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Inducible and Reversible Transgene Expression in Human Stem Cells After Efficient and Stable Gene Transfer

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ABSTRACT

We report here a lentiviral vector system for regulated transgene expression. We used the tetracycline repressor fused with a transcriptional suppression domain (tTS) to specifically suppress transgene expression. Human cells were first transduced with a tTS-expressing vector and subsequently transduced with a second lentiviral vector-containing transgene controlled by a regular promoter adjacent to a high-affinity tTS-binding site (tetO). After optimizing the location of the tetO site in the latter vector, we achieved a better inducible transgene expression than the previous lentiviral vectors using the tetracycline repressor systems. In this new system, the transgene transcription from a cellular promoter such as EF1α or ubiquitin-C promoter is suppressed by the tTS bound to the nearby tetO site. In the presence of the tetracycline analog doxycycline (Dox), however, the tTS binding is released from the transgene vector and transcription from the promoter is restored. Thus, this system simply adds an extra level of regulation, suitable for any types of promoters (ubiquitous or cell-specific). We tested this tTS-suppressive, Dox-inducible system in 293T cells, human multipotent hematopoietic progenitor cells, and three human embryonic stem cell lines, using a dual-gene vector containing the green fluorescent protein reporter or a cellular gene. We observed a tight suppression in the uninduced state. However, the suppression is reversible, and transgene expression was restored at 5 ng/ml Dox. The lentiviral vectors containing the tTS-suppressive, Dox-inducible system offer a universal, inducible, and reversible transgene expression system in essentially any mammalian cell types, including human embryonic stem cells. STEM CELLS *2007;25:779–789*

INTRODUCTION

Recombinant viral vectors based on retroviruses remain the main choice to efficiently transduce primary mammalian cells or cell lines that are refractory to plasmid-mediated transfection [1]. As compared with oncoretroviral vectors (RVs), lentiviral vectors (LVs) offer several advantages. First, LVs can transduce both mitotically active and inactive cells [2–4]. Second, transgenes introduced with the LV backbone integrated into the host genome are more resistant to transcriptional silencing [2–5]. Third, the self-inactivating (SIN) LVs, which permanently disable the viral promoter within the viral long-terminal repeat (LTR) after integration, enable transgene expression to be solely controlled by an internally built promoter [2–10]. Fourth, LVs can better accommodate multiple promoters, either ubiquitous or cell-specific, in the same vector [10].

It is ideal, and quite often necessary, to have regulated or inducible transgene expression in gene function studies or gene therapies [11, 12]. This is particularly important for stem cells to achieve stage-specific or lineage-specific gene expression in progeny cells after stable gene transfer. SIN LVs that confer promoter-specific gene expression should also better accommodate an inducible promoter such as those based on tetracycline (tet)-inducible systems. In the widely used tet-off system, the DNA-binding domain of bacterial tet repressor (tetR) is fused to a mammalian transcription activation domain to make a transcription activator (tTA), which activates a synthetic promoter after binding to the adjacent tet operator (tetO) site [13]. In the presence of tet or its synthetic analog doxycycline (Dox), however, the binding of the tTA is released from the promoter, and the transcription is in the "off" state. The persistent presence of Dox is required to maintain the transgene in the off state, which is obviously inconvenient in many experimental and therapeutic settings. In the later "tet-on" system, a mutated tetR (rtTA) binds to tetO with a low affinity, but its binding is enhanced by Dox [13]. The transgene expression reaches the maximal induced or "on" state when a high concentration of Dox is added. However, the transgene expression is often not completely off in the absence of Dox. One approach to overcome this limitation is to select, expand, and use most favorable stable transfectant clones. However, it is not always possible to generate a large pool of individual stable clones. Although both tet-on and tet-off inducible systems have been incorporated to RV and LV systems [14, 15], the leakiness and low levels of induction have severely limited their wide use. Several groups recently reported

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their improvements over previous tet-on or tet-off LV systems that they previously made [16–18], and data with human stem cells are currently unavailable.

Others have attempted to use the KRAB transcriptional suppression (TS) domain tethered to the tTR to make a tet-suppressive system [19–21]. In the absence of Dox, the tTR-TS fusion protein (tTS) binds the tetO site and suppresses transcription initiation from a nearby polymerase II or III promoter located in both directions within a radius of 2–3 kilobases [19–24]. The binding and suppression will be released by adding Dox. Wiznerowicz and Trono published the evidence of using a LV system for tet-suppressive expression of transgene or small hairpin RNA (shRNA), in which both the tetO site and shRNA are located in the shortened U3 region in the LTR [25]. After viral DNA integration, shRNA and tetO and its promoter sequences are duplicated at the both ends of integrated vector to achieve a higher level of shRNA expression. In the absence of Dox, tTS tightly suppresses the expression of shRNA from the promoter next to the tetO site. When the Dox is added, the suppression is released, and shRNA is expressed from the both sites to achieve RNA interference [25–27]. Using the same vector system, we also observed the green fluorescent protein (GFP) gene interference in multiple cell types when Dox was added, and over 100-fold induction of the polymerase II transgene unit expression from the same vector. However, leaky expression of the polymerase II transgene unit was also observed. One possibility is that the tetO sites are located too far away (2,306 and 1,777 base pairs from the 5′ and 3′LTRs, respectively) from the transcription start site of the internal polymerase II promoter, rendering an incomplete suppression from the bound tTS.

In this study, we examined the effects of the tetO site at various locations in an improved LV. We evaluated the tight and inducible regulation of the transgene expression from two types of polymerase II promoters in several human cell types, including human $CD34^+$ multipotent hematopoietic progenitor cells and human embryonic stem (hES) cells. A tightly controlled, dose-dependent, and reversible transgene expression was achieved after stable gene transfer by LVs.

Methods and Materials

Human Cell Lines

Human 293T and HeLa were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, http://www.invitrogen.com) containing 10% fetal bovine serum (FBS). Three NIH-approved hES cell lines were used in this study. The H1 and H9 hES lines were obtained from WiCell Research Institute (Madison, WI, http://www.wicell.org) at passages 22 and 23, and the I-6 was obtained from Technion-Israel Institute of Technology (Haifa, Israel, http://www.technion.ac.il) at passage 30. All three hES cell lines were propagated on top of mitotically inactive primary mouse embryonic fibroblast (pMEF) feeder cells or human feeder cells as described [28–30]. A human $CD34^+$ hematopoietic progenitor cell line TF1 (ATCC CRL-2003) was cultured as previously described [9, 10, 31]. Briefly, TF1 cells were cultured in RPMI 1640 medium supplemented with 10% FBS and 1 ng/ml granulocyte macrophage-colony-stimulating factor (Peprotech, Rocky Hill, NJ, http://www.peprotech.com). TF1-PNH cell line containing a 7-basepair deletion in the coding region of the human phosphatidylinositol-glycan complementation class A (PIG-A) gene was a gift from Drs. Galina Muskina and Robert Brodsky (Johns Hopkins University). The TF1-PNH cell line, which lacks the PIG-A activity and all the glycosyl-phosphatidylinositol-anchored proteins (GPI-APs) on cell surface, is used to test the restoration of the *PIG-A* gene and GPI-APs [32]. Similarly, we established an H1 hES cell-derived clone, AR1-c1, lacking functional PIG-A proteins and GPI-APs (G. Chen, Z. Ye, L. Cheng, manuscript in preparation). The AR1-c1, H1, and H9 hES cells were karyotypically normal during the course of this study (before and after two rounds of LV transduction). The double-transduced I-6 cells, as well as their parental I-6 hES cells used in this study, were found karyotypically abnormal and then discontinued. The karyotyping (300–400 G banding) was performed by the Prenatal Cytogenetics Laboratory in the Johns Hopkins University Hospital as described previously [28].

