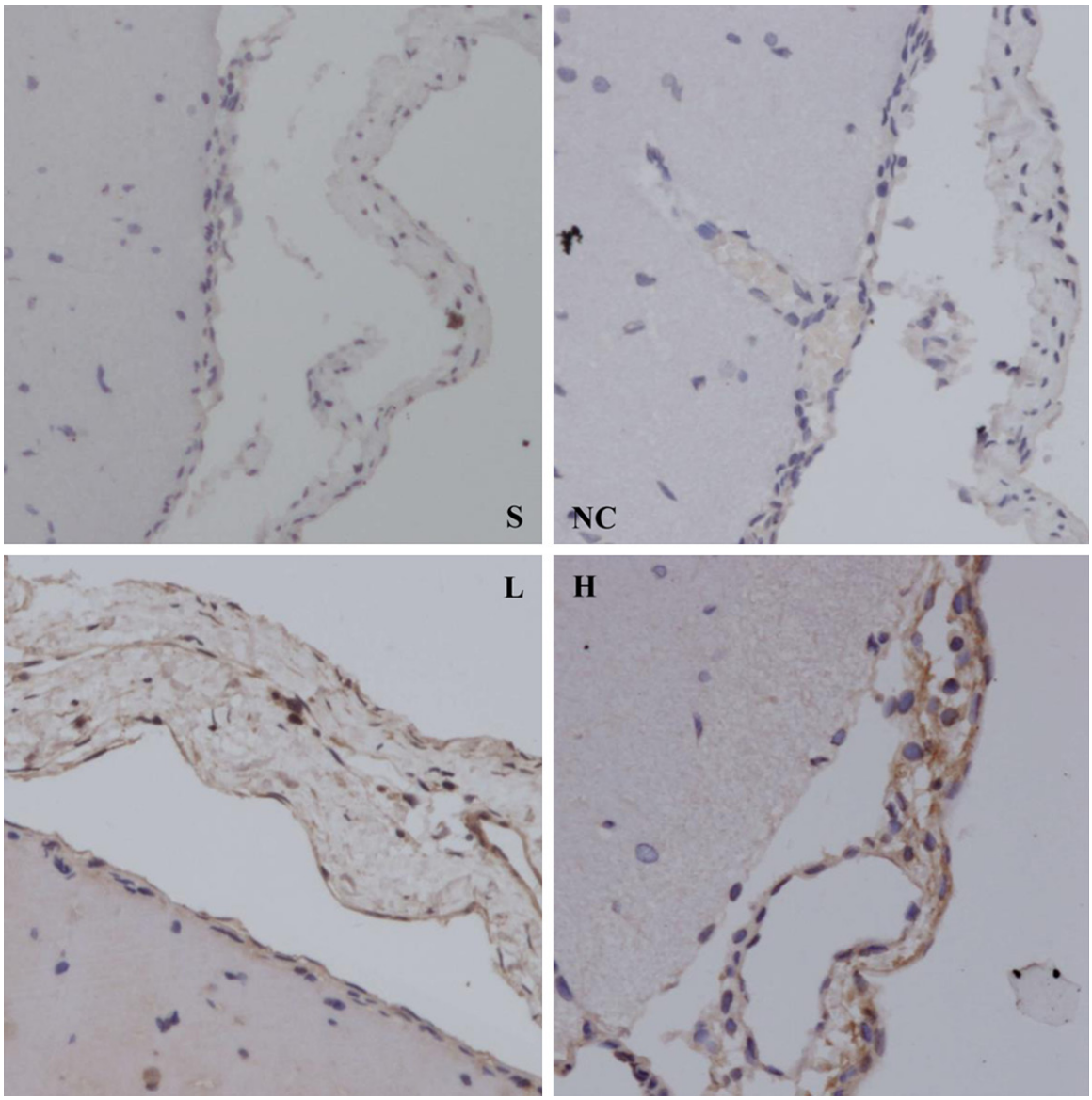

Figure 1. Immunohistochemical results of brain tissues of rats in different groups. The letters S, NC, L and H represent sham, negative control, low- and high-dose groups.

