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ORIGINAL ARTICLE

Novel lentiviral-inducible transgene expression systems and versatile single-plasmid reporters for *in vitro* and *in vivo* cancer biology studies

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Many of the cancer cell lines derived from solid tumors are difficult to transfect using commonly established transfection approaches. This hurdle for some DNA transfection systems has hindered cancer biology studies. Moreover, there are limited tools for studying pathway activities. Therefore, highly efficient improved gene transfer and versatile genetic tools are required. In this study, we established and developed a comprehensive set of new lentiviral tools to study gene functions and pathway activities. Using the optimized conditions, cancer cell lines achieved >90% transduction efficiency. Novel lentiviral doxycycline-regulated pTet-IRES-EGFP (pTIE) systems for transgene expression and TRE reporters used for pathway activity determination were developed and tested. The pTIE Tet-Off system showed *in vitro* doxycycline-sensitive responses with low or undetectable leakage of protein expression and *in vivo* tumor suppression as illustrated using candidate tumor suppressors, *Fibulin-2* and *THY1*. In contrast, the Tet-On system showed dose-dependent responses. The pTRE-EGFP (pTE) and pTRE-FLuc-EF1α-RLuc (pT-FER) reporters with the NFκB p65 subunit consensus sequence showed GFP and firefly luciferase responses, which were directly correlated with TNFα stimulation, respectively. Taken together, these newly developed lentiviral systems provide versatile *in vitro* and *in vivo* platforms to strengthen our capabilities for cancer biology studies.

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INTRODUCTION

Characterizing the functions of tumor suppressor genes (TSGs), oncogenes, and different signaling pathways in cancers is needed to enhance our understanding of cancer pathogenesis and to reveal the molecular basis of tumorigenesis, and, thus, to subsequently identify useful diagnostic markers and therapeutic targets. To evaluate the functions of genes of interest and the promoter activities in mammalian cells, exogenous plasmid DNAs containing luciferase reporter-labeled genes or promoters are commonly transferred using various well-established techniques. These are classified into three classes, namely chemical transfection, mechanical transfection, and viral transduction. Many chemical and mechanical gene transfer tools are available. However, the efficiencies of gene transfer and stable transfection are always the major concerns. Geiling *et al.* [1] reviewed some drawbacks of the use of plasmid-based systems caused by poor DNA uptake and loss of plasmids over time occurring in some cell lines.[1] Therefore, development and establishment of a more universally efficient gene transfer system is needed and is essential for enhancing more extensive *in vitro* and *in vivo* studies.

In general, viral transductions show high efficiency with low toxicity and result in stable gene expression. The first retroviral gene transfer vector was developed by Cepko *et al.*, using murine leukemia viruses, which are γ-retroviruses.[2] However, a retrovirus cannot infect non-dividing cells as it cannot pass through the nuclear membrane, and, thus, only cells undergoing mitosis are infected. The lentivirus is a subclass of retroviruses. It can infect a wide range of cells, including dividing and non-dividing cells. The first generation lentivirus vector was established based on the human immunodeficiency virus (HIV) in 1996.[3] Liver cells and hematopoietic stem cells were successfully infected with lentivirus.[4,5] The virus enters the cells via endocytosis and its RNA genome is reverse-transcribed into DNA, followed by randomly integrating into the host cell genome. Thus, lentiviral transduction provides a method to obtain long-term stable gene expression. In contrast, other groups have also developed some non-primate lentiviral vectors such as the feline immunodeficiency virus (FIV)-based vector and equine infectious anemia virus (EIAV)-based vector.[6,7] However, the most commonly used vector is still HIV-based. Dider Trono's and Inder Verma's groups continued to modify the HIV-based vectors for better transduction and improvement of biosafety.[8–10] The second and third generation lentiviruses contain a 3′ self-inactivating long terminal repeat, preventing the replication of the lentivirus in the cell after integration into the host cell genome. Taken together, lentiviral systems offer a stable transduction of dividing and non-dividing cells for subsequent *in vitro* and *in vivo* studies.[11]

For the studies of TSGs and oncogenes in epithelial cancer cell lines, lipofection is the most common transfection method. For example, CMV-based DNA plasmids, pCR3.1 and pcDNA3.1, carrying candidate TSGs *FBLN2* and *CACNA2D3* were transfected into nasopharyngeal carcinoma (NPC) cell lines using lipofection;[12,13] nevertheless, stable expression of TSGs with growth inhibitory activities in cancer cell lines remains the major challenge.

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Protopopov *et al*. developed an inducible Tet-Off system to study functions of TSGs in various cancer cell lines.[14] The selected cell line was first engineered with a retroviral tTA-expressing vector; the gene of interest was then cloned into the episomal responsive plasmid and was transfected into the chosen cell line. Several candidate TSGs such as *ADAMTS9, CRIP2, CRYAB, FBLN2, MIPOL1, MMP19, PTPRG*, *THY1*, and *TSLC1* were successfully transfected into an engineered NPC cell line, HONE1–2, using lipofection.[12,14–22] Nevertheless, low transfection efficiencies, loss of gene expression *in vivo*, clonal variation, non-physiological expression levels driven by the CMV promoter, and limited tTA-expressing cell lines are the obstacles encountered in these studies. Thus, it is important to establish new transgene expression systems.

In the present study, we aimed to optimize the lentiviral transduction conditions and compared CMV and EF1α promoter activities for a more efficient gene transfer to various cancer cell lines, including HONE1, HNE1, CNE1, CNE2, SUNE1, HK1, C666-1, 81T, EC1, HepG2, A549, BOWES, HeLa, and HT1080, as well as non-cancer cell lines, HEK293T and COS7. We have generated a new inducible lentiviral tetracycline-regulated system (pTet-IRES-EGFP, pTIE) to express a gene of interest for *in vivo* and *in vitro* assays. The TSGs *FBLN2* and *THY1* were used as the transgenes to demonstrate the usefulness of the system. Furthermore, we have also established a lentiviral fluorescent protein reporter system (pTRE-EGFP, pTE) for determining promoter activities, as well as a single lentiviral plasmid of firefly/*Renilla* luciferase reporter (pTRE-FLuc-EF1a-RLuc, pT-FER) for both cell tracking and detection of promoter activities. The NFκB consensus sequence was used to test for these newly developed reporters.

