Selection of an optimal promoter for
gene transfer in normal B cells

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Abstract. Gene transfer into normal quiescent human B cells is a challenging procedure. The present study aimed to investigate whether it is possible to increase the levels of transgene expression by using various types of promoters to drive the expression of selected genes-of-interest. To produce lentiviral particles, the present study used the 2nd generation psPAX2 packaging vector and the vesicular stomatitis virus -expressing envelope vector pMD2.G. Subsequently, lentiviral vectors were generated containing various promoters, including cytomegalovirus (CMV), elongation factor-1 alpha (EF1α) and spleen focus-forming virus (SFFV). The present study was unable to induce satisfactory transduction efficiency in quiescent normal B cells; however, infection of normal B cells with Epstein-Barr virus resulted in increased susceptibility to lentiviral transduction. In addition, the SFFV promoter resulted in a higher level of transgene expression compared with CMV or EF1α promoters. As a proof-of-concept that this approach allows for stable gene expression in normal B cells, the present study used bicistronic lentiviral vectors with genes encoding fluorescent reporter proteins, as well as X-box binding protein-1 and binding immunoglobulin protein.

Introduction

Gene transfer into normal cells serves an important role in basic studies aiming to elucidate the fundamental processes taking place in living organisms, as well as in gene therapy and immunotherapy of human diseases. In the field of immunotherapy there remains a requirement to develop and optimize gene transfer into primary target cells. However, the majority of basic studies are conducted with transformed cells, due to difficulties in achieving stable and efficient levels of gene expression in normal cells. Gene transfer into normal B cells is particularly difficult. Notably, B cells induced to express the antigen of interest can stimulate human antigen-specific cytotoxic T lymphocytes and are useful and convenient source of autologous antigen-presenting cells for examining human immune response. In addition, genetically modified B cells may be used to induce anti-tumor immune response, thus contribute to the development of novel strategies for treatment of cancer. Following gene transfer, B cells have additionally been successfully applied to induce tolerance and prevent autoimmune responses in animal models. Unfortunately, the use of nonviral methods for gene transfer into mature B cells is particularly ineffective, and retroviral transduction of quiescent mature B cells has not been reported in the literature. Therefore, lentiviruses have attracted significant interest, considering their ability to transduce nondividing cells (1,2). Vectors based on murine leukemia virus (MLV) have previously been used to transfer genes into transformed B cells or B-cell precursors (3). However, only 1-4% of B cells were transduced with these vectors, and they were revealed to be ineffective in gene transduction of mature quiescent B cells (4,5). Even with lentiviruses, quiescent B cells require prior stimulation with cytokines, cell surface molecules or other factors to stimulate entry from G₀ to G₁ phase of the cell cycle, in order to become transduced. This is achieved by various methods, including B-cell activation via cluster of differentiation (CD)40, with either antibodies or feeder cells expressing CD40 ligand (CD40L) (6); cytokines, such as interleukin (IL)-4 and IL-21; CpG oligonucleotides (5); anti-CD20 antibodies (7); infection with Epstein-Barr virus (EBV); or a combination of these approaches (4). A major breakthrough was associated with the generation of pseudotyped lentiviral vectors. Lentiviral vectors pseudotyped with glycoprotein from the vesicular stomatitis virus (VSVG) or envelope proteins from g-retroviruses, such as MLV, have been reported to result in broad tropism in the majority of human cells (8); however, they were particularly ineffective in the case of B cells (6). Previous studies have revealed that VSVG-lentiviruses require low-density lipoprotein-receptor, which is inducible in numerous activated cells, but not in B cells (9,10). Improved
lentiviral pseudotypes have been designed by incorporating measles virus (MV) glycoproteins F and H into their surface (MV-lentiviruses) (11-13). These vectors target normal B cells through MV receptors, such as CD46 or signaling lymphocytic activation molecule (14,15). Pseudotyped MV-lentiviruses have been reported to achieve ~50% gene transfer efficiency in resting B cells (11); therefore, further improvements and optimization strategies are required. Efficient delivery of genes into primary human mature B cells may be used to investigate the functions of genes-of-interest (GOIs) in these cells. In addition, efficient gene delivery may result in the development of gene therapies, as well as the development of novel tools for B-cell immortalization, allowing for cloning of antigen-specific B-cell populations, which may be used in the production of monoclonal antibodies. Therefore, the aim of the present study was to further optimize the methods for efficient B-cell transduction by using various gene promoters to increase the levels of transgene expression.