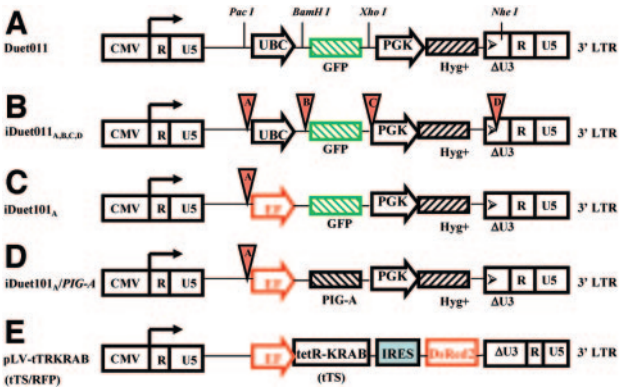

Figure 1. Schematic diagrams of lentiviral vectors used in this study. **(A):** An improved dual promoter vector Duet011. ▶, LoxP recombination site. **(B):** The Duet011 vector with the 7-copy tet operator ($tetO_7$) DNA sequence inserted at various locations (▼) to make tet-inducible vectors iDuet011: A, at the PacI site; B, at the BamHI site; C, at the XhoI site; D, at the NheI site. **(C):** A tet-inducible vector $iDuet101_A$ in which the EF promoter is used to replace the UBC promoter in $iDuet011_A$. **(D):** An $iDuet101_A$ vector in which the human PIG-A gene is used to replace the GFP gene. **(E):** A regulator lentiviral vector used in this study. More details given in Materials and Methods. Abbreviations: CMV, cytomegalovirus; DsRed2, modified DsRed gene encoding a red fluorescent protein; EF, elongation factor 1α; GFP, green fluorescent protein gene; Hyg^+, hygromycin resistance gene; IRES, internal ribosome entry site; LTR, long terminal repeat of lentiviral DNA; PGK, the mouse phosphoglycerate kinase promoter; PIG-A, phosphatidylinositol glycan A; R, repeat region in the viral long terminal repeat; RFP, red fluorescent protein; tTS, a tetracycline repressor (tetR or tTR) fused with the KRAB suppression domain; ΔU3, shortened U3 region lacking viral promoter and enhancer activities; U5, U5 regions in the viral long terminal repeat; UBC, human ubiquitin C promoter.

Lentiviral Vector Constructs

All the LVs except two were constructed in the Cheng laboratory (Fig. 1) following standard recombinant DNA techniques. The pLV-tTR-KRAB-DsRed2 and pLVTH-siGFP LVs were obtained from Dr. Didier Trono [25]. The pLV-tTR-KRAB-DsRed2 (Fig. 1E) is the regulator vector encoding tTR-KRAB (tTS) fusion protein and red fluorescent protein (RFP) [25]. The vector is abbreviated hereafter as tTS/RFP.

The other transgene expression vectors were based on an improved LV backbone and made in our laboratory. The new vector backbone was built in a plasmid that can replicate stably in high copy numbers in *Escherichia coli*, similar to the HR′CS-G vector [7]. The U3 deletion was further extended (−418 nucleotides [nt] to −9 nt; the 3′-R region starts at +1 nt). The central purine tract fragment and a ubiquitin-C promoter (UBC)-driven GFP cassette were added [5]. The new LV is named UG (for unigene). A dual-gene vector was constructed by inserting the mouse phosphoglycerate kinase 1 (PGK) promoter and hygromycin selection gene cassette from the MSCVhygro vector [33]. The LoxP sequence was inserted at the shortened U3 region. The resulting LV is dubbed the Duet (for dual-gene, excisable transgene) vector.

We next inserted the 7-copy tetO ($tetO_7$) DNA sequence into the Duet011 vector at various locations (Fig. 1B). The 309-base pair (bp) *tet*O_7 sequence was PCR-amplified from the template pLVTH-siGFP [25]. The inducible $iDuet011_A$ vector has the $tetO_7$ sequence inserted at the PacI site (with a BstXI linker), immediately upstream of the UBC promoter (Fig. 1A, 1B). The inducible $iDuet011_B$ has the $tetO_7$ sequence at the BamHI site, immediately downstream of the UBC promoter fragment (Fig. 1A, 1B). The inducible $iDuet011_C$ vector was

constructed by inserting the $tetO_7$ sequence at the end of GFP gene at the XhoI site, which is approximately 1,643 base-pairs downstream from the transcription start site of the UBC promoter (Fig. 1A, 1B). The inducible $iDuet011_D$ vector was constructed by inserting the $tetO_7$ sequence at the NheI site in the shortened U3 region of the 3′LTR (Fig. 1A, 1B). After viral DNA integration, the UBC promoter transcription start site is 3,643 base pairs upstream from the $tetO_7$ sequence in the 3′LTR and 2,222 base-pairs downstream from the $tetO_7$ sequence in the duplicated 5′LTR.

An EF1α-containing inducible vector, $iDuet101_A$ (Fig. 1C), was constructed by replacing the UBC promoter in $iDuet011_A$ with the EF1α promoter [9]. The inducible $iDuet101_A$/*PIG-A* vector (Fig. 1D) was constructed by cloning a human *PIG-A* cDNA (~1.5 kb coding sequence) into $iDuet101_A$ to replace the GFP gene. Construction details of all novel LVs are available upon request. They are in the process of being deposited to the Johns Hopkins University special collection at the American Type Culture Collection (Manassas, VA, http://www.atcc.org).