MATERIALS AND METHODS

Cell culture

HONE1, HNE1, CNE1, CNE2, SUNE1, HK1, A549, HeLa, BOWES, HT1080, HEK293T, and COS7 cell lines were cultured in DMEM with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Pen/Strep).[23–31] C666, 81T, and EC1 were cultured in RPMI1640 with 10% FBS and 1% Pen/Strep.[32–34] HONE1, HNE1, CNE1, CNE2, SUNE1, and C666 are poorly or undifferentiated epithelial NPC cell lines. C666 is the only Epstein–Barr virus-positive NPC cell line and HK1 is the only well-differentiated epithelial NPC cell line. 81T and EC1 are undifferentiated esophageal squamous cell carcinoma cell lines. A549, HeLa, and BOWES are the lung, cervical, and melanoma epithelial cell lines, respectively. HT1080, a fibrosarcoma cell line, HEK293T, and COS7 cells are well-known cell lines often used in studies, which were chosen to be transduction controls in this study.

Plasmids

The lentiviral pWPI plasmid containing the EF1α-MCS-IRES-EGFP cassette was used in the present study (Addgene plasmid 12254). The lentiviral pLVX-CMV-puro plasmid containing the CMV-MCS-PGK-PuroR cassette was developed by Clontech (Palo Alto, CA, USA). The CMV promoter was replaced by EF1α promoter from pWPI to generate pLVX-EF1α-puro plasmid. To verify the transduction efficiency, EGFP was cloned into both pLVX-CMV-puro and pLVX-EF1α-puro plasmids from pEGFP-N1 (Clontech, Palo Alto, CA, USA). For construction of the pTet-IRES-EGFP (pTIE) plasmid, the TetO promoter sequence was excised from pETE-Bsd plasmid[14] and ligated into the pWPI plasmid, in which the EF1α promoter had been removed. The tTA sequence was excised from pLNCtTA-hCMV plasmid[14] and the rtTA sequence was excised from pTET-ON plasmid (Clontech). The tTA and rtTA were ligated into pLVX-EF1α-puro, separately. For the construction of the transcriptional response element (TRE)-fluorescent protein reporter, pTRE-EGFP (pTE) plasmid, a pair of oligonucleotides containing five repeats of NFκB consensus sequence (5′TGGGGACTTTCCGC3′) linked with a TATA box sequence was used to replace the CMV promoter in the pLVX-CMV-EGFP-puro plasmid. The TATA box is used as a minimal promoter for reporter gene expression. For the construction of pTRE-FLuc-EF1α-RLuc (pT-FER) plasmid, firefly luciferase (FLuc) was excised from pGL3-Basic (Promega, Madison, WI, USA) and was used to replace EGFP in the pTE plasmid, resulting in a pTRE-FLuc plasmid. *Renilla* luciferase (RLuc) was subcloned from pRL-TK-*Renilla* (Promega) to pLVX-EF1α-puro plasmids, and the EF1α-RLuc fragment was further excised and subcloned into pTRE-FLuc before PGK promoter, as a result of establishment of a pT-FER reporter plasmid.

Lentivirus preparation and transduction

The lentiviral plasmids were mixed with the packaging plasmid, psPAX2 (Addgene plasmid 12260), and the envelope plasmid, pMD2.G (Addgene plasmid 12259), in a ratio of 4:3:1 (8/6/2 μg for a T-75 flask). The HEK293T cell line was used as the packaging cell line and was seeded for 2 days before transient transfecting with FuGENE HD transfection reagent (Promega, Madison, WI, USA) according to the manufacturer's instructions. Cells were kept for 3 days after transfection without changing medium. The conditioned medium, containing virus, was briefly centrifuged to remove cell debris and was then filtered through a 0.45- μm pore size filter (Pall, Port Washington, NY, USA). Transduction was achieved by mixing 1 ml virus, 1 ml fresh medium with cells, and 8 μg ml^{-1} polybrene (Sigma-Aldrich, St Louis, MO, USA) in six-well plates. The transduction mixture can be scaled up/down depending on the quantity of transduced cells needed. Medium was changed the next day to reduce the stress of cells. For those lentiviral plasmids containing the puromycin resistance gene, medium containing 0.5 μg ml^{-1} of puromycin (InvivoGen, San Diego, CA, USA) was added to the cells at passage one. Transduced cells containing fluorescent protein markers were examined under fluorescence microscopy day 5 post-transduction to confirm the efficiency of transduction. Passages two to three were considered as the early stable pool.

Fluorescence-activated cell sorting (FACS) analysis

Lentiviral-transduced cells were trypsinized, pelleted, washed twice with 1x PBS, and analyzed in a BD FACSCantoII Analyzer (BD Biosciences, San Jose, CA, USA) equipped with a 488-nm solid state laser. The GFP signals were detected with an emission filter of 530/30 nm. For each sample, 20 000 events were counted. The parental non-GFP cells were used as a negative control to set the threshold for GFP expression level. Data analysis was performed using FlowJo 7.6.1 software (TreeStar, Ashland, OR, USA).

Fluorescence microcopy analysis

The GFP signals were visualized and the images were captured using Nikon Eclipse Ei fluorescence microscopy (Nikon Instruments, Melville, NY, USA) with Nikon CFI Plan Fluor 10x objective (Nikon Instruments, Melville, NY, USA) and SPOT RT3 flexible cooled CCD camera (Diagnostic Instruments, Sterling Heights, MI, USA). The camera settings including gain and exposure time were kept constant during the analyses using SPOT 4.6 software (Diagnostic Instruments, Sterling Heights, MI, USA).

Western blotting analysis

Whole-cell lysates were collected using radio immuno precipitation assay (RIPA) buffer (30 mM Tris, pH 8.0, 150 mM sodium chloride, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS) with Complete Protease Inhibitor Cocktail (Roche Applied Science, Penzberg, Upper Bavaria, Germany). Western blotting was then performed as previously described[12] using anti-FBLN2 (a gift from Dr Takako Sasaki, Oita University, Japan), anti-GFP (Cell Signaling Technology, Danvers, MA, USA), and anti-THOC1 (nuclear matrix protein p84) antibodies (GeneTex, Irvine, CA, USA) to evaluate equal loading.