Materials and methods

**Cell culture.** The Raji human Burkitt lymphoma cell line (American Type Culture Collection, Manassas, VA, USA) and human embryonic kidney (HEK)-293T cell line (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) were cultured in RPMI 1640 medium (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany). HT-1080 cells (American Type Culture Collection) were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich; Merck Millipore). The B95.8 human EBV-producing cell line was kindly provided by Professor U. Wojda (International Institute of Molecular and Cellular Biology, Warsaw, Poland); these cells were cultured in RPMI 1640 medium until they reached ≥2 ml/ml density. Subsequently, the supernatant was collected and filtered through a 0.45 µm syringe filter (Promega Corporation, Madison, Wisconsin, USA), aliquoted and stored at -80°C until further use. All media were supplemented with 10% heat-inactivated fetal bovine serum (FBS; HyClone; GE Healthcare Life Sciences, Logan, UT, USA), 100 µg/ml streptomycin, 100 U/ml penicillin and 250 ng/ml amphotericin B (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂, and were passaged approximately every other day.

**B-cell isolation from blood and in vitro culture.** Peripheral blood mononuclear cells (PBMCs) from healthy donors (recruited between September and November 2013, 3 males and 3 females aged 25-45 years old, blood collected via venipuncture from forearm) were isolated from full blood using Histopaque-1077 (Sigma-Aldrich; Merck Millipore) according to the manufacturer's protocol. B cells were isolated from PBMCs using the magnetic EasySep™ Human B Cell Enrichment Kit (STEMCELL Technologies Canada, Inc., Vancouver, BC, Canada) according to the manufacturer's protocol. Cells were cultured in Iscove's modified Dulbecco's medium (IMDM; Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 10% heat-inactivated FBS, 100 µg/ml streptomycin, 100 U/ml penicillin, and 250 ng/ml amphotericin B at 37°C in a humidified atmosphere containing 5% CO₂. The present study was approved by the Institutional Review Board of the Medical University of Warsaw (Warsaw, Poland) and was conducted according to the Declaration of Helsinki. Each patient provided written informed consent for the procedures. For co-culture experiments, HT-1080 pLVX-CD40L cells were incubated with mitomycin C (10 µg/ml; Sigma Aldrich; Merck Millipore) for 3 h at 37°C, and were reseeded into 6-well plates at a density of 4x10^5/well. A total of 5x10^4 B cells at a density of 2.5x10^5/1 ml were then placed onto the HT-1080 pLVX-CD40L cells in IMDM supplemented with 25 ng/ml IL-21 (PeproTech, Rocky Hill, NJ, USA).

**Plasmids.** A human CD40L coding sequence was amplified by polymerase chain reaction (PCR) from cDNA obtained from healthy donor PBMCs, and was cloned into a pLVX-internal ribosome entry site (IRES)-Puro vector (Takara Bio Europe, Saint-Germain-en-Laye, France) according to a standard protocol (16). Briefly, cells were washed with PBS, pelleted, and resuspended in 1 ml TRizol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) to extract total RNA, according to the manufacturer's protocol. First strand cDNA synthesis was then performed: 0.5 µg total RNA was primed with oligo(dT) using AMV reverse transcriptase (EURL Ltd., Gdansk, Poland). PCR was performed using Mastercycler personal (Eppendorf Instrumente GmbH, Hamburg, Germany) and Color Opti Taq polymerase (EURx Ltd.) using the following PCR cycling conditions: Initial denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec, elongation at 72°C for 60 sec, and a final elongation step at 72°C for 10 min. Amplification products were analyzed by 1% agarose gel electrophoresis. The following primers containing restriction sites for Xhol and BamHI on the forward and reverse primer, respectively were used: Forward, 5'-CCG ACTCGAGACCATGTGAGACATACACACAAACTCT GCCC-3' and reverse, 5'-GGGGGATCCGAGAGAATTTGAGTAATGCCAAGGGC-3'. The sequence of the construct (pLVX-CD40L-IRES-Puro) was confirmed by Sanger sequencing using BigDye™ Terminator Version 3.1 Ready Reaction Cycle Sequencing kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The human immuno-deficiency virus (HIV)-spleen focus-forming virus (SFFV)-monomeric red fluorescent protein (mRFP)-woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) plasmid was a generous gift by Professor Els Verhoeyen (Ecole Normale Supérieure de Lyon, Lyon, France) according to a standard protocol (17). The sequences encoding MCS-IRES-RFP, BiP-IRES-RFP, MCS-IRES-GFP and XBpl-IRES-GFP were synthesized by Epoch Life Science, Inc. (Missouri City, TX, USA) and provided as inserts in pBluescript SK vectors. The inserts were subsequently cloned by ligation into the BamHI and XhoI sites of the HIV-SFFV-mRFP-WPRE plasmid. Sequences of the constructs were confirmed by Sanger sequencing.