Production and Characterization of Novel LVs

All recombinant lentiviruses were produced by transient transfection of 293T cells, using the cytomegalovirus (CMV) ΔR8.91 and pMD.G helper plasmids [9, 10]. After overnight incubation, the culture medium was replaced with DMEM containing 1% FBS. The medium containing secreted viral particles was collected daily and filtered through a 0.45-μm filter (Corning, Acton, MA, http://www.corning.com). Viral titers were measured by an HIV-1 p24 enzyme-linked immunosorbent assay kit RETROtek (Zeptometrix, Buffalo, NY, http://www.zeptometrix.com) as described before [9]. By this assay, all Duet vectors we constructed have similar titers, equivalent to ~10^7 transducing units/ml using a validated EF.GFP vector as a control [9]. Concentrated virus (20× to 100×) was obtained by Amicon Ultra-15 centrifugal filter device (Millipore, Billerica, MA, http://www.millipore.com) and used to transduce hES cells in some experiments.

Lentiviral Transduction

293T and HeLa cells were transduced with a multiplicity of infection (MOI) of 20 as previously described [9, 10]. For transduction of TF1 and TF1-PNH cells, 1 ml LV supernatants and 8 μg/ml polybrene (Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com) were mixed with 0.2 million cells with an MOI of 50 and incubated for 8 hours [9, 10]. For transduction of hES cells cultured under a feeder-free condition, undifferentiated cell clumps were passaged onto Matrigel (BD Biosciences, San Diego, http://www.bdbiosciences.com) and cultured in pMEF-conditioned medium (pMEF-CM) for 1–2 days [30]. At the time of transduction, the mixture of 50 μl of concentrated (30×) LV supernatant and 6 μg/ml polybrene in pMEF-CM was applied to hES cells for 8 hours (MOI, 30–100). Transduced cells were then cultured with daily changes of pMEF-CM. Three days after transduction, cells were replated on fresh pMEF feeders.

Flow Cytometric Analysis of Transgene Expression

To analyze regulated transgene expression, target cells were first transduced with the tTS/RFP LV and sorted for RFP^+ cells by a FACSvantage flow cytometer (BD Biosciences). The expanded cells were then transduced with a second LV carrying GFP or PIG-A transgene. To analyze GFP expression, cells were cultured in the presence of Dox (Clontech, Palo Alto, CA, http://www.clontech.com; 0.5 μg/ml unless otherwise indicated) for the indicated days and then analyzed by flow cytometry. To analyze the restoration of GPI-APs on cell surface of TF1-PNH cells, fluoresceinated aerolysin (FLAER; Protox Biotech, Victoria, BC, Canada, www.ProtoxBiotech.com), an Alexa488-labeled inactive variant of aerolysin that binds to GPI-APs selectively, was used [34]. Cells were stained by FLAER for 15 minutes before fluorescence-activated cell sorting analysis. To analyze the restoration of GPI-APs on the surface of hES cells that lack endogenous PIG-A, we also used antibodies against human CD90/Thy-1 and CD59 (both mouse IgG). To analyze the regulated GFP expression in hES cells, single-cell suspension was stained by SSEA-3 or SSEA-4 (Developmental Studies Hybridoma Bank, Iowa City, IA, http://www.uiowa.edu/~dshbwww), followed by staining with anti-rat IgM or mouse IgG antibodies conjugated with Alexa647 (Invitrogen). Similarly, Tra-1–60 (mouse IgM; Chemicon, Temecula, CA, http://www.chemicon.com) was used to identify undifferentiated hES cells with anti-mouse IgM antibodies conjugated with Alexa647 as the secondary antibody.

RESULTS

Construction of Optimized Lentiviral Vectors for Dual-Gene, Excisable Transgene Expression

We improved the LV backbone for dual-gene expression, based on our previous work to express two transgenes simultaneously [10]. Unlike previous versions we used [9–10], the new vector backbone is based on a plasmid that can replicate stably in high copy numbers in *E. coli*. In addition, we added the following features. (a) The U3 deletion was further extended to reduce residual U3 viral promoter/ enhancer activities that may remain in the previous versions [2–10]. (b) The internal CMV promoter/enhancer in the previous versions was replaced with more ubiquitous ones, such as those from cellular housekeeping genes EF1 α and UBC. (c) A second expression cassette containing the PGK promoter and the hygromycin selection (hyg^+) gene was added, which allows a preselection of transduced cells independent of transgene functions. (d) A loxP site is added into the shortened 3′ U3 region, allowing the Cre recombinase-mediated excision of both transgenes from the integrated vector [35]. This new type of dual promoter/dual-gene expression vectors is dubbed Duet (for Dual-gene, excisable transgene) LVs. An example (Duet011) is shown in Figure 1A.

Effects of the tetO Site on tTS-Mediated Transgene Suppression

The improved Duet011 vector also allows us to insert the tetO site at various locations (Fig. 1B). A 7-copy tetO ($tetO_7$) DNA sequence was inserted immediately upstream to the UBC promoter (site A) to make $iDuet011_A$, downstream to the UBC promoter fragment (site B) to make $iDuet011_B$, downstream to the GFP transgene (site C) to make $iDuet011_C$, or in the shortened U3 region of 3′LTR (site D) to make $iDuet011_D$. The latter would be an analog to the pLVTH-siGFP, in which the $tetO_7$ in the 3′LTR will be duplicated after viral integration [25]. The location of tetO did not affect viral titers.

We next compared these four inducible vectors in 293T cells expressing the tTS suppressor (+tTS) or lacking tTS (−tTS), with the parental Duet011 vector as a control (Fig. 2A). In the 293T-tTS group (Fig. 2A, left column), GFP transgene levels without Dox are shown, and adding Dox had no effects (data not shown). For the cells transduced by the parental Duet011 vector lacking the $tetO_7$ site (Fig. 2A, top row), GFP expression in the presence or absence of Dox is also similar in +tTS cells, as expected. However, we noticed that the mean fluorescent intensity (MFI), as well as the percentage of GFP-positive cells, was less in +tTS cells than in −tTS cells. The mechanism of this tetO-independent but tTS-dependent suppression needs further investigation. When Dox was added into +tTS cells, $iDuet011_A$ and $iDuet011_C$ conferred 64% and 71% of GFP^+ cells, respectively (Fig. 2A, right column). The percentage by $iDuet011_D$ (last row) was significantly lower (33%) compared with $iDuet011_A$ and $iDuet011_C$; so was the MFI of the positive cells. Unexpectedly, the $iDuet011_B$ vector essentially failed to express the GFP transgene in the presence of Dox, and the expression level was also much lower in parental 293T cells (Fig. 2A, third row). Since the viral titer of $iDuet011_B$ measured by the HIV

