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Coordinate Lentiviral Expression of Cre Recombinase and RFP/EGFP Mediated by FMDV 2A and Analysis of Cre Activity

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ABSTRACT

The site-specific recombination mediated by Cre recombinase has been utilized extensively in genetic engineering and gene function studies. Efficient delivery of a Cre enzyme with enzymatic activity and the ability to monitor the enzyme expression are required in applications, and lentiviral constructs with a fluorescent protein (FP) to report the Cre expression are suitable for most studies. However, the current lentiviral vector systems have some deficiencies in precise reporting the Cre expression through fluorescence. To solve the problem, we generated a lentiviral system with Cre and RFP or EGFP bridged by an FMDV 2A sequence in an open reading frame expressed by a CMV promoter. We then examined the capabilities of the constructs to package with VSVG into infectious virus and to mediate expression of the Cre enzyme and fluorescent reporter. Furthermore, we monitored the bioactivities of the expressed products. We demonstrated the coordinate expression of the enzyme and the reporter. The expressed Cre was efficient at removing LoxP-flanked fragments in cells and did not show obvious cellular toxicity, and the expressed FPs allowed direct observation under fluorescent microscope. Therefore, the conjugation of CMV-Cre-2A-FP represents a significant improvement to the current lentiviral Cre delivery systems for obtaining a required Cre activity while accurately monitoring its presence. Our study also provides information concerning application of the established vector system. J. Cell. Biochem. 113: 2909–2919, 2012. © 2012 Wiley Periodicals, Inc.

KEY WORDS: FMDV 2A; CRE RECOMBINASE; FLUORESCENT REPORTER; LENTIVIRAL VECTOR

Cre/LoxP is the most widely used molecular system for removing large portions of DNA in mammalian cells [Sauer and Henderson, 1988; Gu et al., 1994]. This system has been utilized extensively in genetic engineering for both in vitro and in vivo gene function studies [Sauer, 1993; Kuhn et al., 1995], and there is increasing interest in adapting this system to other types of research [Brault et al., 2007; Bordonaro, 2009; Wang, 2009; Oh-McGinnis et al., 2010]. A lentiviral vector system that coexpresses Cre and a fluorescent reporter is desirable for most Cre/lox applications. Such vector systems are currently available, but the fluorescent protein (FP) gene is either directly fused to or bridged by the IRES sequence to the *Cre* [Oh et al., 2003; Ahmed et al., 2004; Douin et al., 2004]. Direct fusion can impair the biological functions of the gene product [Casanova et al., 2002; de Felipe et al., 2006; Cronican et al., 2010] and may result in inconsistent levels of *EGFP* and *Cre* expression or expression only in a subset of cells [Ahmed et al., 2004]. With IRES-conjugation, the *Cre* and reporter genes can be expressed separately. However, the inconsistent translation of CAP-dependent and -independent genes often results in substantially lower expression of the second gene [Mizuguchi et al., 2000]. Therefore, the level of gene expression is defined by the IRES-driven translation of bicistronic mRNA with regard to the composition and arrangement of the gene [Hennecke et al., 2001]. Indeed, the fluorescent signal, that is, mediated by the IRES is often inefficient for many studies [Douin et al., 2004]. Therefore, it is often difficult to determine the presence and level of Cre expression by fluorescence with those vector systems.

Foot and mouth disease viruses (FMDV) belong to the family Picornaviridae. These viruses contain a single, long open reading frame encoding a polyprotein approximately 225 kDa in size. The polyprotein undergoes rapid intramolecular (*cis*) cleavage at the C-terminus of the 2A region [Ryan et al., 1991]. This self-cleavage, which has been found later being actually the result of a ribosomal-skip mechanism [Donnelly et al., 2001], is a unique property of the

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short 2A sequence (NFDLLKLAGDVESNPG), and the FMDV polyprotein context is not required for cleavage ("skip"). Therefore, the upstream and downstream sequences surrounding the 2A region can be completely replaced with heterologous genes [Ryan and Drew, 1994]. When used to link two genes in one ORF under a single promoter, the 2A peptide breaks at its C-terminus and separates into two genes with almost equimolar products [de Felipe et al., 2006]. Therefore, using FMDV 2A has obvious advantages over IRES to mediate coexpression of a second gene [Chinnasamy et al., 2006]. To date, the FMDV 2A or 2A-like sequences have been successfully used for coexpression of functional heterologous proteins in various cells and tissues [Furler et al., 2001; de Felipe et al., 2006; Hasegawa et al., 2007; Wang, 2009]. However, to our knowledge, it has not been exploited in a Cre-delivery lentiviral vector system.

In the present study, we generated a lentiviral vector system with Cre and RFP or EGFP along with an intervening FMDV 2A sequence. We then examined the capability of the constructs to package into infectious vector viruses and to mediate coexpression of the Cre enzyme and fluorescent reporter as well as the bioactivities of the coexpressed products. It appeared that coordinate expression of Cre with RFP or EGFP was achieved. The fluorescent signal was easily observed under a fluorescent microscope, and the Cre was able to effectively remove floxed fragments in the cells whether or not the fragments had integrated into the genome. Furthermore, Cre expression did not affect cell viability. Therefore, the vector system with the CMV-Cre-2A-FP conjugation has obvious advantages over the current Cre-delivery lentiviral systems in that it is able to deliver effectively required Cre activity with comparable molar level of functional FP to report its expression. In addition, we monitored the dynamic expression and action of the Cre enzyme mediated by the vector system, providing valuable information for the experimental design in studies using this or similar vector systems.