**Lentiviral transduction of target cells.** For lentiviral production, HEK-293T cells were seeded into 6-well plates (4x10^5/well) and were co-transfected with 2 µg gene-of-interest (GOI)-containing vectors and components of 2nd generation packaging vectors: 1.5 µg psPAX2 packaging vector and 1 µg pMD2.G envelope vector (both vectors were obtained from Addgene) with 1 µg of the HIV-1 packaging plasmid (pMD2.G) and 1 µg of the psPAX2 packaging plasmid. 24 h after transfection, supernatants were harvested and used to infect HT-1080 cells.
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from Professor Didier Trono; École Polytechnique Fédérale de Lausanne, Lausanne, Switzerland), using GeneJuice® transfection reagent (EMD Millipore, Billerica, MA, USA), according to the manufacturer's protocol. The following lentiviral vectors were used in this study: pLVX-IRES-Puro and pLVX-IRES-ZsGreen1 (both from Clontech, Takara Bio Europe), pGIPZ (Open Biosystems; GE Healthcare Life Sciences), pLVTHM (AddGene, Inc., Cambridge, MA, USA). 72 h post-transfection, the lentivirus-containing medium was collected, filtered and added to the culture of target HT-1080 or human B cells with a multiplicity of infection (MOI) of 2. MOI was determined using the Lenti-X p24 Rapid Titer kit (Clontech, Takara Bio Europe), according to the manufacturer's protocol. To select GOI-containing cells, either puromycin selection or sorting for fluorescent-positive cells using a FACSAria III cell sorter (BD Biosciences, La Jolla, CA, USA) was performed 7 days post-transduction.

HT-1080 cells modified with pLVX-CD40L-IRES-Puro construct (HT-1080 pLVX-CD40L) were cultured in DMEM supplemented with 1 µg/ml puromycin (Sigma Aldrich; Merck Millipore).

Staining of surface antigens. Cells were incubated with saturating amounts of fluorochrome-conjugated antibodies (Table I) for 30 min at room temperature. Prior to analysis cells were washed and resuspended in PBS. Cells were analyzed using a FACScan (BD Biosciences).

EBV infection and generation of lymphoblastoid cell lines (LCL). The supernatant from B95.8 cells was collected and stored as aforementioned, and was thawed on the day of infection. Human B cells freshly isolated from buffy coats, as aforementioned, were seeded at a density of 5 mln/ml in IMDM in a T25 flask. Subsequently, 2 ml fresh RPMI 1460 medium was added, followed by 2 ml EBV-containing B95.8 supernatant. 24 h post-infection the cells cultured at 37°C began to form clusters.

Fluorescent microscopy. Cell cultures were analyzed for the expression of fluorescent proteins (GFP and RFP) using an inverted fluorescence microscope (Eclipse TE-2000; Nikon Corporation, Tokyo, Japan) equipped with Plan Fluor 10x/0.30 Ph1 DLL, S Plan Fluor ELWD 20x/0.45 Ph1 ADM and Plan Fluor ELWD 40x/0.60 Ph2 ADL objectives, together with UV-2E/C, B-2E/C, G-2E/C and Y-2E/C fluorescence filters. Images were captured using a QICAM FAST1394 12-bit digital CCD camera (Q Imaging, Surrey, BC, Canada) and were analyzed using Image-Pro Plus 7.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

ELISA. The concentration of immunoglobulin (Ig)G in cell culture supernatants was evaluated using Human IgG ELISA kit (Koma Biotech, Seoul, Korea), according to the manufacturer's protocol. This assay allows for detection of IgG in a sample at the range of 1.95-125 ng/ml.