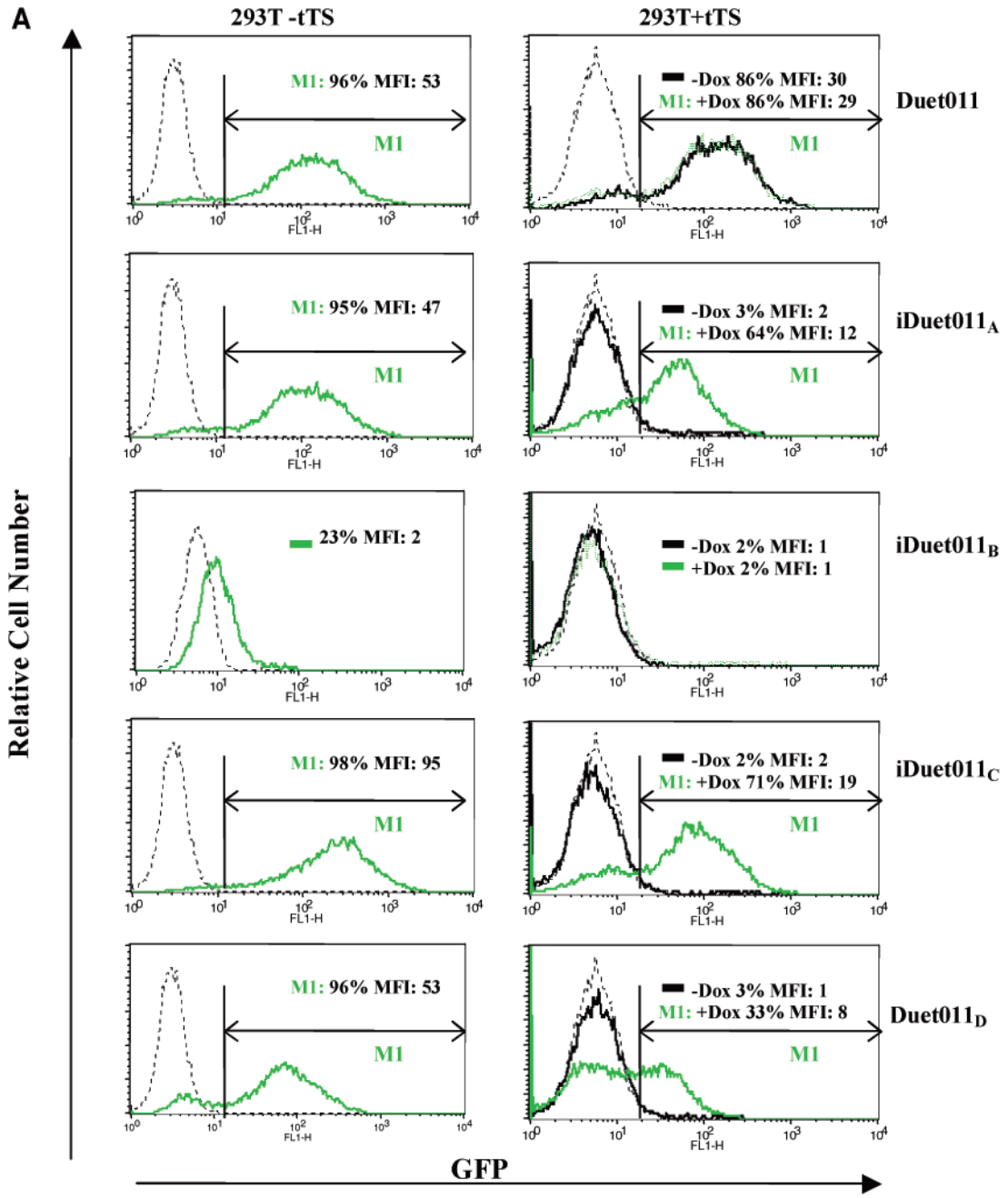

Figure 2. Dox-induced GFP transgene expression in human 293T and TF1 cells. **(A):** GFP transgene expression in 293T cells expressing the tTS (+tTS) and parental 293T cells lacking tTS (−tTS) regulator. Various indicated Duet011 viruses (with the human ubiquitin C promoter) were used to transduce both 293T cell types in parallel. Three days after transduction, cells were incubated with 0.5 μg/ml Dox (+Dox) or without Dox (−Dox) for 7 days and analyzed for transgene (GFP) expression after stable gene transfer. The background level of 293T cells (+tTS or −tTS) that were not transduced by a GFP vector was set at ≤1%, and its MFI was set at 1. The MFI value of all other samples was expressed as a ratio of their actual MFI to that of the control. **(B):** Regulated GFP expression in 293T cell transduced with the iDuet101$_A$ vector (containing the EF1α promoter). Three days after transduction, cells were incubated with Dox for 7 days and then analyzed. **(C):** Regulated GFP expression in TF1 cell transduced with the same iDuet101$_A$ vector. Three days after transduction, cells were incubated with Dox for 4 days and analyzed. The data are representative of three independent experiments. Abbreviations: Dox, doxycycline; GFP, green fluorescent protein gene; MFI, mean fluorescent intensity; tTS, transcriptional suppression domain.

p24 level was similar to those of its iDuet011 counterparts, and the GFP expression was also low in the transfected 293T cells during viral production (data not shown), we suspect that there is a block in GFP transgene expression. One possibility is that the tetO$_7$ sequence inserted (between the intron in the extended UBC promoter fragment but before GFP) is transcribed from the UBC promoter and inhibitory to GFP translation from the mRNA. The exact mechanism of GFP expression blocking remains unsolved.

For the best transgene regulation, it is also critical to assess the basal level expression (in +tTS cells) in the absence of Dox. Essentially, few cells were GFP-positive after transduction by

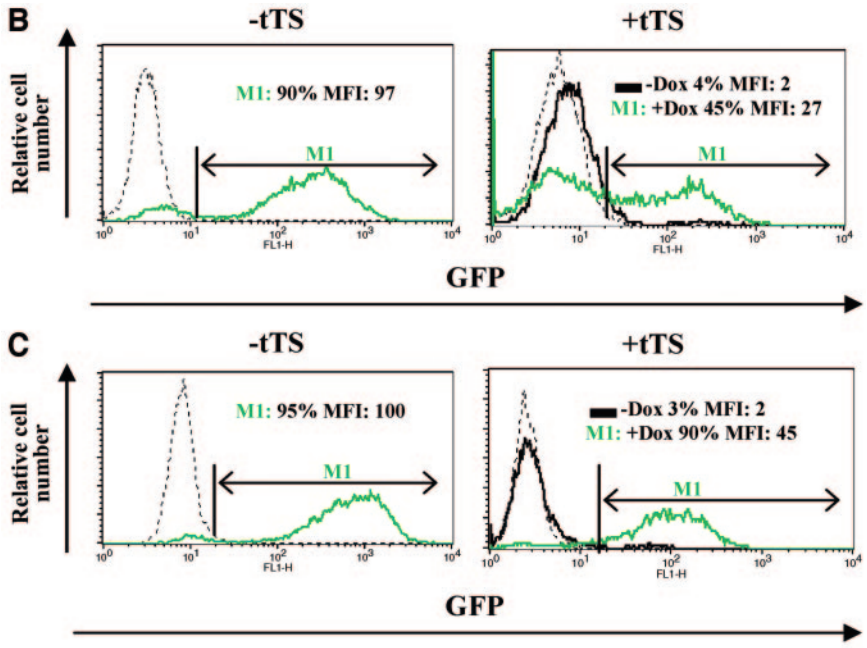

Figure 2. Continued

any of the four iDuet011 vectors when Dox was absent, and the MFI of the whole population did not increase more than twofold over the background of 293T cells. Similar results were also observed in HeLa cells expressing tTS (data not shown). Thus, the double copy strategy that places the $tetO_7$ site in each of the two LTR regions is not necessary to suppress transgene expression from an internal promoter. Based on the negligible or very low basal activity in the off state and the high level of transgene expression in the on state, both $iDuet011_A$ and $iDuet011_C$ vectors appeared adequate.