MATERIALS AND METHODS

CONSTRUCTION OF LENTIVIRAL VECTORS

High-fidelity PCR was performed using the Vent polymerase (NEB product, Cat. No.: M0254L) to amplify the target genes with specific primers containing an extra 5′-stretch designed to generate the end constructs. The ORF (open reading frame) of *Cre* contained an optimized codons was amplified from pcDNA 3.1-Cre plasmids (kindly provided by Professor Ouyang Hongsheng at Jilin University, China) and the ORFs for *RFP* and *EGFP* were amplified from the pPRIME-CMV-dsRed-FF3 and pPRIME-CMV-EGFP-FF3 plasmids (kindly provided by Professor Stephen J. Elledge at Harvard Medical School, USA). The reaction was prepared in a total volume of 25 μl containing 16.5 μl of ddH_2O, 2.5 μl of Vent buffer (10×), 0.8 μl of a dNTP mix (10 μM of each), 1 μl of template plasmid (20 ng), 2 μl of forward primer (10 pmol/μl), 2 μl of reverse primer (10 pmol/μl), and 0.2 μl of Vent polymerase (2 U/μl). The PCR program used was 94°C for 5 min followed by 30 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 1 min (for RFP/EGFP) or 3 min (for Cre) and a final step at 72°C for 10 min. Upon completion of amplification, an extra incubation step of 10 min at 72°C with two units of *Taq* polymerase (Fermentas product, Cat. No.: EP0071) was performed to obtain the A-overhang ends required for T/A cloning. The PCR products were examined and separated by electrophoresis on a 1% agarose gel. The target bands were then isolated with a gel extraction kit (OMEGA product, Cat. No.: D2500-02) and cloned into the pMD-18T simple vector (Takara product, Code No.: D103A) following the manufacturer's instructions. The DNA fragment for FMDV 2A was generated by annealing two of the synthesized oligos of the top and bottom strands based on the peptide sequence SRAPVKQTLNFDLLKLAGDVESNPGP [Ryan et al., 1991; Szymczak et al., 2004]. Briefly, the two oligos were mixed at a ratio of 1:1 in TE (pH 8.0) and heated at 95°C for 30 s to eliminate any secondary structures of the oligos. The mixture was then brought to 72°C for 2 min and 25°C for 2 min to anneal the two oligos into dsDNA. Upon completion, the reaction tube was chilled on ice or stored at 4°C until use. The primers and 2A oligos are listed in Table I; and the produced molecular clones are listed in Table II.

To prepare the lentiviral constructs, the verified the *Cre*, *RFP*, and *EGFP* clones in the pMD-18T-simple vectors were first digested with *Apa*I and *Not*I, respectively. The fragments containing *RFP* or *EGFP* were then ligated into the linearized *Cre*-containing vector to generate T-Cre-RFP/EGFP. Next, the FMDV 2A DNA was inserted into T-Cre-RFP/EGFP between the *Cre* and the *RFP* or *EGFP* to generate T-Cre-2A-RFP/EGFP. Finally, the Cre-2A-RFP/EGFP fragment was cutout with *Age*I and *Not*I and ligated into the lentiviral vector pPRIME-CMV-EGFP-FF3 that had been linearized with the same enzymes. This resulted in the creation of the lentiviral plasmids pPRIME-CMV-Cre-2A-RFP/EGFP. T4 DNA ligase (Fermentas product, Cat. No.: K1422) was used for the ligations. The molar ratio of insert to vector was 3:1, and the ligation reactions were performed at 22°C for 10 min.

LENTIVIRUS PRODUCTION AND TITRATION

All of the lentiviruses were generated in human kidney 293T cells (ATCC product) with transient transfection using PEI reagent (Sigma product, Cat. No.: 40872-7). Briefly, 293T cells were seeded onto 100-mm dishes with DMEM medium (HyClone product, Cat. No.: SH30022.01) containing 10% FBS (Invitrogen product, Cat. No.: 16000044) to obtain ~80% confluence on the second day. For each dish, a mixture of 12 μg of the main plasmid, 9 μg of PHR, and

TABLE I. Primers and 2A Oligos Used for Molecular Cloning

AgeI-Cre-*Xba*I-*Apa*I-*Not*I-F	5′-ACCGGTGCCACCATGGCCAATCTCCTGACCGT-3′
AgeI-Cre-*Xba*I-*Apa*I-*Not*I-R	5′-GCGGCCGCGGGCCCTCTAGAATCGCCATCTTCCAGCAG-3′
ApaI-dsRed-*Not*I-F	5′-GGGCCCATGGCCTCCTCCGAGGACG-3′
ApaI-dsRed-*Not*I-R	5′-GCGGCCGCCTACAGGAACAGGTGGTGG-3′
ApaI-EGFP-*Not*I-F	5′-GGGCCCATGGTGAGCAAGGGCGAGG-3′
ApaI-EGFP-*Not*I-R	5′-GCGGCCGCCTACTTGTACAGCTCGTCCA-3′
2Aoligo-F	5′-CTAGAGCCCCTGTGAAGCAGACCCTGAATTTCGATCTGCTGAAGCTGGCCGGCGACGTGGAGTCTAATCCTGGGCC-3′
2Aoligo-R	5′-CAGGATTAGACTCCACGTCGCCGGCCAGCTTCAGCAGATCGAATTCAGGGTCTGCTTCACAGGGGCT-3′

TABLE II. Molecular Clones Produced in This Study

Description	Sequences
T-AgeI-Cre-*Xba*I-*Apa*I-*Not*I	5′-ACCGGTCCACC-Cre ORF-TCTAGAGGCCCGCGGCCGC-3′
T-ApaI-EGFP-*Not*I	5′-GGGCCC-EGFP ORF-GCGGCCGC-3′
T-ApaI-RFP-*Not*I	5′-GGGCCC-RFP ORF-GCGGCCGC-3′
XbaI-2A-*Apa*I	5′CTAGAGCCCCTGTGAAGCAGACCCTGAATTTCGATCTGCTGAAGCTGGCCGGCGACGTGGAGTCTAATCCTGGGCC-3′

Note: All ORFs are described in the text; the flanking adapters sequence containing restriction sites are informed; T stands for pMD-18T-simple vector.

3.5 μg of VSVG was transfected into the cells with PEI. Eight hours after the transfection, the medium was replaced with DMEM containing 2% FBS and lentivirus-containing medium was collected every 24 h for 3×. Cellular debris was cleared by low-speed centrifugation and passage through a 0.45-μm filter (Millipore product, Cat. No.: SLHV033RB). The collected medium was concentrated by ultracentrifugation (90 min at 4°C, 75,000*g*, rotor JA30.50 from Beckman) and resuspension of the viral particle containing pellets into 200 μl of DMEM medium.

The titers of the pPRIME-CMV-Cre-2A-EGFP/RFP-FF3 and pLentiLox3.7-Neo-containing viruses were measured as follows: 8×10^4 HeLa cells (ATCC product) were plated in 24-well plates and infected with packaged vector virus in the presence of polybrene (8 μg/ml, CHEMICO product, Cat. No.: TR-1003-G). After 12–16 h, the medium was washed off, and fresh medium was added. Every infection was performed in three replicate wells, and the infectious unit (IU) was determined by the number of fluorescent cells at 48 h after infection.