In vivo tumorigenicity assay

The *in vivo* stability of the newly developed inducible lentiviral system was tested using the *in vivo* tumorigenicity assay, in which 1×10^7 cells in 0.2 ml serum-free DMEM medium of lentiviral-transduced HONE1 cell lines were subcutaneously injected into 6- to 8-week-old female immunodeficient athymic BALB/C *nu/nu* nude mice. Twelve sites were injected into six mice for each tested cell line. A total of 200 μg ml^{-1} doxycycline and 2.5% sucrose was added into drinking water 1 week before injection, and the water with and without doxycycline was changed twice a week. Tumor volumes were measured once a week using calipers and calculated with the formula: length × breadth × height (mm^3). The ethics for the animal usage was approved by the Committee on the Use of Live Animals in Teaching and Research (CULATR) at the University of Hong Kong.

Reporter assay

HONE1 and HK1 were transduced with pTE reporter virus and the stable cells were plated in a six-well plate. The cells were allowed to grow in a serum-free medium for 24 h before 50 ng ml^{-1} TNFα stimulation. The GFP signal was captured and compared with 24 h serum-free-medium-treated cells. For testing the pT-FER reporter system, HONE1 was transduced with pT-FER virus. The 5×10^4 stable cells were plated in a 96-well plate and allowed to grow for 2 days before 24 h stimulation of TNFα at concentrations ranging from 20, 50 to 100 ng ml^{-1}. For detecting the signals of firefly luciferase and renilla luciferase, 15 µl of firefly luciferin substrate and 4 µl of coelanterazine substrate from Promega dual reporter assay kit (Promega) were added into the wells, respectively, and the bioluminescence was immediately measured by Xenogen IVIS 100 *in vivo* imaging system (PerkinElmer, Waltham, MA, USA).

RESULTS

Optimization for lentiviral transduction of epithelial cancer cells

To optimize the transduction efficiencies in various cancer cell lines, three variables were tested in this study: the quantity of virus used, detachment of cells before transduction, and the numbers of cells used. The pWPI lentiviral system developed by Trono's group was used for the purpose of optimization. The pWPI vector is a bicistronic vector, which allows for simultaneous expression of a transgene and the enhanced green fluorescent protein (EGFP) marker to facilitate tracking of transduced cells (Figure 1a). In addition, the EF1α versus CMV promoter activities were compared using the pLVX-EF1α and -CMV vectors, and EGFP was cloned into these vectors (pLVX-EF1α-EGFP and -CMV-EGFP) to indicate the successful infection.

We first determined whether the infection levels correlates with the amount of virus media used. Figure 1b shows that infection levels improved with increased quantities of input virus, as evidenced by the percentage of GFP-positive cells that was sufficient to infect nearly 90% of the HONE1 cells. Next, to test whether cell detachment from the culture dish would affect the transduction efficiencies, the cells were seeded to around 50% confluence and were then treated with or without trypsin. The virus was subsequently added to cells with or without trypsinization (Figure 1c). The fibrosarcoma HT1080 cells served as controls. There was no obvious difference in transduction efficiencies after trypsinization of 81T, A549, BOWES, HT1080, CNE1, and HeLa cell lines; higher transduction efficiencies were achieved in trypsinized HK1, EC1, and HepG2 cells (Figure 1c and Supplementary Figure S1), suggesting that transduction efficiency is cell line-specific. To investigate the influence of cell density in transduction, a total of 2×10^5 (around 20% confluence) to 1.6×10^6 (around 80% confluence) trypsinized HONE1 cells in a six-well plate were infected with pWPI lentivirus. Results clearly demonstrated that the percentage of GFP-positive cells is inversely proportional to the number of cells used for transduction and the GFP expression level dropped significantly when more trypsinized cells were used for transduction (Figure 1d). There is threefold