Induced GFP Expression from the tet-Regulated EF1α Promoter

To further determine whether the tet-suppressive system is also applicable to other promoters, we used the EF1α promoter to replace the UBC promoter to make the $iDuet101_A$ vector (Fig. 1C). We first tested the $iDuet101_A$ vector's ability to regulate GFP reporter expression in 293T+tTS cells (Fig. 2B). The result showed that the tTS similarly suppressed the GFP transgene expression from the EF1α promoter. In the presence of Dox, 45% of cells expressed GFP. The MFI of induced GFP^+ cells is 27, which is more than twofold stronger than that from the UBC promoter (MFI = 12, from the $iDuet011_A$ in Fig. 2A). This is consistent with our previous observations that the EF1α promoter is 2–3-fold stronger than the UBC promoter in proliferating human cell types (described below).

We also tested the tet-suppressive LV in human multipotent hematopoietic progenitor line TF1, which is $CD34^+$ and capable of differentiating into multiple types of erythroid and myeloid lineage [31]. The $iDuet101_A$ vector was used to transduce TF1 cells expressing or lacking tTS. Three days later, we added Dox and monitored it for 4 days (Fig. 2C). In the presence of Dox, 90% of cells were induced to express GFP, which is close to the 95% in −tTS TF1 cells (revealing the maximum by the GFP transduction). In the absence of Dox, a small fraction (~3%) expressed GFP at a low level (MFI of the whole cell population is approximately twofold compared with the untransduced cells). This is much lower than the MFI [45] when Dox was added. Based on data in both 293T and TF1 cells, we used the $iDuet101_A$ vector containing the regulated EF1α promoter for the regulated expression of other transgenes.

Induced PIG-A Transgene Expression

The *PIG-A* gene is required for the synthesis of GPI-APs, a group of cell surface proteins [32]. The human *PIG-A* gene was found mutated in paroxysmal nocturnal hemoglobinuria (PNH) patients [32, 34, 36]. A somatic mutation in the *PIG-A* gene occurs at the HSC level in a given PNH patient, resulting in a clonal mutation found in all the hematopoietic cell lineages. PNH patients with complete *PIG-A* deficiency lack dozens of GPI-APs, such as CD55 and CD59, two surface molecules serving as complement inhibitors. We constructed $iDuet101_A$/*PIG-A*, in which the human *PIG-A* cDNA replaced the GFP gene in the $iDuet101_A$ (Fig. 1D). The TF1-PNH cell line, which lacks functional PIG-A protein and all GPI-APs, was used to test the new *PIG-A* inducible vector. As before, we transduced the $iDuet101_A$/*PIG-A* vector into the TF1-PNH cells that had been transduced with the tTS/RFP regulator vector and sorted for RFP-expressing cells. The restoration of PIG-A proteins was monitored by the presence of GPI-APs on cell surface using a specific fluorescent probe [34].

After Dox was added to the double-transduced cells, 41% of cells transduced by the $iDuet101_A$/*PIG-A* vector (Fig. 3, upper rows) were stained positive for GPI-APs, with MFI = 17 (Fig. 3A). In contrast, only 5% of cells stained positive by a control vector (Fig. 3C), likely reflecting the background staining for cell surface GPI-APs. In the absence of Dox, 7% of cells transduced by $iDuet101_A$/*PIG-A* stained positive (Fig. 3B), similar to the background staining (~5%) in Figure 3C and 3D by the control vector. We further confirmed the presence of GPI-APs after restoration by the $iDuet101_A$/*PIG-A* vector and Dox induction by staining for the protein portion of CD55 and CD59 that are GPI-APs expressed on cell surface of TF1-PNH cells (data not shown). Taken together, these results indicate that our system allows inducible expression of a cellular gene to restore biological functions.

Time Course and Dose Dependence of Induced Gene Expression

We next investigated the kinetics of transgene expression after adding Dox. To this end, tTS-expressing TF1-PNH cells that ex-

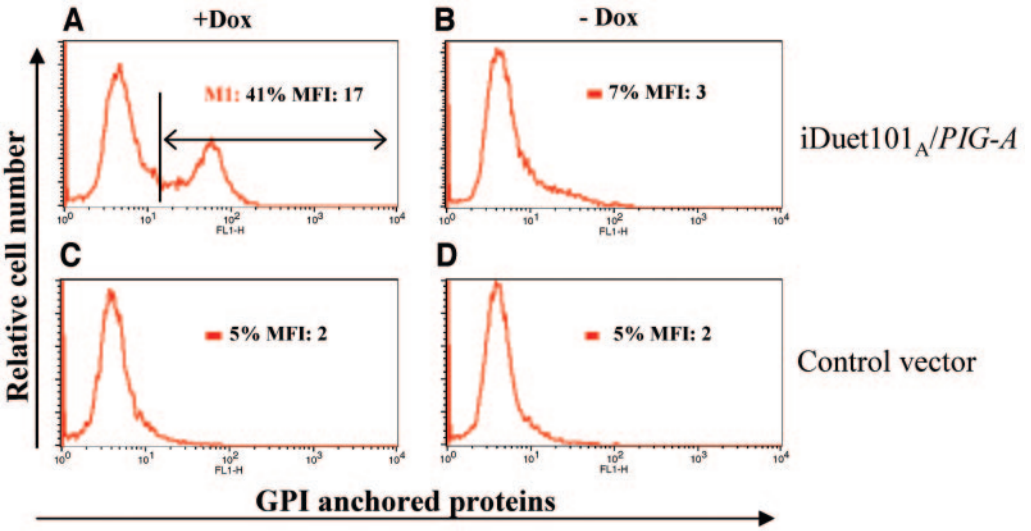

Figure 3. Dox-induced PIG-A expression and restoration of GPI-anchored proteins on cell surface. The TF1-PNH cells lacking a functional copy of the *PIG-A* gene and GPI-anchored proteins are used to test Dox-induced *PIG-A* transgene expression. TF1-PNH cells expressing tTS (+tTS) were transduced with either the iDuet101$_A$/*PIG-A* virus **(A, B)** or a control virus lacking PIG-A expression **(C, D)**, respectively. Three days after transduction, 0.5 μg/ml Dox was added to the culture medium and incubated for 4 days as indicated by +Dox **(A, C)**. The cultures without Dox induction were used as controls indicated by −Dox **(B, D)**. Cells were harvested for analysis of PIG-A expression and thus GPI-anchored protein restoration on cell surface by FLAER, an Alexa488-labeled probe that binds to GPI anchors selectively. After 15 minutes of incubation, cells were subjected to fluorescence-activated cell sorting analysis. A background staining (5%) was observed with a relative MFI of 2–3, as compared with that of by Alexa488 alone (1% and MFI = 1) with parental TF1-PNH cells. The data are representative of four independent experiments. Abbreviations: Dox, doxycycline; GPI, glycosyl-phosphatidylinositol; MFI, mean fluorescent intensity.