QUANTIFICATION OF FLUORESCENT INTENSITY

293T cells 40 h after infection or transfection with the indicated vectors were used for analysis of fluorescent intensity by Fluorescence activated cell sorting (FACS) and colorimetric instruments respectively. For FACS analysis, a mixture of fresh medium and the medium from the packaging dishes (1:1) was used to infect the cells cultured in a 6-well plate; the harvested cells were washed twice with PBS and kept on ice. The cell sorting data were obtained from BECKMAN COULTER Epics XL and analyzed with software Flowjo.7.6.2. For colorimetric analysis, molar comparable amount of the unpackaged lentiviral constructs were co-transfected with of 0.2 μg of β-galactosidase (β-gal) expression plasmids into the cells cultured in 48-well plates. The transfection was performed with TurboFect (Fermentas product, Cat. No.: R0531), four wells repeats for each of the combinations. The cells were than lysed by adding 150 μl/well of RIPA Lysis reagent (Beyotime product, Cat. No.: P0013B) on ice for 15 min followed by 20 min centrifugal at 15,000*g*. The supernatants were then used for measurements of β-gal OD and fluorescence values. For the β-gal OD value, 20 μl of the supernatant samples were taken and added with 130 μl of ONPG (Thermo product, Cat. No.: 34055); and the $OD_{(405\ nm)}$ was then determined using an instrument of Thermo Multiskan MK3. For the fluorescence values, 50 μl of the supernatant samples were used, and the values were determined using an instrument of Thermo Varioskan. The emission and excitation light wavelength are 488 and 507 nm for the GFP, 558 and 583 nm for the RFP, respectively. The fluorescence values are then normalized by their corresponding β-gal OD values for the comparison.

ANALYSIS OF GENE EXPRESSION

Five hundred thousand 293T cells were seeded in each well of 6-well plates and were infected with the packaged vector virus (MOI of 2). Eight micrograms per milliliter of polybrene was added after adding the virus to improve the infection efficiency. The cells were collected two days later. For RT-PCR, the collected cells were homogenized with TRIzol (Invitrogen product, Cat. No.: 15596-018) for RNA isolation, cDNA was made using the ReverTra Ace®qPCR RT Kit (TOYOBO product, Cat. No.: FSQ-101). Normal PCRs was used to verify the messenger presence of the inserted genes and were performed as described in the cloning section except that the Vent and Vent buffer were replaced by *Taq* (1.5 U) and *Taq* buffer (Fermentas product, Cat. No.: EP0071), and the extension times were reduced to 40 s for RFP/GFP and 1 min for Cre. Real-time PCRs were used to compare messenger level and were performed with a BIO-RAD Mini Thermal Cycler using the SYBR Green qPCR Mix Regent (TOYOBO product, Cat. No.: QPS-201) under standard conditions. Fold changes relative to the house-keep gene β-actin were recorded and used for comparison. Primers for all targeted genes were designed to have a single, sharp peak in the melting curve analysis (Table III). For Western Blot, harvested cells were lysed in RIPE buffer at 4°C for 30 min and centrifuged at 15,000*g* for 15 min for the protein sediment. Twenty-five micrograms of proteins was loaded and Western Blotting was performed according to standard protocol. Monoclonal mouse anti-Cre antibody (Sigma, Cat. No.: C7988), monoclonal mouse anti-GFP antibody (Millipore, Cat. No.: MAB3580) or rabbit Polyclonal anti-RFP antibody (GeneTex, Cat. No.: GTX59862) were used to detect the target genes, and mouse anti-α-tubulin monoclonal antibody (Sigma, Cat. No.: T9026) was used to detect the human α-tubulin as internal control. Corresponding HRP-conjugated goat anti-rabbit IgG (Thermo Fisher Scientific, Cat. No.: SA1-9510) or HRP-conjugated goat anti-mouse IgG (Millipore, Cat. No.: AP308P) served as secondary antibody. Band density analysis was performed by using the software BandScan 5.0. For dynamic expression, cells were

TABLE III. Primers Designed for Quantitive PCR

Description	T_m (°C)	Amplifcation efficiency (%)	Sequences
β-actin-qPCR-F	60	100	CATGTACGTTGCTATCCAGGC
β-actin-qPCR-R	60		CTCCTTAATGTCACGCACGAT
GFP-qPCR-F	60	98.95	CGACGTAAACGGCCACAAGTTCAG
GFP-qPCR-R	60		CCGTAGGTCAGGGTGGTCACGAG
RFP-qPCR-F	60	98.60	GCCACTACCTGGTGGAGTTC
RFP-qPCR-R	60		TAGTCCTCGTTGTGGGAGGT
Cre-qPCR-F	60	100	GGGAGAATGCTCATCCACAT
Cre-qPCR-R	60		GGAAATCCATCTCTCGACCA

either transfected with the plasmids (2 μg/well, TurboFect) or infected with packaged viruses (MOI = 8) and split every 2 days after the treatments. Microscopic examination and photograph were performed before each splitting and the expression was evaluated by the fluorescent cells in contrast to the total cell numbers in the same scope.

DETERMINATION OF Cre ACTIVITY

Parallel cultures of 293 cells, including the stable 293 cell line that contains the pLentiLox3.7-Neo (green fluorescence) plasmid, were cultured in 12-well (4×10^5 cells/well) and 6-well plates (8×10^5 cells/well). The transfection and infection were performed as follows: (a) the "stable cells" were transfected with pPRIME-CMV-Cre-2A-RFP-FF3 plasmids (1 μg/well in the 12-well plate or 2 μg/well in the 6-well plate) for 18 h; (b) 293 cells were infected with pPRIME-CMV-Cre-2A-RFP-FF3 lentiviruses (MOI = 8) for 6 h and then transfected with the pLentiLox3.7-Neo plasmid (0.45 μg/well of 12-well plate or 0.9 μg/well of 6-well plate); and (c) 293 cells were cotransfected with the pPRIME-CMV-Cre-2A-RFP-FF3 plasmid (0.55 μg/well of 12-well plate or 1.1 μg/well of 6-well plate) and the pLentiLox3.7-Neo plasmid (0.45 or 0.9 μg/well). The cells were split every 2 days. The cells in the 12-well plate were used for images and counting of the cells; and half of the cells in the 6-well plate were collected for DNA isolation, which was used to determine whether the floxed CMV-GFP-Neo fragments were still present in the genomic DNA or in the pLentiLox3.7-Neo vector. The primer pair utilized in the PCRs for checking the Cre action were 5′-AGCTCGGCTACTCCCCTGCC-3′/5′-AACAGCCGAGCCCC-TGTCCA-3′; and the PCR program was 95°C at 4 min followed by 30 cycles of 30 s at 95°C, 30 s at 63°C and 3 min at 72°C, followed by a 7-min incubation at 72°C.

CELL SURVIVAL ASSAY

A stable HeLa cell line containing the pLentiLox3.7-Neo plasmid was used. Briefly, 4×10^5 cells/well were seeded onto a 12-well plate, three wells for each test and control treatments. The test wells were transfected with the pPRIME-CMV-Cre-2A-RFP plasmid and control wells with the pPRIME-CMV-dsRed-FF3 plasmid. The transfected cells were then subjected to G418 (500 μg/ml) selection. Each time the cells in the control wells reached about 90% confluence, the floating cells in both the test and control wells were carefully washed away with PBS. Cells for the control wells were split normally, and cells in the test wells were kept growing wish fresh medium added. Until day 14 when the floating cells in the test wells were not more than those in the control wells, the surviving cells in each well were counted for statistical analysis (*t*-tests).