Western blotting. Cells were washed with PBS, pelleted, lysed in a custom-made lysis buffer (50 mM HEPES pH 7.4, 10% Triton X-100, 150 mM NaCl, 10% glycerol, 5 mM EDTA) and separated by 10% SDS-PAGE. Protein concentration prior to electrophoresis was measured using the Bradford Protein Assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Total proteins (10-20 µg) were loaded onto each well. Separated proteins were transferred onto Protran nitrocellulose membranes (Schleicher & Schuell BioScience GmbH, Dassel, USA). Membranes were blocked for 1 h at room temperature with 5% non-fat milk in TBS-0.1% Tween (TBST; see below). Membranes were then incubated with anti-XBP-1s purified immunoglobulin (clone AC-15; 1:50,000; Dako; Agilent Technologies, Santa Clara, CA, USA).

Table I. Antibodies used for cell sorting.

| Antibody | Fluorochrome | Clone | Cat. no. | Dilution | Company          |
|----------|--------------|-------|----------|----------|------------------|
| CD19     | FITC         | 4G7   | 345776   | 1:10     | BD Biosciences   |
| CD20     | FITC         | L27   | 345792   | 1:10     | BD Biosciences   |
| CD27     | PE           | L128  | 340425   | 1:10     | BD Biosciences   |
| CD138    | FITC         | MI15  | 552723   | 1:10     | BD Biosciences   |
| CD20     | PE           | 2H7   | 555623   | 1:10     | BD Biosciences   |
| IgD      | FITC         | Polyclonal | F0189   | 1:10     | Dako             |
| IgM      | FITC         | Polyclonal | F0058   | 1:10     | Dako             |
| IgG      | FITC         | Polyclonal | F0185   | 1:10     | Dako             |
| IgA      | FITC         | Polyclonal | F0188   | 1:10     | Dako             |
| CD40L    | PE           | 89-76 | 555700   | 1:10     | BD Biosciences   |

CD, cluster of differentiation; Ig, immunoglobulin; FITC, fluorescein isothiocyanate; PE, phycoerythrin. BD Biosciences, La Jolla, CA, USA; Dako; Agilent Technologies, Santa Clara, CA, USA.

CD, cluster of differentiation; Ig, immunoglobulin; FITC, fluorescein isothiocyanate; PE, phycoerythrin. BD Biosciences, La Jolla, CA, USA; Dako; Agilent Technologies, Santa Clara, CA, USA.
Sigma Aldrich; Merck Millipore) for 45 min at room temperature.

Results

Normal B cells co-cultured with CD40L-expressing feeder cells start to proliferate after 7 days of culture. The present study aimed to select optimal constructs and promoters for genetic modification of B cells. As a terminal cell type, B cells can usually only be cultured for a relatively short time. Therefore, to prolong the culture period B cells were co-cultured with feeder cells expressing human CD40L. Initially, HT-1080 cells were modified with an expression vector encoding CD40L (Fig. 1A). The CD40L coding sequence was amplified by PCR from the cDNA of healthy donor PBMCs, after which it was cloned into the pLVX-IRES-Puro vector and subsequently used to modify HT-1080 cells. Feeder cells were pretreated with mitomycin C to prevent their proliferation before starting the co-cultures. B cells co-cultured with feeder cells formed clumps of proliferating cells that were viable for ≥14 consecutive days (Fig. 1B).

