Neuroscience Research 63 (2009) 149–154

Contents lists available at ScienceDirect

Neuroscience Research

journal homepage: www.elsevier.com/locate/neures

Technical note

High-level transgene expression in neurons by lentivirus with Tet-Off system

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ARTICLE INFO

Article history:
Received 7 August 2008
Received in revised form 9 October 2008
Accepted 23 October 2008
Available online 6 November 2008

Keywords:
Lentivirus
Tet-Off
Neuron
High-level
Specific
In vivo

ABSTRACT

We developed novel lentiviral vectors by using "Tet-Off system" and succeeded in achieving high-level and neuron-specific gene transduction *in vivo*. One week after viral injection into the rat neostriatum, the GFP expression was almost completely neuron-specific and about 40 times higher than the expression of a conventional lentiviral vector. High transcriptional activity and neuronal specificity were sustained for up to 8 weeks. Furthermore, neuronal processes of the infected neurons were efficiently visualized by adding a plasma membrane-targeting signal to GFP. These results suggest that the present method is valuable for strong gene transduction and clear visualization of neurons *in vivo*.

1. Introduction

Recombinant viral vectors are now considered to be powerful tools for gene transfer in the field of basic and clinical neurosciences, since they can be directly delivered to any specific region of the animal brain at any time. Among the available viral vectors, lentiviral vectors offer unique advantages of stably integrating transgenes into the genome of mature neurons and of providing the basis for sustained gene expression. Lentiviral vectors, however, have a disadvantage in that transgene expression using them is generally weaker than the other viral vectors (Kanter-Schlifke et al., 2007; Wickersham et al., 2007), especially with cell type-specific promoters (Hioki et al., 2007). Thus, it has been assumed that lentiviral vectors are inappropriate for strong labeling of neurons and therefore unsuitable for *in vivo* imaging studies. Recent advances in optical imaging techniques and sophisticated transgenic technology enable the monitoring of neuronal activities (calcium increase, neurotransmitter release, or membrane depolarization), control of the electrical activities (photostimulation), and observation of structural dynamics of dendritic spines and axon terminals *in vivo* (Miesenbock and Kevrekidis, 2005; Misgeld and Kerschensteiner, 2006; Svoboda and Yasuda, 2006; Zhang et al., 2006). Studies using transgenic mice expressing fluorescent proteins under the control of a modified Thy1-promoter element have made huge contributions in this field (Feng et al., 2000). These mice express high levels of fluorescent proteins and label subsets of neurons as observed in Golgi staining without immunostaining. This allows the observation of structural dynamics of dendritic spines in pyramidal cells of the neocortex, *in vivo*, over periods ranging from minutes to months (Grutzendler et al., 2002; Trachtenberg et al., 2002). Although lentiviral vectors can infect a wide variety of species other than mice, cell type-specific gene expression using them is usually low because of the weak activity of the specific promoter. Thus, it is worthwhile to overcome the problem of low-level gene expression in these vectors, shorten the survival time for the sufficient expression, and apply them in *in vivo* imaging studies.

2. Materials and methods

The experiments were conducted in accordance with the Committee for Animal Care and Use of the Graduate School of Medicine at Kyoto University and that for Recombinant DNA Study in Kyoto University. All efforts were made to minimize animal suffering and the number of animals used.

2.1. Plasmids construction

The lentiviral vector was derived from human immunodeficiency virus 1 (HIV-1; Invitrogen, Carlsbad, CA), and constructed as follows. Human synapsin I (SYN) promoter (primer set PF1/PR1; see Supplementary table; Hioki et al., 2007), GFP

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doi:10.1016/j.neures.2008.10.010