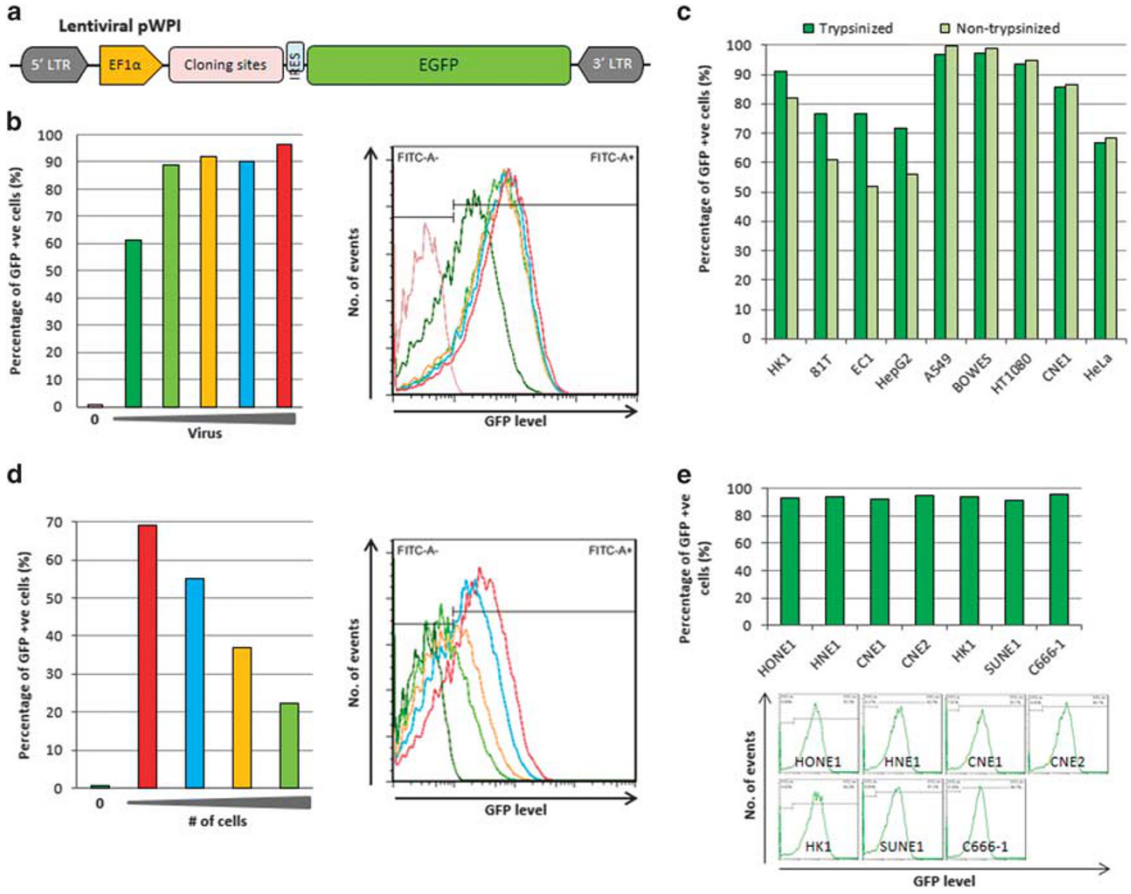

Figure 1. Optimization of lentiviral transduction. (**a**) Schematic diagram of the pWPI lentiviral plasmid (Addgene plasmid 12254) showing LTR (long terminal repeat), EF1α (elongation factor 1 alpha) promoter, IRES (internal ribosome entry site), EGFP (enhanced green fluorescence protein). (**b**) Optimizing quantities of virus for transduction. Tested volumes of virus were 0, 0.5, 0.75, 1, 1.25, and 1.5 ml. (**c**) Evaluating effect of trypsinization before transduction. (**d**) Testing cell densities for use in transduction. Tested cell numbers are 2×10^5, 4×10^5, 8×10^5, and 1.6×10^6 cells. (**e**) Transduction of seven NPC cell lines using optimized transduction conditions (0.75 ml virus after trypsinization of 2×10^5 cells). Bar charts indicate the percentages of GFP-positive cells and FACS analyses show GFP expression levels.

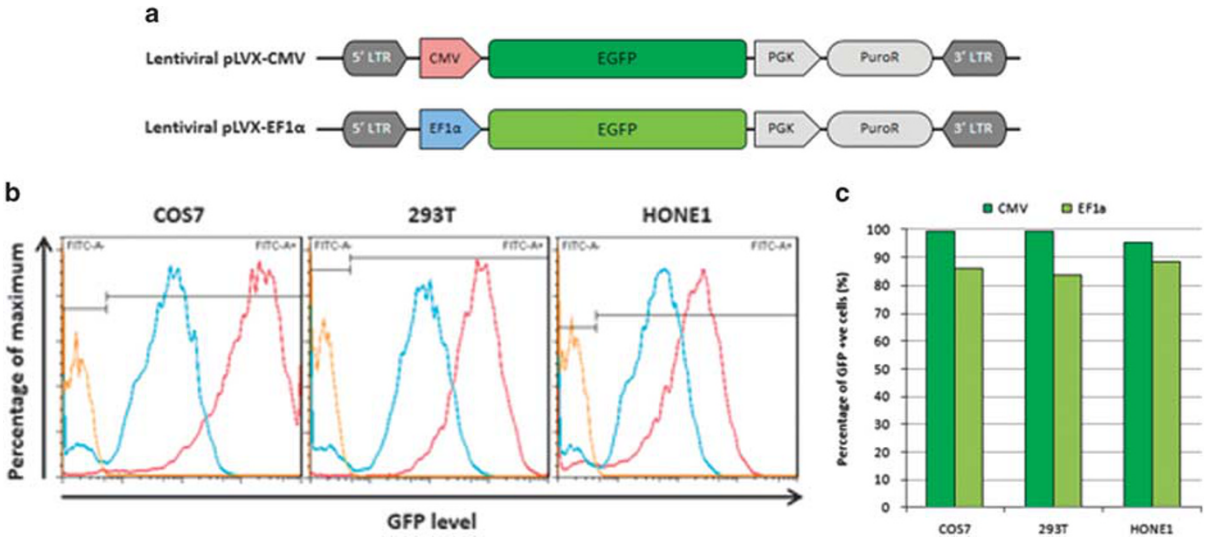

Figure 2. Cell line-dependent promoter activities. (**a**) Schematic diagrams of pLVX lentiviral plasmids, pLVX-CMV and pLVX-EF1α with LTR (long terminal repeat), CMV (cytomegalovirus) promoter, EF1α (elongation factor 1 alpha) promoter, EGFP (enhanced green fluorescence protein), PGK (phosphoglycerate kinase 1) promoter, and PuroR (puromycin resistance) marker. (**b**) FACS analysis showing differential GFP expression levels driven by CMV and EF1α promoters in three tested cell lines. (**c**) Differencing percentages of GFP-positive cells driven by different promoters.

difference in transduction efficiencies between the use of high cell number versus low cell number, suggesting that fewer numbers of cells enhance the transduction efficiency. Using the optimized conditions for lentiviral transduction, the seven NPC cell lines, HONE1, HNE1, CNE1, CNE2, HK1, SUNE1, and C666-1, were successfully and efficiently infected with the pWPI vector (91.2 to 95.7% GFP-positive; Figure 1e). The GFP signals are normally detected 2–4 days post-infection. According to the current findings, it is critical to use a high virus to cell number ratio. Although prior detachment of cells is not necessary for lentiviral transduction, it is still recommended for adjustment of cell numbers for transduction (Figure 1c and d).

Using the same optimized conditions as for pWPI transduction, the GFP expression levels driven by EF1α and CMV promoters in HONE1, HEKT293T, and COS7 cells were compared (Figure 2 and Supplementary Figure S2). The results show that the GFP expression levels driven by CMV promoter fluctuated depending on cell lines (Figure 2b), although the percentages of GFP-positive cells are as high as 99.3% (Figure 2c). Nevertheless, the GFP expression levels driven by the EF1α promoter were highly consistent (Figure 2b) and the percentages of GFP-positive cells are highly similar among three tested cell lines (83.7 to 88.3%, Figure 2c). The results suggested that gene expression driven by CMV promoter may be too strong and are highly cell line-dependent.