RESULTS

GENERATION OF LENTIVIRAL VECTOR WITH Cre-2A-EGFP/RFP CONJUGATION AND EXAMINATION OF ITS PACKAGING ABILITY

With an aim to solve the problems in the current vector system for delivering a Cre recombinase and reporting its expression, we generated a lentiviral vector system with *Cre* and *RFP* or *EGFP* along with an intervening FMDV 2A through a multistep cloning procedure sequence (see Materials and Methods Section for detail). Restriction enzyme and sequencing verification confirmed successful in construction of the designed lentiviral plasmids. A primary transfection test showed the plasmids were able to mediate fluorescence that could be easily observed under microscope, confirming that the expressed fluorescent proteins were functional.

To examine the packaging ability of the constructs, we packaged the constructs with PHR and VSVG, which encodes the vesicular stomatitis virus envelope glycoprotein, by transfecting them into 293T cells. We took advantage of the fluorescent reporter to monitor the packaging process and used the EGFP-monocistronic construct (pPRIME-CMV-EGFP-FF3) as a control. Efficient transfection (>70%) was assured by optimization of the PEI reagents (Fig. 1a). The packaged vector viruses of all three constructs were equally infectious (Fig. 1b). The production of infectious vector viruses had an average titer of 4.6×10^6 IU/ml for the pPRIME-CMV-Cre-2A-RFP construct, 3×10^6 IU/ml for the pPRIME-CMV-Cre-2A-EGFP construct and 5×10^6 IU/ml for the control (Fig. 1c). Although there is a tendency of slight reduction of package titers, the resulting reduction in titers were not significant ($P > 0.05$, $n = 3$). Since the packaging efficiency can be reduced by the increased length of the inserts, the Cre-2A-FP conjugation appeared to have little adverse effects on lentiviral packaging; and as the titers of the tested vectors were of the same order of magnitude as titer of the control, we think the constructs was able to produce a sufficient amount of infectious virus to meet the requirements for most studies.

COORDINATE EXPRESSION OF Cre RECOMBINASE AND RFP/EGFP

In the packaging and titration experiments, we noticed that the fluorescence mediated by our constructs could be directly observed as that mediated by of the monocistronic constructs (Fig. 1). We then performed quantifications of fluorescent signals in the cells. FACS analysis of the cells infected with the vector viruses in the original packaging medium showed the fluorescent cells infected with the virus containing Cre-2A-EGFP/RFP vectors had an average fluorescence intensity (MFI) about 58% to the cells infected with the control vectors that express the same FP alone. However, due to the large dispersion of fluorescent intensity in the cells (standard deviations were near to or bigger than the mean value); the differences did not appear statistical significant (Fig. 2a); and it could not excluded that the lower infection rate, which may result in less copies of viruses in the infected cells, had enlarged the differences. To overcome the difficulties in getting comparable infection rate and repeat experiments for the FACS method, we conducted further measurements of the fluorescent intensity relative to the β-gal expression in the co-transected cells with colorimetric instruments. The experiments showed again reduced MFI for the bicistronic vectors. The relative fluorescence of the bicistronic vectors to the monocistronic vectors were 62% and 64% respectively for the EGFP and the RFP constructs; and the reduction of the fluorescent signals appeared statistical significant (Fig. 2b).

To gain further insight into the expression actions of the packed vector viruses, we examined further the Cre and FP expressions in the cells infected with packaged vector virus at RNA level by RT-PCRs and at protein level by Western blot. In the RT-PCRs, we first confirmed the presence of messenger both for the Cre and the FP in

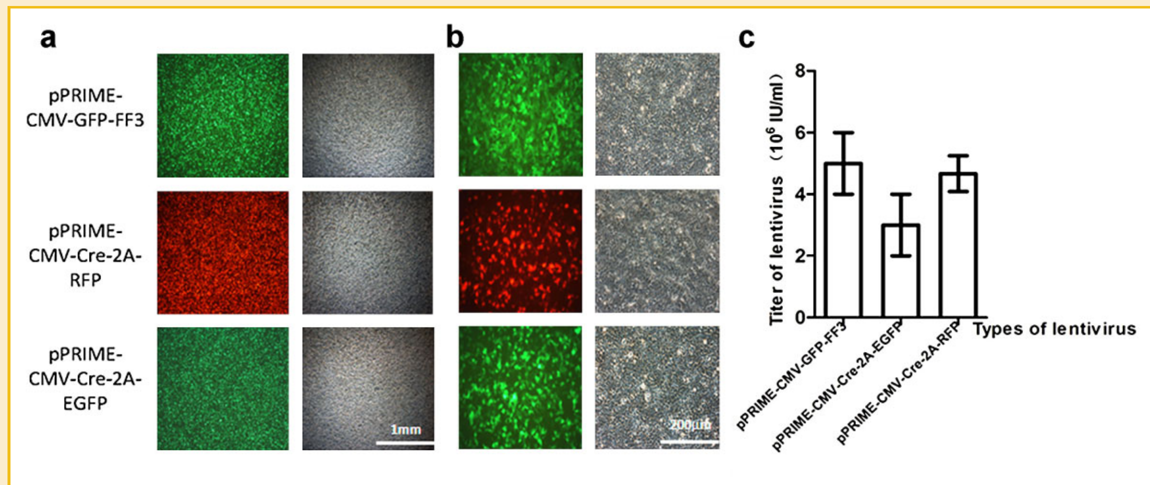

Fig. 1. Lentiviral packaging and titration of the packaged vector viruses. Fluorescence in panels (a) and (b) show respectively the transfection of the packaging cells (293T) performed using PEI reagents and infection of HeLa cells by the packaged vector viruses, which were utilized for the titration. The black and white photos show the total number of cells that were imaged in the left fluorescent panel. The photos were taken 40 h after transfection or infection. Column diagram (panel c) shows the titration results of the packaged vector virus titers ($P > 0.05$, n = 3).