To get rid of genomic DNA, a reaction system was prepared with 500 ng total RNA and 4 μL 4×gDNA Wiper Mix, using RNase-free water to bring the final volume to 16 μL. The reaction was kept at 42°C. Two minutes later, 4 μL 5×qRT Super Mix II was added to the RNA sample. This new reaction system was kept at 25°C for 10, then 42°C for 30 min, and finally, 85°C for 5 min. For PCR, 7.5 μL 2×PCR Pre-Mix, 1 μL 100 μM forward primer (5'-AGGAGCGCACCATCTTCTT-3'), 1 μL 100 μM reverse primer (5'-CTCGTTGGGGTCTTTGCTCA-3') and 1 μL cDNA were mixed and diluted with RNase free water to 15 μL. The PCR conditions were: initial denaturation at 95°C for 5 min, after 35 recycles of degeneration (95°C, 30 s), annealing (58°C, 25 s) and extension (72°C, 30 s), extension at 72°C was performed for 3 min.

Statistical analysis

The gray intensity of each band in Western blotting and RT-PCR results was measured with Adobe Photoshop (Adobe Systems Inc., Mountain View, California, USA) three times, and all data were processed by DPS v6.55 software and expressed as mean ± SD (standard deviation). Differences were analyzed by stu-

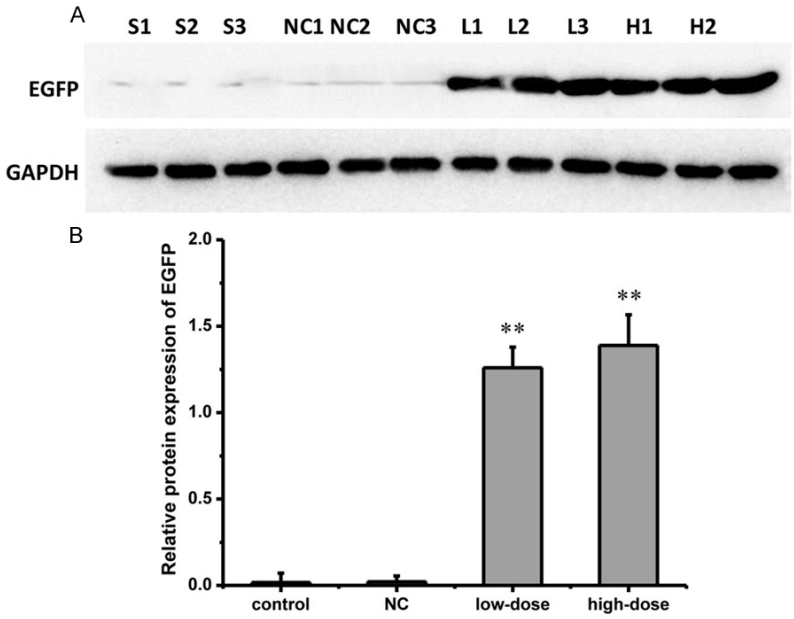

Figure 2. Protein level of EGFP in brain tissues of rats in different groups. A. Representative electrophoretograms of Western blot analysis. B. Semi-quantitative results of EGFP expression through analyzing the gray intensity. The letters S, NC, L and H represent sham, negative control, low- and high-dose groups, respectively. $**P < 0.01$, represented a very significant difference, compared with negative control.