B cells activated with CD40L and IL-21 are resistant to transduction. To determine the levels of transgene expression in B cells three bicistronic plasmids were used that encode various GFPs under the control of different promoters, namely pGIPZ [cytomegalovirus (CMV) promoter; turbo GFP, which is an improved variant of the green fluorescent protein CopGFP], pLVTHM [elongation factor 1 alpha (EF1α) promoter; GFP] and pLVX-IRES-ZsGreen1 (CMV promoter; ZsGreen1, which is a human codon-optimized variant of ZsGreen) (Fig. 2A). Independent of the vectors used in the present study, a very low level of transgene expression was detected in B cells; expression did not exceed 10%, as assessed with flow cytometry 7 days post-transduction (Fig. 2B). Notably, concomitant transduction of Raji lymphoma cells with pLVX ZsGreen1, pGIPZ and pLVTHM proved to be effective; the levels of transgene expression ranged between 14.22 and 40.25% (Fig. 2C).

EBV infection of B cells generates LCLs. Since Raji cells carry the latent EBV genome and are positive for EBV nuclear antigen 1, and EBV infection has previously been described to increase the sensitivity of B cells to genetic modification (17), the present study infected normal B cells with EBV to determine whether this would allow for increased levels of transgene expression with lentiviral vectors. Freshly isolated human B cells were mixed with supernatants collected from B95.8 human EBV-producing cells, in order to allow B cell infection and immortalization. Transformation of B cells with EBV resulted in the generation of LCLs. These cells were highly variable in shape and grew in clumps. The cells expressed antigens characteristic for normal B lymphocytes, such as CD19 and CD20 (Fig. 3A). Furthermore, EBV-infected B cells displayed memory phenotype, as they were positive for CD27. The expression of CD138, which is an antigen that is present during activation and differentiation of B cells and is specific for terminally differentiated B cells, was variable in tested LCLs (Fig. 3A). In addition, the membrane expression of IgG was determined, and the results indicated that LCLs do not express membrane IgD. However, ~60% of LCLs expressed membrane IgG, whereas ~40% expressed membrane IgA and membrane IgM (Fig. 3B). These results confirmed the findings of previous studies (18,19), and suggested that EBV infection

![Figure 1](image1.png)  
Figure 1. Culture of normal B cells with CD40L-expressing feeder cells. HT-1080 cells were modified to express CD40L, as confirmed by (A) flow cytometry. (B) HT-1080 pLVX-CD40L cells were used as feeder cells to stimulate the proliferation of normal B cells. Images were captured using a Nikon Eclipse TE2000-E microscope, magnification, x200. CD40, cluster of differentiation 40 ligand; PE, phycoerythrin.
Figure 2. Lentiviral modification of normal B cells and Raji cells. (A) Three vectors (pLVX ZsGreen1, pLVTHM and pGIPZ) were used for lentiviral modification of (B) normal B cells or (C) Raji cells. The percentage of GFP-positive cells was determined using flow cytometry. Co-cultures of normal B cells and feeder cells were additionally stained with anti-CD20 antibody to distinguish B cells from feeder cells. GFP, green fluorescent protein; CMV, cytomegalovirus; EF1α, elongation factor 1 alpha; CD20, cluster of differentiation 20; PE, phycoerythrin.

Figure 3. Characteristics of Epstein-Barr virus-infected B cells. Surface expression of (A) CD19, CD20, CD27 and CD138, and (B) Igs (IgA, IgD, IgG and IgM) in lymphoblastoid cell lines was determined by flow cytometry with fluorochrome-conjugated antibodies. CD, cluster of differentiation; Ig, immunoglobulin; FITC, fluorescein isothiocyanate; PE, phycoerythrin.
expands the CD27+ memory cell pool, and the majority of these cells exhibited a switch in isotype to IgG or IgA. In addition, LCLs were tested for the secretion of Igs; the mean output was 0.004 ng IgG per cell per day (data not shown).
SFFV promoter provides the best level of transgene expression in Raji cells and LCLs. Since CMV and EF1α promoters allowed for ≤40.25% transgene expression in Raji cells further experiments were conducted using the SFFV promoter. The results demonstrated that SFFV was much stronger in driving transgene expression in human hematopoietic cells compared with other promoters, including CMV or EF1α (12). Notably, the level of transgene expression in Raji cells was >89% when the SFFV promoter was used (Fig. 4). Subsequently, the present study analyzed the levels of transgene expression in Raji cells, LCLs and normal CD40L-stimulated B cells using the HIV-SFFV-mRFP-WPRE vector. The SFFV promoter resulted in high levels of transgene expression in Raji and LCL cells, as determined by fluorescent microscopy; however, normal CD40L-stimulated B cells were still resistant to modification (Fig. 5).