cells infected with virus containing the bicistronic constructs by normal RT-PCRs (Fig. 3a). We then performed realtime RT-PCRs for quantification and comparison of messenger levels in cells infected with variant vector viruses. As a result (Fig. 3b), the average messenger levels of Cre and FP relative to the housekeeping gene β-actin were 0.3250 folds for EGFP of the CMV-EGFP construct, 0.3157 folds for Cre and 0.3129 folds for EGFP of the CMV-Cre-2A-EGFP, 0.2773 folds for RFP of the CMV-RFP, and 0.2747 folds for Cre and 0.2734 folds for RFP of the CMV-Cre-2A-RFP. Thus the bicistronic constructs have only a tendency of <4% decreases of messenger level relative to their monocistronic counterparts (2.43% for less RFP and 3.74% less for GFP); while the messenger levels of the Cre and FP from the same bicistronic constructs showed only <1% difference (0.50% less for RFP and 0.90% less for GFP), and non-statistical significance appeared for those differences. These results indicated that both Cre-2A-RFP and Cre-2A-EGFP were fully transcribed and were almost all transcribed as a bicistronic messenger, which assures equimolar transcription of Cre and FP. In the Western blot, we extended a little the colorization so as to identify target proteins of low concentration. As a result (Fig. 3c), while both the Cre and the FP proteins were well presented in separated bands, no fusion band of Cre-RFP or Cre-EGFP was detected, indicating the "cleavage" was rather complete; and probably because of the extended colorization, we did not detect neither significant decrease of FP production for the bicistronic constructs (<4% difference according to the band scan analysis). Since different antibodies were required for detection of the different products, we were not able to make quantitative comparison of the Cre and FP production. Altogether, these data showed that the bicistronic vectors allowed coordinate expression of *Cre* and *RFP* or *EGFP* with comparable levels and the fluorescent signal was strong enough to allow direct observation and imaging of transfected or infected cells, although the signal could be weaker comparing to monocistronic vectors.

DYNAMIC EXPRESSION OF THE CONSTRUCTED LENTIVIRAL VECTORS

To obtain information on the dynamic transgenic expressions of the vector system, which should be valuable for the design of studies using the vector system, we monitored and compared the fluorescence cells mediated by our vectors and a widely used commercial lentiviral vector pLentiLox3.7-Neo. Expressions of the transgenes were evaluated by the ratio or number of fluorescent cells in contrast to the total cells in the same wells of treatments. We find that the expressions among the repeat wells were quite constant at same time points with the same treatment and that the expressional dynamics of the all the vector types were highly comparable. Figure 4 included photos of cells in the experiment with pLentiLox3.7-Neo (green fluorescence) and pPRIM-CMV-CRE-RFP (red fluorescence), representing the typical expression patterns of the two vector types at given time points. It appeared that the fluorescent signal started to appear 12–18 h after transfection or infection and maintained high levels for 36–60 h, and then gradually lowered until day 6. A small percentage of the cells (up to 10–20%) maintained strong expression after day 6, which may be attributed to the stable expression from vectors integrated into the cell's genome. The result indicated also that infection with a sufficient titer well-packaged vector viruses was better than transfection to achieve a high ratio of transient and stable expression (Fig. 4c vs. a and d vs. b).

ANALYSIS OF ENZYMATIC ACTIVITY OF THE CRE RECOMBINASE

To examine the Cre activity mediated by the constructed lentiviral system, we performed cell assays to monitor the Cre actions in removing a floxed CMV-GFP-Neo fragment, which had been introduced into the 293 cells with the pLentiLox3.7-Neo vector. In one assay, a stably cell line (simplified as "stable cells" in the following text) that we had previously established with the pLentiLox3.7-Neo vector were used. In the other two assays, the

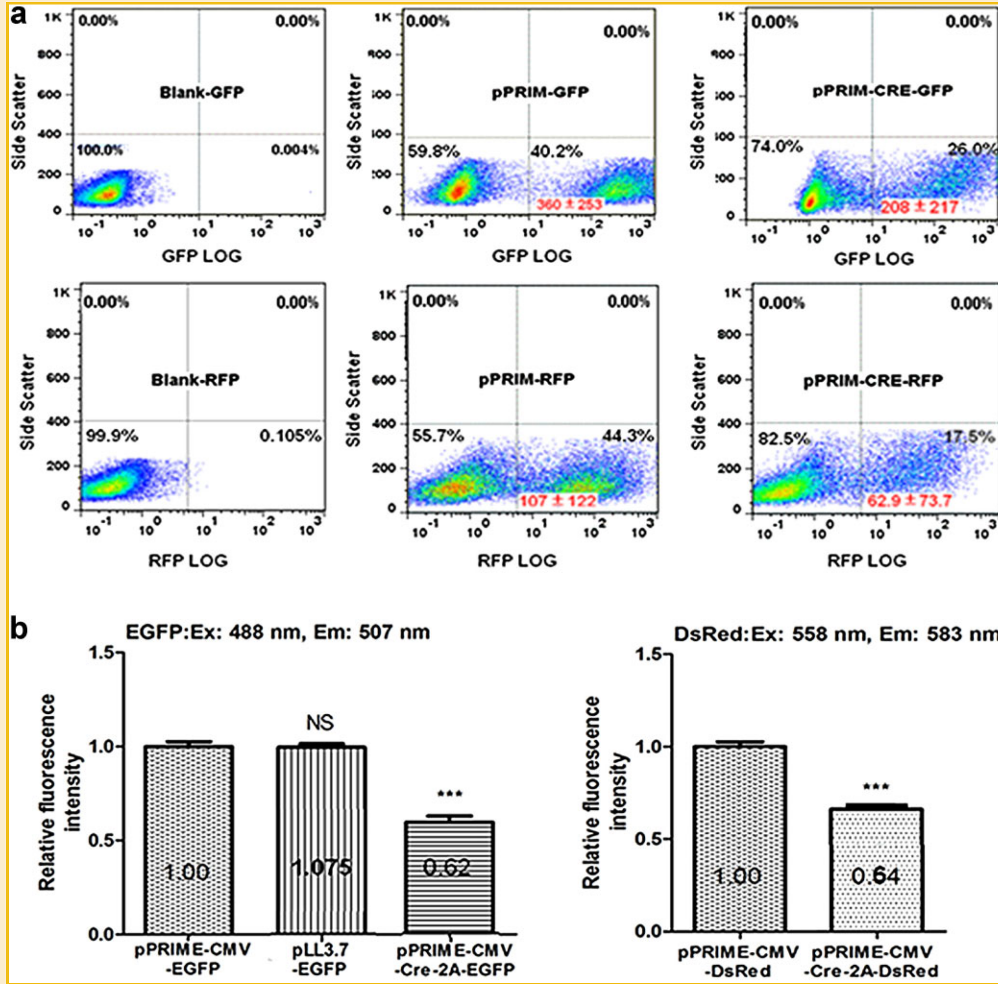

Fig. 2. Quantification of fluorescent intensity. 293T cells 40 h after infection or transfection with the indicated vectors were used for the analysis. a: FACS analysis of fluorescent intensity in infected cells with packaged vector viruses: the Blank-GFP and -RFP show the effectiveness of the gate-settings for the test samples as determined by the uninfected cells, percentage of the cells in each gate and the average fluorescent intensity and their standard deviation (highlighted in red) of the cells in the corresponding gates are indicated. b: Fluorescent intensity relative to the β-galactosidase expression in the co-transfected cells: the data from co-transfection with the bicistronic constructs were compared with data from the co-transfection with corresponding FP monocistronic constructs, which was set as "1" (*t*-test n = 4).