Doxycycline-sensitive pTIE Tet-Off system and dose-dependent Tet-On system

The TetO sequence from the episomal pETE plasmid[14] was used to replace the EF1α promoter in pWPI to generate the pTIE plasmid, whereas the tTA and rtTA sequences were obtained from pLNCtTA-hCMVplasmid[14] and pTet-On plasmid and were inserted into the pLVX-EF1α plasmid, resulting in Tet-Off and Tet-On systems, respectively. We first used GFP expression as an indicator to test for usefulness of this newly developed inducible vector with the tTA-expressing HONE1 as the recipient cell line (Figure 3a). As shown in Figure 3b, pTIE-transduced tTA-engineered HONE1 shows obvious GFP signals, when this transgene is switched on without doxycycline treatment. Addition of 200 ng ml^{-1} doxycycline significantly reduces the GFP signals to the pTIE-alone control level (Supplementary Figure S3). Furthermore, to determine the minimal doxycycline levels required to shut off the GFP expression in HONE1 cells, concentrations of doxycycline ranging from 12.5 to 200 ng ml^{-1} were tested (Figure 3b). Given that the basal GFP expression of pTIE-alone is 0.44%, the GFP signal was reduced from 55.5% to low levels of 2.8% by all tested concentrations (Figure 3c and Supplementary Figure S3). A representative result of the inhibition by all concentrations of doxycycline is shown in Figures 3b and c and Supplementary Figure S3. As can be observed from these findings, 12.5 ng ml^{-1} doxycycline is sufficient to abrogate the transgene expression in HONE1 cells using this Tet-Off system; this system is quite sensitive to the effects of doxycycline.

To further test the usefulness of the current pTIE Tet-Off system *in vitro*, the NPC TSG *FBLN2* was subcloned into the pTIE vector (pTIE-*FBLN2*) and infected into HONE1 cells with subsequent analysis by western blotting (Figure 3d). The FBLN2 protein expression was detected in the absence of doxycycline, while its protein expression was undetectable in the presence of doxycycline with various doses (not dose dependent) (Figure 3d). To evaluate the usefulness of pTIE in *in vivo* models, THY1, a previously reported TSG[20,35] was used as a functional readout. *THY1* was subcloned into the pTIE vector (pTIE-*THY1*) and infected into HONE1, and the transgene expression was analyzed by western blotting to show that doxycycline could inhibit the gene expression with undetectable leakage (Supplementary Figure S4). As shown in Figure 3e, the subcutaneous *in vivo* tumor growth kinetics showed significant suppression of tumor growth when the transgene was expressed (without dox). On addition of doxycycline, *THY1* expression was completely abrogated, hence allowing tumors to grow at a faster rate that is comparable to the vector-alone controls (with or without dox). Taken together, these results confirm the tight regulation of *THY1* expression and its tumor-suppressive function by doxycycline *in vivo*. Furthermore, two additional NPC cell lines, CNE2 and SUNE1, were also used to test the inducible system *in vitro* (Supplementary Figures S5 and S6). Both tTA-expressing CNE2 and SUNE1 cells transduced by pTIE also showed doxycycline-sensitive responses.

Unlike the Tet-Off system, the Tet-On system (EF1α-rtTA+pTIE) shows a dose-dependent response to doxycycline, as indicated by