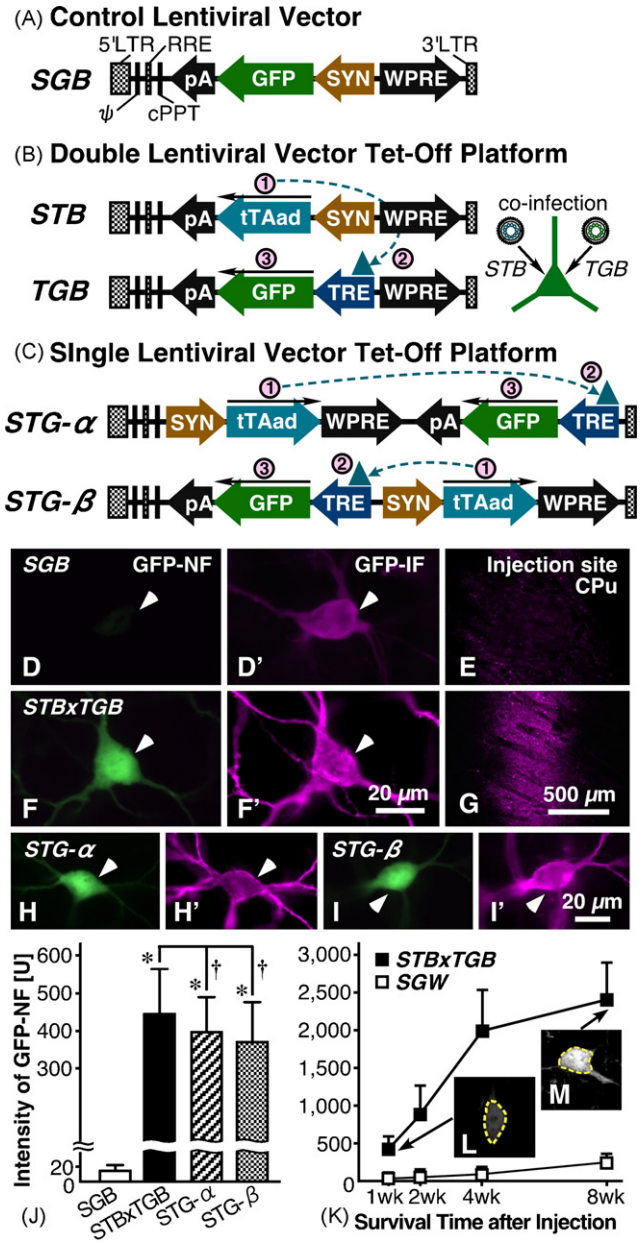

Fig. 1. High-level transgene expression of neostriatal neurons by lentiviral vectors using the "Tet-Off system." (A) Control vector *SGB* expresses GFP under the control of the human synapsin I (SYN) promoter. (B) "Double Lentiviral Vector Tet-Off Platform" is composed of 2 elements—regulator and response lentiviral vectors. The regulator vector, namely, *STB*, expresses an improved version of tetracycline-controlled transactivator (tTAad) under the control of the SYN promoter. The response vector, namely, *TGB*, produces GFP under a modified Tet-Response Element composite promoter (TRE-tight). The tTAad expressed in neuronal cells binds to TRE-tight, and strongly activates the transcription of GFP. (C) In "Single Lentiviral Vector Tet-Off Platform," the regulator and response elements are combined in a single lentiviral genome. In the present study, we designed 2 lentiviral vectors, namely, *STG-α* and *STG-β*. (D–I′) We injected 2.0 μl of *SGB*, a mixture of *STB* and *TGB* (*STB* × *TGB*) (1.0×10^8 TU/ml), *STG-α* (1.0×10^6 TU/ml), and *STG-β* (1.0×10^6 TU/ml) into the rat neostriatum. One week after the injections, we observed GFP-NF and GFP-IF labeled with AlexaFluor 594 under a fluorescence microscope. The arrowheads indicate GFP-expressing cell bodies. (J) One week after lentivirus injection into the rat neostriatum, we captured the digital images of GFP-NF by using a confocal laser-scanning microscope under identical conditions, and saved them as 12-bit TIFF files in a grayscale ($n = 30$ neurons). Then, we measured average intensity per pixel of GFP-NF in the soma [U] (L, M) by using the ImageJ software (http://rsb.info.nih.gov/ij), and performed a multiple comparison test by one-way ANOVA followed by Bonferroni's *post hoc* test. GFP production by *STB* × *TGB*, *STG-α*, and *STG-β* was dramatically increased by 42.6-, 29.2-, and 27.5-fold, respectively, as compared with the GFP production by *SGB* (*, $p < 0.001$). Furthermore, the GFP expression level when *STB* × *TGB* was used was significantly higher than that observed when *STG-α* or *STG-β* was used (†, $p < 0.001$). (K) We further examined the time course of GFP expression levels with *STB* × *TGB* from 1 to 8 weeks, by measuring average intensity per pixel of GFP-NF in soma [U] ($n = 30$ neurons). In the previous study (Hioki et al., 2007), we produced lentivirus coding SYN promoter, GFP and WPRE in the positive strand of the genome (*SGW*), and demonstrated that the expression level of GFP under the control of SYN promoter was directly proportional to the time course from 1 to 8 weeks. Average intensity per pixel of GFP-NF in the soma with *SGW* was re-calculated from the previous data (Hioki et al., 2007). Each symbol represents the mean ± S.D. cPPT, central polypurine tract; LTR, long terminal repeat; ψ, HIV-1 packaging signal; pA, a polyadenylation signal derived from the bovine growth hormone (BGH) gene; RRE, HIV-1 Rev response element; WPRE, woodchuck hepatitis virus posttranscriptional regulatory element. Scale bar = 500 μm (E, G); 20 μm (D–D′, F–F′); 20 μm (H–I′).