press either GFP or PIG-A transgene were sorted after induction by Dox. Sorted, double-positive cells were expanded in the absence of Dox for two passages (~6 days). Then, Dox was added and transgene expression was monitored daily (Fig. 4). In sorted TF1-PNH cells that co-express tTS and harbor the *PIG-A* transgene, the *PIG-A* transgene expression diminished after Dox withdrawal since GPI-APs were nearly undetectable (Fig. 4A, ii). Upon Dox induction, the percentage and MFI gradually increased to the maximal (99%) within 2 days (Fig. 4A, iv). Similar data were obtained by evaluating GFP expression in the iDuet101$_A$-transduced cells, although it took 3 days to reach the maximal level of GFP expression (Fig. 4B). In these sorted TF1-PNH cells, the cycle of induction (+Dox) and suppression (−Dox) can be repeated many times, and nearly 100% of cells expressed transgene after Dox induction even after months of continuous cell cultures (data not shown). We also examined the Dox dose-response curve required for transgene expression (Fig. 4C). In both cases, percentages of transgene-expressing cells reached a level close to the maximum with as low as 5 ng/ml of Dox after 3 days of treatment. The requirement for a low-level Dox to turn on the transgene expression will likely facilitate the in vivo regulated transgene expression.

Induced Transgene Expression in Human Embryonic Stem Cells

It has previously been demonstrated that hES cells can be efficiently and stably transduced by LVs without losing their pluripotency and differentiation potentials [37–40]. Using our previous versions of unconcentrated LVs containing the GFP reporter gene driven by the EF1α or UBC promoter, we could routinely achieve an efficiency such that ≥50% of cells stably expressed GFP in undifferentiated hES cells without overt toxicity (data not shown). Our data are consistent with several recent publications using similar LVs [37–40], which is in sharp contrast to the reported stable transfection efficiencies of $\leq 10^{-5}$ using plasmid vectors and various transfection methods [41, 42]. However, we also observed a gradual reduction of GFP$^+$ cells after extended culture in the absence of further selection (typically ~0.5% per day), although there were always GFP$^+$ cells present (data not shown). For basic research, such as elucidating functions of a specific gene, and for translational research, such as enhancing cellular functions, a regulated transgene expression in hES cells is often desirable. Therefore, we tested the above tet-suppressive, Dox-inducible system in hES cells to see whether we could achieve regulated transgene expression in hES cells after stable gene transfer. As before, hES cells were first transduced by the tTS/RFP regulator vector. After sorting and expansion, tTS-expressing hES (+tTS) cells were used in various assays. Although these tTS-transduced cells grew and behaved similarly to untransduced hES cells, some of them were more likely to lose RFP expression during the necessary expansion or prolonged cell culture. This is likely due to the use of DsRed2-encoded as terameric RFP protein, which appears to be less stable in hES cells than GFP (data not shown). Nonetheless, we transduced the tTS-transduced as well as parental H1 hES cells with the iDuet011$_C$ vector (Fig. 5), which contains the inducible UBC promoter controlling GFP reporter and the hygromycin-resistant gene (Fig. 1B). In the absence of Dox, approximately 30% of H1 parental hES cells became GFP$^+$ after two passages (data not shown), but very few cells (~1%) became positive in the tTS-hES cells (Fig. 5A) even after several more passages lasting for 24 days (Fig. 5B). After Dox induction, however, ~20% of tTS-H1 cells became GFP$^+$ (Fig. 5C–5E). We next used hygromycin selection to enrich GFP-transduced tTS-H1 cells. After 12 days of selection, up to 39% of tTS-H1 cells expressed GFP, although cells lacking tTS/RFP expression were also increased to 23% (Fig. 5F). The induced GFP transgene was shut down again by Dox withdrawal (Fig. 5G, 5H). GFP$^+$ cells in the tTS/RFP$^+$ cell population (Fig. 5G, 5H, upper right quadrants) were reduced to background level (~1.4%), although some GFP$^+$ cells could be detected in hES cells lacking tTS/RFP expression (Fig. 5G, 5H, lower right quadrants). Moreover, GFP transgene could be induced again by Dox (Fig. 5I, 5J). After 6 and 8 days, GFP$^+$ cells increased again, to 28% and 32%, respectively.