Constructs with IRES and fluorescent markers enable the selection of transduced LCLs. Based on the HIV-SFFV-mRFP-WPRE vector, the present study constructed vectors to allow expression of one transgene of interest, and in addition, a fluorescent marker protein for identification and selection of transduced cells. In order to enable the coordinated and efficient expression of a transgene and fluorescent marker directly from the SFFV promoter, attenuated IRES was introduced in front of the fluorescent marker. Since a gene located behind the IRES is expected to be translated with lower efficiency than the one in front, such conformation ensures high-level expression of the GOI, rather than the fluorescent marker gene. As transgenes, the present study introduced genes encoding XBP-1s and BiP proteins (Fig. 6). Flow cytometric analysis and sorting of Raji cells (Fig. 7A) or LCLs (Fig. 7B) was conducted 10 days post-transduction. Notably, the levels of transgene expression with GFP-encoding vectors was higher in Raji cells compared with in LCLs, whereas the levels of transgene expression with RFP-encoding vectors were comparable between the cell lines (Fig. 7). Finally, after sorting, it was demonstrated that LCLs were successfully modified to overexpress the XBP-1 s protein, as confirmed with western blotting (Fig. 8).

Discussion

The present study aimed to identify a eukaryotic promoter allowing for efficient and stable transduction of normal human B cells with GOI. Quiescent cells are notoriously resistant to various methods of gene transfer. Normal human B cells are particularly difficult to transduce. Nonviral transfection approaches are completely ineffective, and the use of viral vectors requires complex genetic engineering to generate...
pseudotyped lentiviruses. The present study aimed to evaluate the levels of transgene expression with lentiviral vectors containing various promoters, including CMV, EF1α and SFFV. However, despite satisfactory (CMV and EF1α) and notable (SFFV) levels of transgene expression (Fig. 4), allowing for modification of ~90% of Raji cells, <10% of normal human B cells were transduced with vectors containing these promoters (Fig 2B). Since Raji cells are immortalized B cell-derived lymphoma cells carrying the latent EBV genome, the present study aimed to investigate whether EBV infection of normal B cells will make them more sensitive to lentiviral transduction. Notably, infection of B cells with EBV resulted in the generation of LCLs that could be effectively transduced with lentiviruses carrying the SFFV promoter (Fig. 5). Therefore, these results confirmed the findings of a previous study, which indicated that B cells stimulated with EBV are easily modified with lentiviruses, whereas B cells activated with CD40L and cytokines are resistant to transduction (17). To produce lentiviral particles the 2nd generation packaging vector psPAX2 and the VSVG-expressing envelope vector pMD2.G were used. Using VSVG-lentiviruses the present study managed to modify lymphoma cells and LCLs with high efficacy; however, the present study failed to transduce quiescent normal B cells. It has previously been reported that lentiviruses pseudotyped with the Edmonston MV hemagglutinin and fusion glycoproteins (Hgps and Fgps) allowed for efficient transduction of quiescent human B and T cells (12). These findings strongly suggested that the transduction efficiency may be determined at the level of vector entry. It may be hypothesized that bicistronic vectors, when used with lentiviral vectors pseudotyped with the Edmonston MV Hgps and Fgps can serve as a potent transduction tool for normal B cells.

As proof-of-concept, the present study used bicistronic vectors containing genes encoding BiP or mRFP1, as well as fluorescent reporter proteins mRFP or GFP, and demonstrated that SFFV promoter-based lentiviruses may be used for stable transduction of LCLs with GOI. In conclusion, the present study demonstrated that it is possible to achieve an efficient and stable lentiviral transduction of LCLs using SFFV promoter constructs. This approach may be used in future studies aimed at studying various processes associated with B-cell physiology. Such bicistronic vectors expressing fluorescent proteins (GFP or mRFP) also allow for tracking of modified cells in ex vivo or in vivo studies.

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