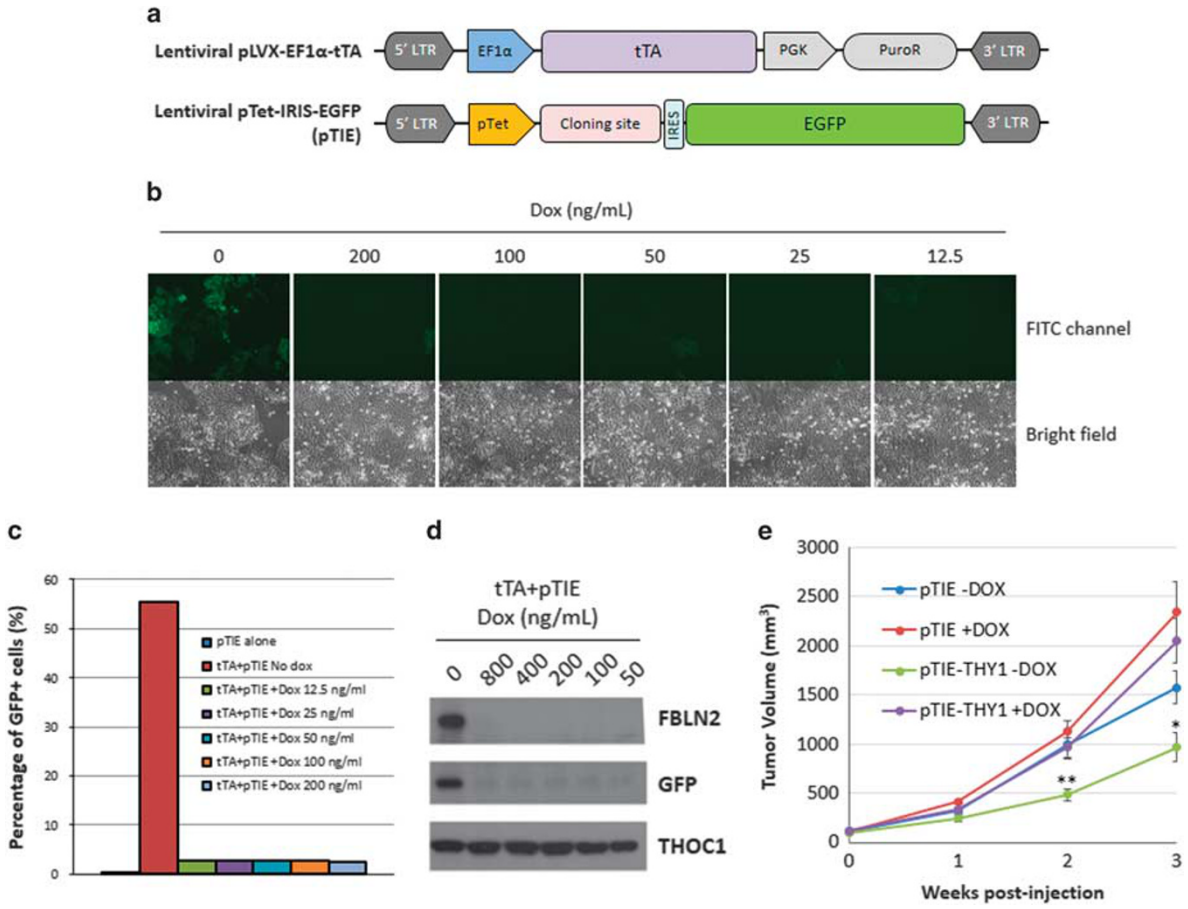

Figure 3. Doxycycline tightly regulated pTet-IRES-EGFP (pTIE) Tet-Off system. (**a**) Schematic diagrams of the Tet-Off system. Two plasmids are shown, EF1α-tTA and pTIE with LTR (long terminal repeat), EF1α (elongation factor 1 alpha) promoter, tTA (tetracycline transactivator) protein; PGK (phosphoglycerate kinase 1) promoter, PuroR (puromycin resistance) marker, pTet (tetracycline responsive) promoter, IRES (internal ribosome entry site) and EGFP (enhanced green fluorescence protein). (**b**) Doxycycline-sensitive responses. HONE1-tTA cells were transduced with pTIE and treated with different concentrations of doxycycline (200, 100, 50, 25, and 12.5 ng ml^{-1}). (**c**) Determining the percentages of GFP-positive cells after treatment of doxycycline by FACS analysis. (**d**) Western blot showing *in vitro* protein expression of FBLN2 and GFP by treating pTIE-*FBLN2*-transduced HONE1-tTA cells with different concentrations of doxycycline (800, 400, 200, 100, and 50 ng ml^{-1}). THOC1 serves as a equal loading marker. (**e**) *In vivo* tumor measurements of THY1 in nude mice by subcutaneous injection of pTIE-*THY1* transduced HONE1-tTA cells with and without 200 μg ml^{-1} doxycycline treatment. Each data point represents an average tumor volume taken from 12 tumor sites, with six mice allocated per cell population ± s.e.m. * Represents $P < 0.05$ and ** represents $P < 0.01$ when compared to pTIE-*THY1* with doxycycline treatment.

the GFP expression in HONE1 cells (Figure 4). Higher concentrations of doxycycline resulted in correspondingly higher detectable GFP signals. No distinct peak of GFP-expressing cells was detected by FACS analysis after addition of high dosages of doxycycline (Supplementary Figure S7), indicating a wide spectrum of the induced GFP expression levels. The relationship of the response of GFP signal and the doxycycline concentration is a sigmoid curve. The maximum percentage of GFP cells observed was 46.6%, when the cells were treated with 1600 ng ml^{-1} doxycycline (Figure 4b and Supplementary Figure S7). The GFP expression almost reached a plateau, when the concentrations were >1600 ng ml^{-1}. Similarly, the FBLN2 protein expression levels were induced by various doxycycline concentrations in a dose-dependent manner, although the maximum expression is still lower than that observed with the Tet-Off system, when the transgene was expressed (Figure 4c).

Lentiviral reporter system

We have also developed a versatile lentiviral fluorescent protein reporter (pTRE-EGFP, pTE) and single plasmid firefly/*Renilla* luciferase reporter system (pTRE-FLuc-EF1α-RLuc, pT-FER) (Figure 5a and b). Under puromycin selection, stable transfectants of pTE-infected HONE1 and HK1 cells were obtained and basal GFP expression levels were determined under serum-free treatment (Figure 5c). Addition of 50 ng ml^{-1} TNFα (a well-known NFκB inducer) for 18 to 24 h elevated the GFP expression in the transfectants of both HONE1 and HK1 cells, showing that this new reporter successfully detected the p65 promoter activities in NPC cell lines. We further constructed a single lentiviral plasmid for simultaneous use as the reporter and control. The pT-FER system was developed in which firefly luciferase and *Renilla* luciferase signals represent promoter activity and cell number, respectively (Figure 5b). Figure 5d clearly showed that firefly luciferase signals directly correlated with stimulation by a range of TNFα concentrations, while the *Renilla* luciferase signal remained constant.

DISCUSSION

The efficacy of transfection of various epithelial cancer cell lines including NPC cell lines is variable and sometimes difficult to