(Clontech, Palo Alto, CA; primer set PF2/PR2) and a polyadenylation signal derived from bovine growth hormone gene (BGHpA; primer set PF5/PR5) were amplified by polymerase chain reaction (PCR) and inserted into HincII, EcoRV and EcoRI/BamHI sites of pBluescript II SK (+) (pBSIISK; Stratagene, La Jolla, CA), respectively, and named as pBSIISK-SYN-GFP-BGHpA. SYN promoter (primer set PF1/PR1), an improved version of the tetracycline-controlled transactivator (tTAad; Urlinger et al., 2000) and BGHpA (primer set PF6/PR6) or woodchuck hepatitis virus posttranscriptional regulatory element (WPRE; primer set PF8/PR8; a gift from Dr. Hope TJ; Zufferey et al., 1999) were inserted into HincII, EcoRI/BamHI and SpeI/NotI or BamHI/NotI sites of pBSIISK, respectively, resulting in pBSIISK-SYN-tTAad-BGHpA or pBSIISK-SYN-tTAad-WPRE. GFP (primer set PF3/PR3) or GFP with a palmitoylation signal (palGFP; primer set PF4/PR3; Kameda et al., 2008) and BGHpA (primer set PF7/PR6) were inserted into BamHI/MluI and MluI/NotI sites of pTRE-Tight (Clontech), respectively, and named as pTRE-GFP-BGHpA or pTRE-palGFP-BGHpA. To generate a Gateway entry vector, a XhoI-to-BamHI fragment from pBSIISK-SYN-tTAad-BGHpA, a XhoI-to-NotI fragment from pBSIISK-SYN-tTAad-WPRE or a XhoI/EcoRV fragment from pTRE-GFP-BGHpA or pTRE-palGFP-BGHpA was inserted into the BamHI/XhoI, XhoI/NotI or DraI/XhoI sites of pENTR™1A (Invitrogen), respectively. We modified the destination vector, pLenti6/BLOCK-iT™-DEST (pLenti6; Invitrogen), by inserting central polypurine tract (cPPT; oligonucleotide set OF10/OR10) into Eco47III/HpaI sites of pLenti6. We further inserted oligonucleotide set OF15/OR15 or WPRE (primer set PF9/PR9) into XbaI/KpnI sites, resulting in pLenti6P or pLenti6PW. Then, the inserts from the entry vectors were transferred to the modified destination vector pLenti6PW by homologous recombination with LR clonase (Invitrogen), resulting in *SGB*, *STB*, *TGB* and *TpGB* (Fig. 1A and B). The fragments SYN-tTAad-WPRE and TRE-GFP-BGHpA were amplified by PCR from pBSIISK-SYN-tTAad-WPRE (primer set PF11/PR11 for *STG-α* or PF12/PR12 for *STG-β*) and pTRE-GFP-BGHpA (primer set PF13/PR13 for *STG-α* or PF14/PR14 for *STG-β*), and inserted into BamHI/EcoRV sites of pENTR™1A by using In-Fusion method (In-Fusion™ 2.0 PCR Cloning Kits; Clontech). Then, the inserts from the entry vectors were transferred to the modified destination vector pLenti6P, resulting in *STG-α* and *STG-β* (Fig. 1C).

2.2. Production and concentration of VSV-G pseudotyped lentivirus

Production of VSV-G pseudotyped lentivirus was performed according to the manufacturer's instructions (Invitrogen), with some modifications. The destination plasmid (*SGB*, *STB*, *TGB*, *TpGB*, *STG-α* or *STG-β*) was cotransfected with the mixture of the packaging plasmids (pLP1, pLP2 and pLP/VSVG; Invitrogen) into the 293FT producer cell line (Invitrogen), using Lipofectamine™ 2000 (Invitrogen). The medium was replaced at 8 h after transfection with UltraCULTURE medium (Lonza, Allendale, NJ) containing 4 mM L-glutamine (Invitrogen), 2 mM GlutaMAX (Invitrogen), 0.1 M Non-Essential Amino acids (Invitrogen), and 1 mM sodium pyruvate (Invitrogen). After 60 h from replacing the medium, the viral particles in the culture supernatant were collected, filtered through 0.45-μm filters (Millipore, Corning, NY) following low speed centrifugation (3000 × *g*, 15 min), and then concentrated with Amicon Ultra-15 Ultracel-100K (Millipore). This viral vector was replication-deficient and had the least chance for production of parent viral particles in the infected cells.

2.3. Titering lentiviral vectors

We added 10-fold serial dilutions of *SGB*, *STG-α*, or *STG-β* into the 293F cells. Since SYN promoter expresses transgene in the 293F cells (unpublished observation), we could observe GFP expression and determine the titers of *SGB* (3.3×10^9 transducing units/ml), *STG-α* (1.7×10^6 TU/ml), and *STG-β* (4.3×10^6 TU/ml). To measure the titers of *TGB* and *TpGB*, we prepared a new cell line with the Flp-In™ System (Invitrogen) according to the manufacturer's instructions. A HindIII/BamHI fragment from pBSIISK-SYN-tTAad-BGH was inserted into the HindIII/BamHI sites of pcDNA5/FRT (Invitrogen), resulting in pcDNA5/FRT/tTAad. The pcDNA5/FRT/tTAad was cotransfected with pOG44 (Invitrogen), Flp recombinase expression plasmid, into the Flp-In™ 293 cells (Invitrogen). By selection using hygromycin B, we generated the stable cell line expressing the tTAad under the control of CMV promoter. We added