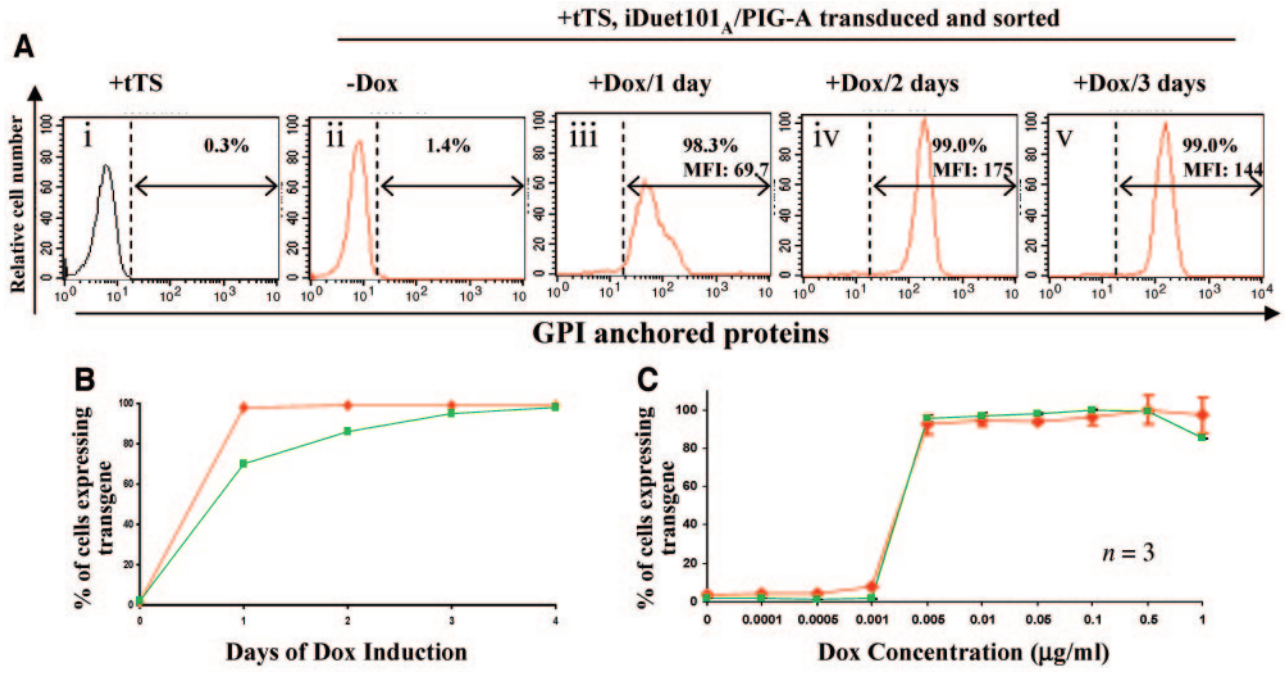

Figure 4. Time course and dose-response curve of Dox induction of PIG-A and green fluorescent protein (GFP) transgene expression. **(A):** Time course of phosphatidylinositol glycan A (PIG-A) expression and GPI-anchored protein (GPI-AP) restoration after Dox induction. TF1-PNH expressing tTS cells (i) were transduced with the iDuet101$_A$/PIG-A virus, induced by Dox, and sorted for PIG-A/GPI-AP$^+$ cells. Sorted cells were then expanded in the absence of Dox for several passages before they were induced again with Dox (0.5 μg/ml). Cells were harvested at various days and analyzed for the presence of GPI-APs on cell surface by FLAER staining, as in Figure 3. In the absence of Dox induction, the basal level of staining (1.4%) was observed (ii). After Dox induction, percentages of positive cells and their MFI gradually increased with time. After 2 days (iv), 99% of cells were positive with a relative MFI of 175 over the background level or when Dox was absent. **(B):** Time course of Dox induction. Mean percentages of GPI-AP$^+$ cells in **(A)** were plotted as a function of time (red diamonds) from triplicate samples. Similarly, TF1-PNH cells expressing tTS cells were transduced with the iDuet101$_A$ virus, and GFP$^+$ cells (green squares) were analyzed as in **(A)**. **(C):** Dependence of GFP or PIG-A expression level on Dox concentration. Dox was added at the indicated concentrations to two types of sorted cells and incubated for 4 days. Cells were harvested and analyzed for percentages of GFP-expressing cells (green line) or PIG-A expressing cells (red line), respectively. Mean and SD values of triplicates were plotted. Abbreviations: Dox, doxycycline; GPI, glycosylphosphatidylinositol; MFI, mean fluorescent intensity; tTS, transcriptional suppression domain.

Similar results were obtained with the iDuet101$_A$ vector that contains EF1α promoter driving GFP and the tetO-binding site at the position A. In the absence of Dox induction, a slightly higher level of background GFP$^+$ cells was observed: ~4% and 6% in tTS/RFP$^+$ and tTS/RFP$^-$ cell population of the tTS-H1 cells (Fig. 6A). After Dox induction and hygromycin selection, ~57% and 32% of cells were GFP$^+$ in tTS/RFP$^+$ and tTS/RFP$^-$ cells, respectively (Fig. 6B). The induced GFP$^+$ cells expressed a high level of Tra-1–60, an undifferentiated marker for hES cells (Fig. 6C). The double-transduced hES cells (H1 here and H9 below) also maintained a normal karyotype (supplemental online Fig. 1).

Alternatively, the cell population in upper right quadrants could be sorted either before or after Dox induction to get rid of unwanted cell populations. An example of an experiment with sorted GFP$^+$ cells is provided in supplemental online Fig. 2. We did not do so in the sequential experiment described in Figure 6, so that tTS/RFP$^-$ cells are always present and provide an internal reference. The induced transgene (GFP) expression was shut down after Dox withdrawal (Fig. 6D) and re-induced by Dox addition (Fig. 6E, after 6 days), although the GFP$^+$ cells did not reach the maximal level of 57% (Fig. 6B). We also tested this inducible system in two other hES cell lines. H9 hES cells were transduced by the tTS/RFP regulator LV first, sorted, and expanded as we did before. Then tTS-H9 hES cells were transduced by the iDuet101$_A$ vector (Fig. 6F–6H), as we did for H1 hES cells (Fig. 6A–6E). After Dox induction and hygromycin selection, ~15% of the cells became GFP$^+$ cells and expressed tTS/RFP. The low percentage is due to the fact that tTS-transduced H9 cells quickly lost the tTS/RFP during hygromycin selection, so that most of GFP$^+$ cells are from the tTS/RFP-negative population (Fig. 6H). Similar experiments were also conducted with the I-6 hES cell line, and the data are shown in supplemental online Fig. 3.

We next tested the regulated expression of a cellular gene using this tTS-suppressive and Dox-inducible LV system in hES cells. This was made possible by using the iDuet101$_A$/*PIG-A* vector and an H1-derived hES cell line AR1-c1 lacking the endogenous *PIG-A* expression. Similar to the TF1-PNH cells (Figs. 3, 4), AR1-c1 hES cells lack the functional PIG-A proteins and thus lack all GPI-APs on cell surface (G. Chen, Z. Ye, L. Cheng, et al., manuscript in preparation). Similar to the wild-type H1 hES cells using the iDuet101$_A$ (GFP-expressing) vector (Fig. 6A–6E), we transduced the AR1-c1 hES cell line with the tTS/RFP regulator vector, sorted by RFP, and expanded the cells for several passages. After transduction by the iDuet101$_A$/*PIG-A*, the double-transduced AR1-c1 hES cells were induced with Dox and cultured on feeder cells. After induction for one passage (5 days), the hES cells (which are GFP$^+$) were analyzed for the expression of GPI-APs (Fig. 7). CD90/Thy-1, a GPI-AP expressed at a high level in undifferentiated hES cells, was used as the first GPI-AP marker. With Dox induction, 55% of hES cells expressed CD90, compared with 7% when Dox was absent (Fig. 7A, 7B). Of note, nearly all the GFP$^+$ cells expressed undifferentiated hES cell marker Tra-1–60 (Fig. 7C, 7D) and SSEA-4 (Fig. 7E, 7F), confirming the nature of undifferentiated hES cells after double transduction and Dox induction. Similar results were obtained with other GPI-APs, such as CD59 (Fig. 7H), although the staining intensity was weaker than that of CD90 (Fig. 7G). In summary, our studies used three differ-