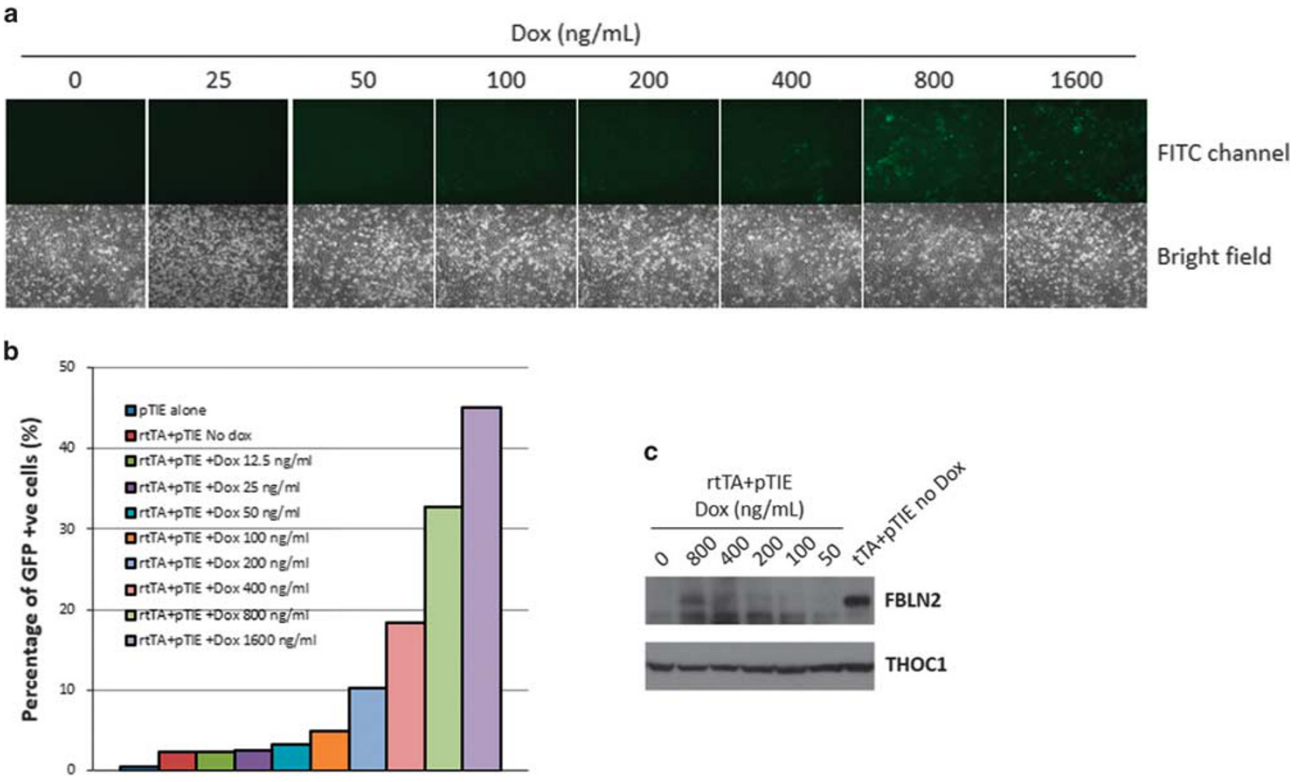

Figure 4. Dose-dependent pTIE Tet-On system. The reverse tetracycline transactivator (rtTA) sequence was used to replace tTA in EF1a-tTA plasmid. HONE1-rtTA cells were then transduced with pTIE. (**a**) Differential GFP expression by treatment with a range of doxycycline concentrations (25, 50, 100, 200, 400, 800, and 1600 ng ml^{-1}). (**b**) The percentages of GFP-positive cells after treatment of doxycycline followed by FACS analysis. (**c**) Western blot showing protein expression of FBLN2 by treating pTIE-*FBLN2*-transduced HONE1-rtTA cells with different concentrations of doxycycline (800, 400, 200, 100, and 50 ng ml^{-1}). Expression of FBLN2 by the Tet-Off system without doxycycline treatment is used as a positive control. THOC1 serves as a equal loading marker.

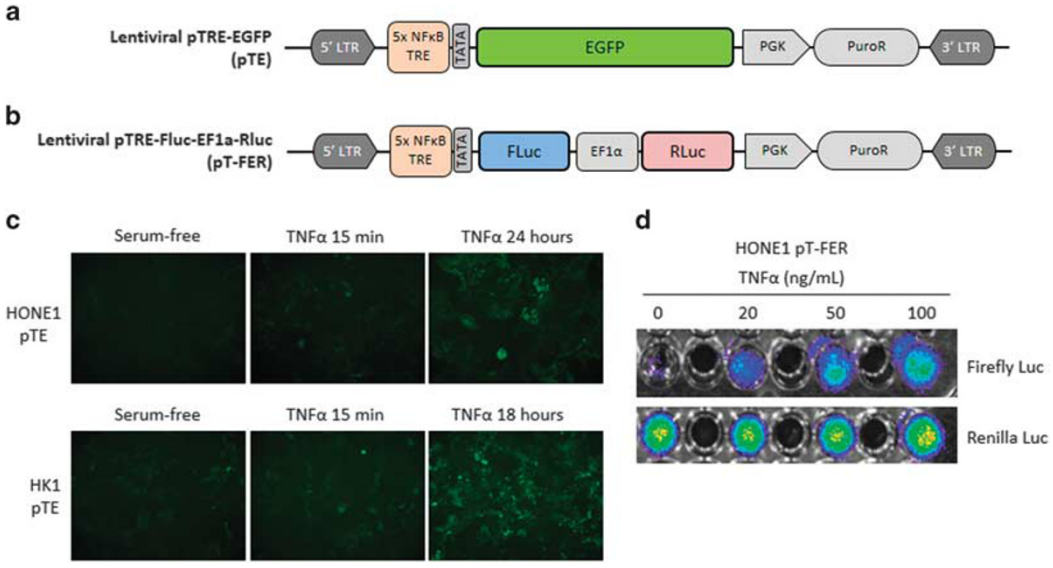

Figure 5. Versatile lentiviral reporter systems. NFκB p65 subunit consensus sequence (5x NFκB) was used to test the systems. (**a**) Schematic diagram of pTRE-EGFP (pTE) reporter with LTR (long terminal repeat), TRE (transcriptional response element), TATA box is used as a minimal promoter to drive transgene expression, EGFP (enhanced green fluorescence protein), PGK (phosphoglycerate kinase 1) promoter, and PuroR (puromycin resistance) marker. (**b**) Schematic diagram of pTRE-FLuc-EF1α-RLuc (pT-FER) reporter with FLuc (firefly luciferase), EF1α (elongation factor 1 alpha) promoter, and RLuc (*Renilla* luciferase). (**c**) GFP signals observed with the pTE reporter after TNFα stimulation. HK1 and HONE1 cells were transduced with pTE reporter and maintained in serum-free conditions for 24 h before addition of 50 ng ml^{-1} TNFα. GFP signals were monitored at 15 min and 24 h post TNFα addition. (**d**) Luciferase signals by the pT-FER reporter with TNFα stimulation. HONE1 cells were transduced with the pT-FER reporter. Bioluminescence signals of firefly luciferase and *Renilla* luciferase were acquired after 20, 50, and 100 ng ml^{-1} TNFα stimulation for 24 h.

achieve high efficiencies. However, transduction efficiency and clonal variation can be improved and minimized, respectively, and we found that the newly developed lentiviral vectors worked well with different cancer cell lines of interest. Besides three variables tested in this study, Denning *et al.* (2013) also showed that other conditions such as the sera and polycations should be considered for virus production and viral transduction, respectively. The formation of adherens junction was shown to prevent virus entry into host cells, and the lentiviral transduction efficiency was reduced with the formation of the cell adherens junction.[36–39] JNK1 could trigger the adherens junction formation and become activated, when the lentivirus enters into the cytoplasm. Inhibiting JNK1 and subsequent abrogation of adherens junction formation promote lentivirus entry and improve transduction efficiency. The transduction was also shown to occur more readily for cells in the periphery of the epithelial colonies than that for the cells in the center.[36,38] These observations are in line with our findings that reduced cell confluence results in high transduction efficiency because of reduced likelihood of adherens junction formation (Figure 1d). As can be seen, our results demonstrate an easy and convenient method for effective transduction for various cancer cell lines.