pLentiLox3.7-Neo was either transfected 12 h after infection with the pPRIME-CMV-Cre-2A-RFP virus or cotransfected with the pPRIME-CMV-Cre-2A-RFP plasmid. An obvious reduction of green fluorescence was observed, which indicated that the floxed GFP-Neo fragment had been excised (Fig. 5a–c). In the experiments with the stable cells, the green fluorescent signal was reduced in the majority of the cells 48 h after transfection and gradually disappeared over 6 days. Only a small portion of the cells maintained a strong green fluorescent signal comparable to that observed in the controls (Fig. 5a). In the other two experiments, when the pLentiLox3.7-Neo plasmids were transfected 12 h after infection with the pPRIME-CMV-Cre-2A-RFP-FF3 vector, GFP expression was almost completely blocked, and only a few scattered green fluorescent cells observed (Fig. 5b). When these two plasmids were cotransfected, the number of fluorescent cells was also greatly reduced and the fluorescence in most of the cells disappeared by 96 h after cotransfection (Fig. 5c). By counting the number of cells in the experiment with the stable cells, we found the percentage of the cells that maintained strong fluorescence corresponded to the transfection efficiency (Fig. 5d). Thus, this small portion of cells may have been present because they were not transfected, rather than failing to express Cre activity. To directly confirm the effectiveness of Cre activity, we also conducted PCR analysis of the deleted region of the floxed fragment at the DNA level. Total DNA was isolated from cells in parallel wells and used as template, and a pair of primers was designed and used to amplify a portion (about 3 kb) of the constructs containing the floxed fragment (about 2.2 kb). The PCR reactions were optimized to obtain comparable results for a 3- or 0.8-kb products representing the intact or Cre-excised fragments, respectively. As shown in Figure 5e, the PCR products from the DNA of the cells that were transfected or infected with the pLentiLox3.7-Neo plasmids alone contained only the 3-kb bands (Fig. 5e, lanes of controls). In the experiment with the stable cells, the 0.8-kb band was detected at day 2, and the intensity of the 3-kb band remained as

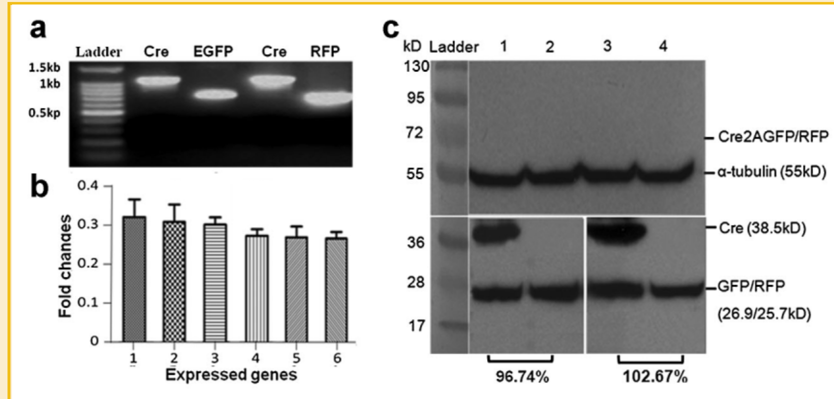

Fig. 3. Coordinate expression of Cre and RFP or EGFP in infected cells. a: Results of RT-PCR: lanes 2 and 3 were from total RNA samples of the 293T cells in that were infected with pPRIME-CMV-Cre-2A-EGFP-FF3; lanes 4 and 5, were from total RNA samples of the 293T cells in that were infected with the pPRIME-CMV-Cre-2A-RFP-FF3. b: Results of real-time RT-PCR: the tested genes included EGFP in mediated by the pPRIME-CMV-EGFP-FF3 (column 1), Cre (column 2), and EGFP (column 3) mediated by the pPRIME-CMV-Cre-2A-EGFP, RFP mediated by the pPRIME-CMV-dsRed-FF (column 4), Cre (column 5), and RFP (column 6) mediated by pPRIME-CMV-Cre-2A-dsRed; fold changes were relative to the housekeep gene β-actin. c: Results of Western blot: lanes 1 and 2 show the Cre and GFP protein expression mediated by the pPRIME-CMV-Cre-2A-EGFP-FF3 and the pPRIME-CMV-EGFP-FF3, respectively; lanes 3 and 4 show the Cre and RFP protein expression mediated by the pPRIME-CMV-EGFP-FF3 and the pPRIME-CMV-dsRed-FF3, respectively. The α-tubulin served as internal control and the position of the expected "uncleaveged" Cre-2A-GFP/RFP protein band was indicated. The top piece of the membrane was blotted with antibodies against Cre and against α-tubulin, and the bottom pieces of the membrane were blotted with antibodies against Cre and against GFP (left) or RFP (right). The percentages under the gel image were quantitative comparison of FP production between the bi-and monocistronic vectors, which were obtained from analysis with BandScan 5.0.

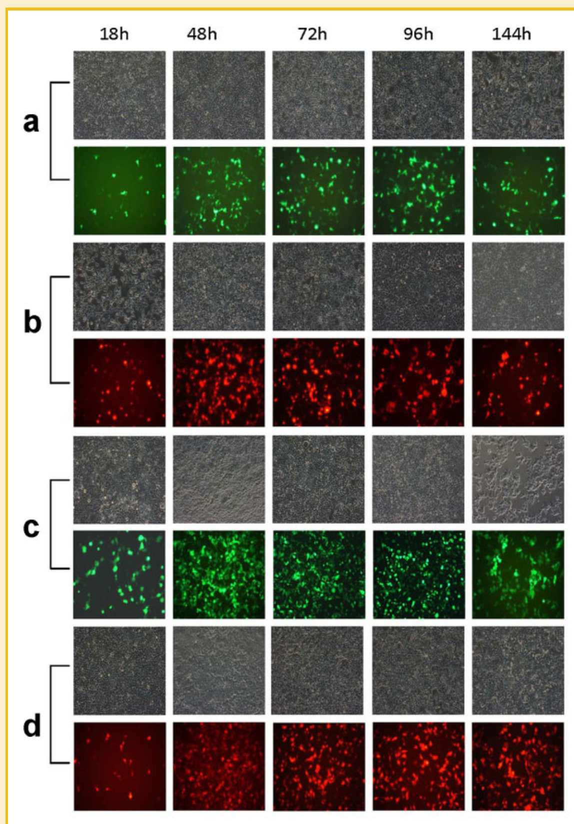

Fig. 4. Dynamic expression of the genes that were inserted into the constructs. Fluorescence indicates the expression mediated by transfection with the pLentiLox3.7-Neo (panel a) and pPRIME-CMV-Cre-2A-RFP-FF3 (panel b) plasmids and by infection with packaged virus containing pLentiLox3.7-Neo (panel c) or pPRIME-CMV-Cre-2A-RFP-FF3 (panel d). The black and white photos show the total number of cells that were imaged in the fluorescent panel.