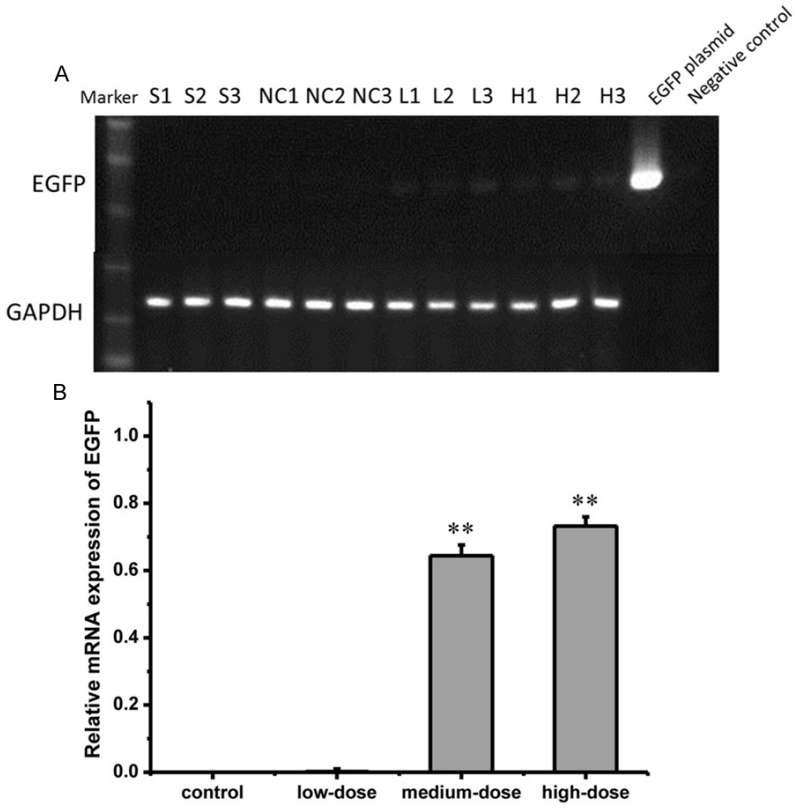

Figure 3. EGFP mRNA level detected by RT-PCR. A. The electrophoretogram of amplified EGFP mRNA. B. Semi-quantitative results of EGFP mRNA level. S, NC, L and H represent sham, negative control, low- and high-dose groups, respectively. $**P < 0.01$, represented a very significant difference, compared with negative control.

dent *t*-test and the significance was accepted at $P < 0.05$.

Results

EGFP expressed in the endocranium of brain

In control group, there was no positive stain shown in the endocranium of the rats (**Figure 1** S). While in all experimental groups, positive staining gradually became obvious with the increasing dosage of EGFP-lentivirus (**Figure 1** NC, L, H). These results indicated that exogenous EGFP could express in the endocranium of rats through EGFP-lentivirus infection.

Protein expression level of EGFP increased with the increasing dosage of EGFP-lentivirus

The results of Western blot analysis showed that EGFP expression couldn't be detected in sham or negative group, while in both lentivirus treatment groups, it significantly ($P < 0.01$) expressed in a dose dependent manner (**Figure 2**). These results suggested that lentivirus infection was a proper method to knock exogenous genes into heterogeneous organism.

MRNA level of EGFP increased with the dosage of EGFP-lentivirus

RT-PCR results at a deeper level validated the above results of Western blot

analysis, also consistent with which, EGFP significantly ($P < 0.01$) expressed in the endocranium in a dose dependent manner after EGFP-lentivirus infection (**Figure 3**).

Discussion

Green fluorescent protein (GFP) is composed of 238 amino acid residues (26.9 kDa) and exhibits green fluorescence after exposure to light in the blue to ultraviolet range [11]. GFP gene is frequently used as a reporter of expression in cell and molecular biology. Enhanced green fluorescent protein (EGFP) is one of the GFP derivatives, and it dramatically improved the spectral characteristics of GFP, increasing the practicality of use by the general researcher [12]. Presently, EGFP's co-packing (with target gene) into lentivirus is a popular method for gene knock in, and it has been widely used [13, 14].

In this study, we surgically injected with the lentivirus carrying EGFP into right transverse sinus, and kept it stay there for 30 min in order to make the target gene managed to express in the endocranium. The present study is a preliminary investigation into the feasibility of gene heterologous expression *in vivo* through lentivirus injection, which is expected as a basis of our following studies. Luckily, the immunohistochemical staining, Western blotting and RT-PCR analyses all pointed out that EGFP could expressed in the endocranium, and the expression level rose with the dosage of the lentivirus.