CMV was shown to be a more powerful promoter in HEK293T cells, as compared to EF1α, and the expression driven by different promoters is highly cell line-specific.[40,41] Our findings further show that the maximum expression levels driven by the EF1α promoter in both the pWPI and pLVX systems are highly similar among different cancer cell lines including seven NPC cell lines, A549, and HT1080 (Figure 1e and Supplementary Figure S1). Recently, we showed that by using the pWPI vector, NPC cell lines could express the candidate tumor suppressor *SAA1*, at a consistent physiological level with a transduction efficiency close to 100%.[42] These results clearly show that gene expression levels driven by EF1α in different cell lines are highly similar and are close to the physiological level expressed by the immortalized nasopharyngeal epithelial cell line. The achievement of physiological expression levels of candidate TSGs is especially important in cancer biology studies. Therefore, pWPI and pLVX-EF1α vectors are recommended for use of expressing transgenes in different cancer cell lines. In general, clonal variations and gene transfer efficiencies in cancer cells can be avoided and improved, respectively, using these lentiviral systems, as the transduction efficiencies of the current lentiviral systems can reach more than 90%. Although the lentiviral elements will be randomly integrated into the host genomes, different batches of lentiviral-infected cells may be used to minimize the positional effects of the integrated transgenes.

Leakage of the tetracycline-responsive promoter, vast overexpression of the gene of interest, and doxycycline-induced inhibitory effects, are the main concerns in using inducible expression systems to study TSGs.[18,21,43–45] Therefore, a modified lentiviral doxycycline- inducible system (pTet-IRES-EGFP, pTIE) to circumvent various drawbacks of other systems was established. To further compare the lentiviral pTIE system with the episomal pETE system,[14] EF1α-tTA-engineered HONE1, CNE2, and SUNE1 cell lines were transfected with the pETE-EGFP plasmid and the GFP signals were captured and analyzed (Supplementary Figure S8), using identical image settings to those used for images shown in Supplementary Figures S5 and S6. Transient transfection of pETE-EGFP resulted in much higher GFP expression levels. The GFP-positive population, however, reached only 22–24%. This finding is in line with another study in which the inducible tetracycline response promoter (pTet) was identified as a strong promoter,[41] resulting in high transgene expression in the cells. Nevertheless, the tested lentiviral systems including pWPI, pLVX, and pTIE systems in the present studies show moderate GFP expression and high stabilities among cell lines (Figures 1, 2, 3 and Supplementary Figures S1–S6 and S8). Haack *et al.* [46] also developed and optimized their inducible lentiviral vectors.[46] They showed that the basal gene expression was unable to be completely eliminated. The doxycycline itself does not affect the basal activities of the pTet promoter, but the *cis* elements in the lentiviral vectors such as woodchuck hepatitis virus (WHP) posttranscriptional regulatory element (WPRE), and the minimal CMV promoter may contribute to the promoter basal activity. Structural modification of the lentiviral vectors may further reduce basal activities. However, in our present Tet-Off system without structural modification, only 0.4 and 2.5% GFP signals were detected in the pTIE-alone and tTA-engineered pTIE-infected cells, respectively (Figure 3). Thus, the newly developed pTIE system only shows a low basal expression, revealing its usefulness in transduced cells. Indeed, our *in vivo* data clearly show that the transgene expression could be tight controlled and there was no leakage of gene expression. As a result, this basal expression seems not to affect the functional effects in the animal.

The TRE luciferase reporter assays are used to determine the activities of promoters of interest. One of the most commonly used systems is the dual-luciferase reporter assay system using two pGL4 plasmids containing separate firefly and *Renilla* luciferases. However, due to the low transfection efficiency and the transient expression nature of this plasmid in epithelial cancer cell lines, the promoter activities may not always be accurately represented. Nevertheless, the lentiviral reporter systems could provide a platform to stably transduce epithelial cell lines, which are difficult to transfect efficiently, for long-term studies. Therefore, these powerful lentiviral reporters now enable us to study pathway activity in a variety of cancer cell lines, as well as to extend studies to *in vivo* models, which remain to be tested.

In summary, the lentiviral systems that have been developed and established in the present study include the following: (1) pWPI and pLVX-EF1α constitutive transgene expression system, (2) pTet-IRES-EGFP (pTIE)-inducible transgene expression system, (3) TRE-EGFP (pTE) reporter system, and 4) single plasmid TRE-FLuc-EF1α-RLuc (pT-FER) reporter system. Infecting cells in suspension and high virus-to-cell ratio are recommended to achieve high transduction efficiency, and the EF1α promoter is suitable for expression studies. The inducible Tet-Off system stably expresses the transgene of interest and is tightly regulated by doxycycline *in vitro* and *in vivo*. In contrast, there is a doxycycline dose-dependent regulation of gene expression in the Tet-On system. For the reporter systems, GFP and firefly luciferase activity are successfully enhanced by TNFα addition in pTE and pT-FER systems, respectively, suggesting that they are indeed useful reporters for functional studies. Moreover, the consensus sequence is easily replaced by other promoters/consensus sequences of interest. Combining these newly developed systems and lentiviral shRNA-mediated knockdown systems, such as the pLKO.1 and Tet-pLKO,[47,48] provides enabling technology to overcome technical challenges in biological studies.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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