strong as that of the band from the control cells. Overtime, the 0.8-kb band become more and more evident, and the 3-kb band reduced to a trace amount by day 6 (Fig. 5e, lanes 3–5). In the other two treatments, the 3-kb bands never appeared as strong as that from the controls and became indiscernible at day 4, whereas the 0.8-kb band reached its highest intensity level at day 2 and started growing fainter by days 2–3 (Fig. 5e, lanes 7–9 and lanes 11–13, respectively). Although these PCRs could not precisely identify the molecular ratio of "Cre-excised" DNA, they demonstrated that 2 days after infection or transfection, the constructs were able to express sufficient levels of functional Cre to remove the fragments surrounded by LoxP sites in cells with or without integrated vector.

In the above cell assays, we did not see obvious decreases of cell viability. Previous studied has revealed that the toxic effects of Cre were not evident until 9 days after introduction of the expression constructs [Loonstra et al., 2001; Silver and Livingston, 2001]. We had maintained culturing of the transfected and infected cells (including 293, 293T, and HeLa cells) up to 4 weeks; but still we had not detected any reduction in cell viability (data not shown).

Because the expression vectors did not affect cell viability, we were able to evaluate the efficiency of Cre activity more precisely through a cell survival assay utilizing the *Neo* gene surrounded by LoxP sites (floxed). In the assay, stable HeLa cells containing the pLentiLox3.7-Neo vector were transfected with the pPRIME-CMV-Cre-2A-RFP or pPRIME-CMV-dsRed-FF3 control, and the cells were than subjected to G418 selection for 14 days (Fig. 6a). The survived cells in the test and control wells were then counted and compared to determine the efficiency of Cre activity. As a result, an average of 20.02×10^6 and 6.47×10^6 cells were obtained from the starting control and test wells, respectively ($P < 0.001$, n = 3; Fig. 6b). Taking into account of the transfection efficiency of the Cre-delivery vectors (71%). The Cre activity from our constructs was

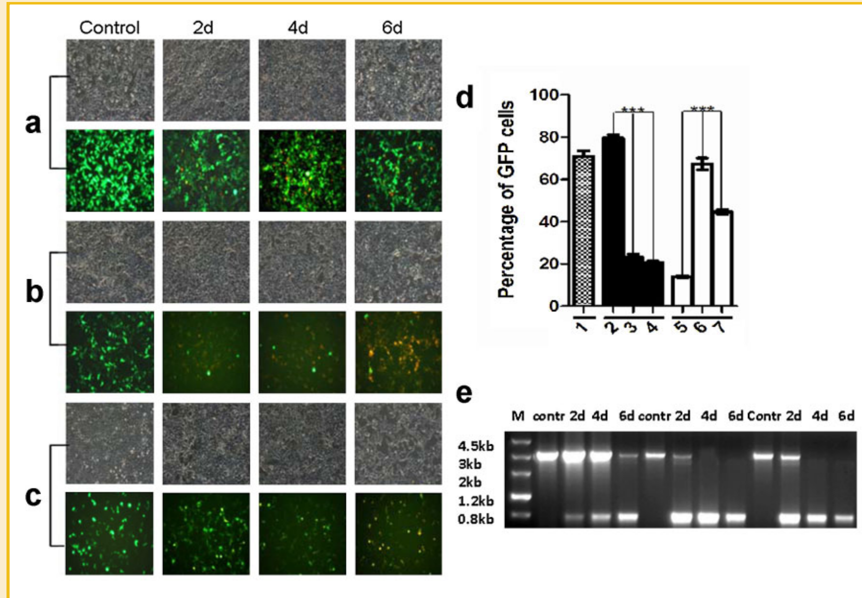

Fig. 5. Effectiveness of Cre in removing the GFP and Neo fragments surrounded by LoxP sites. a: 293 cells from a stable cell clone containing integrated pLentiLox3.7-Neo transfected with pPRIME-CMV-Cre-2A-RFP-FF3; (b) 293 cells transfected with pLentiLox3.7-Neo 12 h after infection with pPRIME-CMV-Cre-2A-RFP-FF3; (c) 293 cells that were cotransfected with pLentiLox3.7-Neo and pPRIME-CMV-Cre-2A-RFP-FF3. The hours indicate the time points after the last transfection. The controls in panel (a) was a photo from the test wells before transfection, and the control in (b) and (c) are photos from parallel cultures that were respectively infected and transfected with pLentiLox3.7-Neo alone on day 2. The black and white photos showed all cells that were imaged in the fluorescent panels. d: Quantification of GFP cells with treatment of (a): 1, efficiency of transfection with pPRIME-CMV-Cre-2A-RFP-FF3; 2–4, percentage of cells with a strong fluorescent signal; 5–7, percentage of cells with a weak fluorescent signal. e: PCR amplification of the DNA region containing the floxed fragment from parallel cultures to those presented in the images: lanes 2–5, 6–9, and 10–13 are from parallel cultures to those presented in panel (a), (b), and (c), respectively.

estimated to have a function of removing floxed fragment from the genome in 95.3% cells.

DISCUSSION

In addition to the well-known "conditional knockout," the Cre/LoxP molecular system is also used in complex manipulations of DNA, such as chromosome engineering and genomic imprinting [Brault et al., 2007; Oh-McGinnis et al., 2010] as well as in more confined inactivation of target genes in a cell- or tissue-specific manner at a definite time [Ray et al., 2000; Lee et al., 2006; Nagy et al., 2009; Speck and Iruela-Arispe, 2009]. To allow direct quantification of Cre expression after transduction, we generated a lentiviral vector system containing a CMV-Cre-2A-FP vector and demonstrated that