In conclusion, the exogenous gene EGFP successfully expressed in the endocranium of rat brain, which is a solid foundation for our future research about knocking in functional genes, including exogenous ones, into rats to investigate their potential efficacy to prevent pachymeningopathy, or making endogenous gene overexpress to discuss the potential mechanism of pachymeningopathy, like dural arteriovenous fistula.

Acknowledgements

This research was financially supported by the National Natural Science Foundation of China. (Grant No. 81200906).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Yi Xu, Department of Neurosurgery, Second Military Medical University, Changhai Hospital, 168 Changhai Road, Yangpu District, Shanghai 200000, China. Tel: +86-13801932229; E-mail: xuyi0307@126.com

References

[1] Stedman TL. The American heritage Stedman's medical dictionary. Houghton Mifflin Harcourt, 2004.

[2] Cooper CJ, Said S, Nunez A, Quansah R, Khalillullah S and Hernandez GT. Dural arteriovenous fistula discovered in patient presenting with recent head trauma. Am J Case Rep 2013; 14: 444.

[3] Luciani A, Houdart E, Mounayer C, Saint Maurice JP and Merland JJ. Spontaneous closure of dural arteriovenous fistulas: report of three cases and review of the literature. Am J Neuroradiol 2001; 22: 992-996.

[4] Gupta A and Periakaruppan A. Intracranial dural arteriovenous fistulas: a review. Indian J Radiol Imaging 2009; 19: 43.

[5] Kim VN, Mitrophanous K, Kingsman SM and Kingsman AJ. Minimal requirement for a lentivirus vector based on human immunodeficiency virus type 1. J Virol 1998; 72: 811-816.

[6] Naldini L, Blömer U, Gallay P, Ory D, Mulligan R, Gage FH, Verma IM and Trono D. In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. Science 1996; 272: 263-267.

[7] Park F, Ohashi K, Chiu W, Naldini L and Kay MA. Efficient lentiviral transduction of liver requires cell cycling in vivo. Nat Genet 2000; 24: 49-52.

[8] Bresler S. Introduction to molecular biology. Amsterdam, Netherland: Elsevier; 2012.

[9] Cattoglio C, Facchini G, Sartori D, Antonelli A, Miccio A, Cassani B, Schmidt M, von Kalle C, Howe S, Thrasher AJ, Aiuti A, Ferrari G, Recchia A and Mavilio F. Hot spots of retroviral integration in human CD34+ hematopoietic cells. Blood 2007; 110: 1770-1778.

[10] Montini E, Cesana D, Schmidt M, Sanvito F, Ponzoni M, Bartholomae C, Sergi Sergi L, Benedicenti F, Ambrosi A, Di Serio C, Doglioni C, von Kalle C and Naldini L. Hematopoietic stem cell gene transfer in a tumor-prone mouse model uncovers low genotoxicity of lentiviral vector integration. Nat Biotechnol 2006; 24: 687-696.

[11] Tsien RY. The green fluorescent protein. Annu Rev Biochem 1998; 67: 509-544.

[12] Heim R, Cubitt AB and Tsien RY. Improved green fluorescence. Nature 1995; 373: 663-664.

[13] Hosoda T, D'Amario D, Cabral-Da-Silva MC, Zheng H, Padin-Iruegas ME, Ogorek B, Ferreira-Martins J, Yasuzawa-Amano S, Amano K, Ide-Iwata N, Cheng W, Rota M, Urbanek K, Kajstura J, Anversa P, Leri A. Clonality of mouse and human cardiomyogenesis in vivo. Proc Natl Acad Sci U S A 2009; 106: 17169-17174.

[14] Seppen J, Barry SC, Harder B and Osborne WR. Lentivirus administration to rat muscle provides efficient sustained expression of erythropoietin. Blood 2001; 98: 594-596.