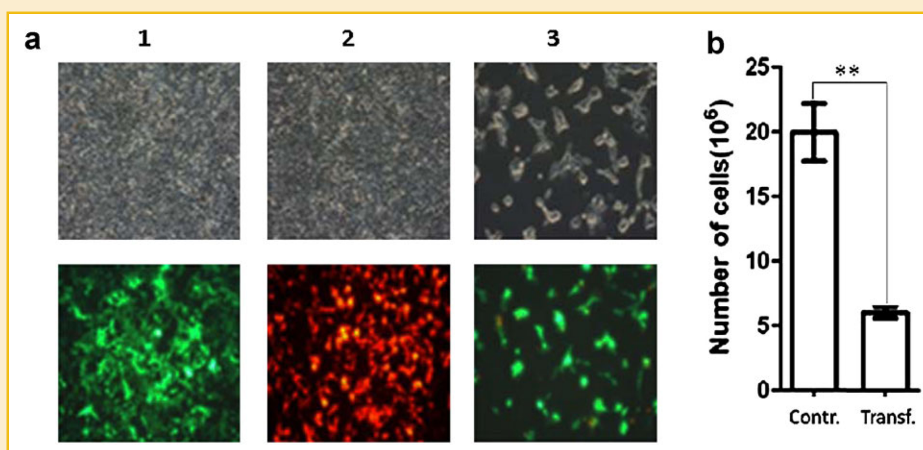

Fig. 6. Cell viability assay after Cre expression. The pLentiLox3.7-Neo stably transfected HeLa cells were used for this assay. a: Images of the cultured cell: panel 1 showed the survived cells in a well that was split from the starting control well (transfected with pPRIME-CMV-dsRed-FF3); panel 2 showed the effective transfection of the cells in the test well (transfected with pPRIME-CMV-Cre-2A-RFP-FF3); and panel 3 showed the survived cells in a test well (transfected with pPRIME-CMV-Cre-2A-RFP-FF3). b: The average number of total surviving cells from the starting control wells and the test wells at the end of the assay ($n = 4$, *t*-test)

the vector mediated coexpression of Cre with enzymatic activity and a fluorescent reporter that allowed the direct observation and quantification of Cre expression.

The FMDV 2A peptide is functional in cells from a wide variety of eukaryotes, including yeast, plants, insects, and mammals [de Felipe and Ryan, 2004]. The obvious advantages of using the 2A sequence as a linker between genes are its small size and its high efficiency in mediating cleavage (skip translation) of artificial polypeptides from a single promoter. This maximizes the ability of the vectors to express two inserted sequences with equimolar concentrations [de Felipe et al., 2006; Hasegawa et al., 2007]. Previously, the 2A peptide has been incorporated into adeno-associated [Furler et al., 2001], retroviral [de Felipe et al., 1999], and lentiviral vectors [Chinnasamy et al., 2006] to mediate coexpression of various genes. While most studies obtained the expected results, incomplete "cleavage" of 2A-mediated fusion products [Groot Bramel-Verheije et al., 2000; Furler et al., 2001; Milsom et al., 2004] or interference of the cleaved 2A peptides with protein function [Ansari et al., 2004; Lengler et al., 2005] had been detected in some cases. Therefore, using 2A to mediate coexpression with another protein can be construct-dependent, and experimental tests are required for each construct [Chinnasamy et al., 2006; de Felipe et al., 2006].

The Cre-2A-FP conjugate had been explored recently in a study using the adeno-associated viral vector system [Tang et al., 2009]. In that study, a modified 2A peptide from a *Thosea asigna* virus was used to bridge a codon-improved Cre recombinase (iCre) and EGFP, and expression of both iCre and FP was successful. The authors reasoned that the addition of four amino acids (APGS) to the C-terminal proline of the 2A sequence may increase the processing efficiency at the cleavage site by creating a constant environment. However, additional amino acids would attach to the final FP, which may not be suitable for all FPs; and the fluorescence had not been quantified and compared directly with single FP expression in that study. On the other hand, as reviewed by de Felipe et al. [2006], earlier studies with the FMDV 2A peptide demonstrated that the cleavage activity could be improved with longer versions of 2A that contain a few extra amino acids or a flexible linker at the N-terminus. In our study, we used a longer version of FMDV 2A containing a short N-terminal linker (SRA), which had been previously reported to mediate complete "cleavage" and stoichiometric production of two fluorescent proteins in multiple cell types [Donnelly et al., 2001; Szymczak et al., 2004]. We obtained full transcription of the bridged genes and rather "complete cleavage," which ensure separation of Cre and FP for their proper functions. Moreover, the fluorescence in cells was strong enough for direct observation and imaging although the MFI were about 2/3 of the monocistronic constructs according to our fluorescent quantifications. On the other hand, we had detected only a tendency of slight decrease of FP production (<4% comparing to the FP monocistronic constructs); and presumably, cleavage of the FMDV 2A would left only a single proline attachment to the N-terminus of the FPs, which is the minimal modification of FP structure for keeping its function and have not been reported to impair FP function. Therefore, the main reason for the lower MFI coming with bicistronic constructs was not identified in this study; probably it was a cumulative result from many factors involved in the experiments. However, it does not impede importantly the usability of the vectors.

Another problem with Cre usage is the toxicity on living cells [Loonstra et al., 2001; Pfeifer et al., 2001; Silver and Livingston, 2001]. Thus, it is crucial to have vector systems that mediate Cre activity and avoid the toxic effects. For this purpose, "self-excising" or "self-deleting" retroviral vectors had been constructed and shown to be successful [Pfeifer et al., 2001; Silver and Livingston, 2001]. However, such vector systems are not applicable for establishing stable Cre expression in cell lines or animals. On the other hand, there are reports of classically constructed vectors that have effective Cre activity without any adverse effects on cell viability both in vitro or in vivo [Kaspar et al., 2002; Ahmed et al., 2004; Tang et al., 2009]. In this study, we demonstrated that our constructs mediated functional expression of Cre and did not affect cell viability. The "no toxic effects" described in previous studies were mainly attributed to a low-level of Cre expression. It is possible that our bicistronic constructs had expressed lower level than monocistronic constructs as indicated by the fluorescent quantification. It is also possible that the Cre activity had been reduced by the attached 2A peptide, as the 2A peptide that remained on the C-terminal of the Cre protein has been reported to result in a slight reduction in enzymatic activity [Ma and Mitra, 2002; Ansari et al., 2004]. However, the influence of the 2A extension on Cre activity requires further investigation, and conjugations with different Cre versions and promoters need to be tested experimentally.

An obvious advantage of using the lentivirus as a vector is that it could be easily engineered to infect a wide variety of cell types with modifications to the envelop of the virus [Naldini and Verma, 2000]. In this study, we showed that the Cre-2A-FP conjugated lentiviral constructs were packaged effectively with the PHR and VSVG and produced infectious viruses. In our hands, the packaged viruses were able to effectively infect not only the 293, 293T, and HeLa cells (presented in Results Section) but also chicken cells including DF-1, DT40, embryonic fibroblasts, and fat precursor cells (Supplementary Fig. 1), as well as embryos (Supplementary Fig. 2). The fluorescent signal mediated by the infection was directly detectable in both in vitro cultured cells and in vivo embryos (Supplemental Figs. 1 and 2). The Cre expression could also effectively remove fragment flanked two Lox5171 sites, which is a mutant of LoxP that cannot recombine [Lee and Saito, 1998] (data not shown).

Finally, in this study we reported also the dynamic expression and action of the Cre enzyme mediated by the vector system, providing information concerning with application of the vector system.

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