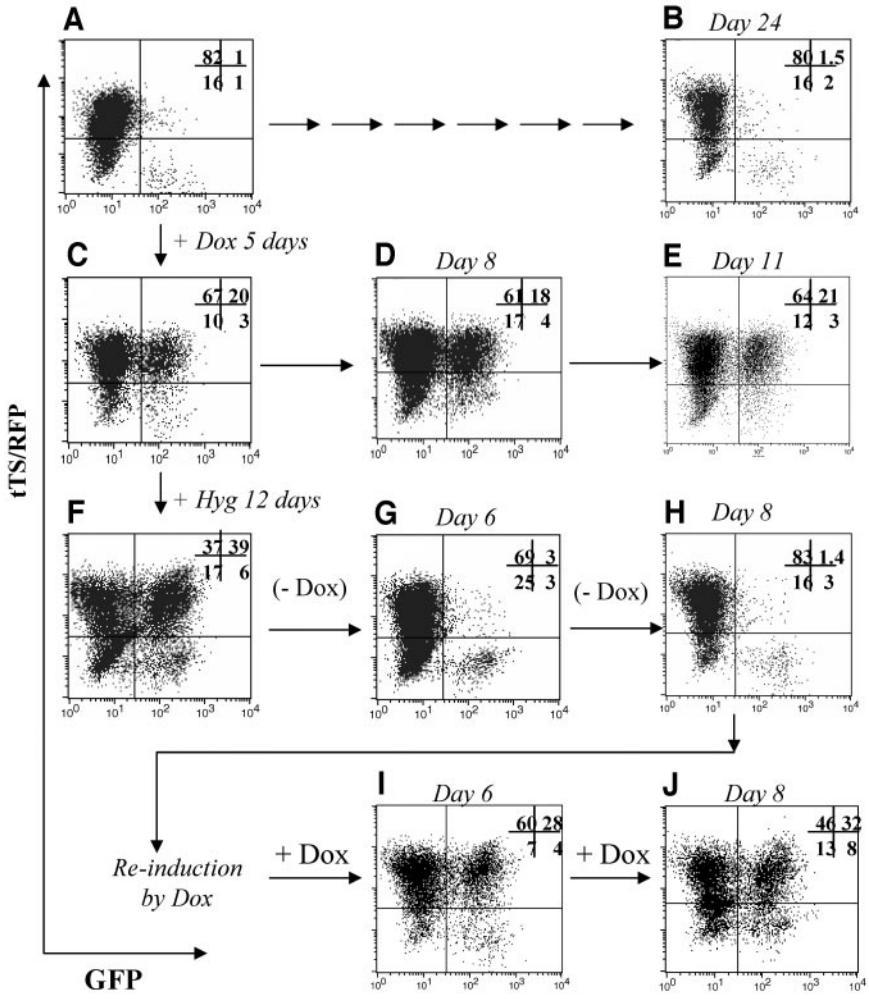

Figure 5. Inducible and reversible regulated transgene expression in human embryonic stem (hES) cells transduced by tTS and iDuet011$_C$ vectors. H1 hES cells were first transduced by the tTS/RFP regulator vector, sorted, and expanded. Then, hES cells were transduced by the iDuet011$_C$ vector, expressing inducible GFP transgene from the human ubiquitin C promoter. hES cells were analyzed by flow cytometry, identified by the expression of the Tra-1-60 undifferentiated marker. Induced GFP expression (*x*-axis) in RFP$^+$ and RFP$^-$ (*y*-axis) hES cells was analyzed, and the percentage of each hES cell population is denoted. In the absence of Dox induction, few hES cells became GFP$^+$ after two passages **(A)** or several more passages **(B)** (additional 24 days). In contrast, ~30% of iDuet011$_C$-transduced cells were GFP$^+$ in the parental H1 hES cells lacking tTS/RFP in parallel experiments (not shown). After Dox induction (0.5 μg/ml), ~20% of cells became GFP$^+$ **(C–E)**. Transduced cells were selected based on the co-expression of Hyg-resistant gene controlled by the PGK promoter. Twelve days after selection (with Dox), 39% of hES cells became GFP$^+$, although tTS/RFP$^-$ cells also increased **(F)**. Upon withdrawal of Dox, transgene (GFP) expression was reversibly shut down **(G, H)**. However, GFP expression can be reinduced upon Dox addition, and up to 32% of hES cells became GFP$^+$ again **(I, J)**. Similar results with the iDuet101$_A$ vector are shown in Figure 6. Abbreviations: Dox, doxycycline; GFP, green fluorescent protein; Hyg, hygromycin; RFP, red fluorescent protein; tTS, transcriptional suppression domain.

ent hES cell lines, two different promoters (EF1α and UBC), and two different transgenes (GFP and PIG-A). The results demonstrate that the LV-based, tTS-suppressive, and Dox-inducible system can effectively regulate transgene expression in hES cells, albeit with some limitations. Plausible explanations and possible improvements in the future are discussed below.

DISCUSSION

In this study, we demonstrate that the LV-based vector system we developed and validated can achieve regulated expression of various transgenes after efficient and stable gene transfer in multiple cell types. In most cell types, the transgene induction is fast (~3 cell cycles to reach the maximum) and dynamic at a low concentration of Dox (~5 ng/ml). This is achieved by incorporating tet-mediated transcription suppression system to our improved dual-promoter (Duet) LVs, building upon a previous success [25]. During the preparation of this article, Szulc et al. reported that they incorporated this system into a single LV to control shRNA and transgene expression [43]. They demonstrated their success in mouse ES cells, primary human CD34$^+$ hematopoietic progenitor cells, and several in vivo mouse models [43]. The key component in all the tet-suppressive systems is the tTS (tetR-KRAB) fusion protein, which functions as a tetO-binding transcription suppressor or silencer. The choice of promoters for transgene expression is very flexible: either a ubiquitous or a specific promoter. This system just adds one more level of transcriptional control (mediated by tTS) on the promoter. The new system maintains the low leakiness and high inducibility of the original tetR used in the tet-off system, with an outcome similar to the tet-on system after Dox induction. Without Dox, the transgene or shRNA expression is fully suppressed in transduced cells in vivo or in vitro. With a very low concentration of Dox, the transgene or shRNA is turned on even after a prolonged suppression state. Together, the LV-based, tet-suppressive systems developed by us and others offer a better approach to achieve regulated transgene expression in vivo and in vitro after efficient and stable gene transfer. To avoid unnecessary confusion with the original tet systems that are commonly known as tet-off and tet-on systems, we propose to abbreviate this relatively new system as "tet-SUPER" (for the suppression or repression [the last three letters in super in reverse order, rep] mediated by tTS.

The tTS/RFP regulator used here was previously constructed by Wiznerowicz and Trono [25], but other components of the tet-SUPER LV system described here differ from previous and recently published LV systems in two respects [25, 43]. First, we demonstrated that it is more important to place the tetO site in proximity to the regulated promoter. The double copy strategy that places the tetO in the shortened U3 region of the LTR is not essential or advantageous, especially for the internal promoter that is distant from the LTRs. Second, we believe that the two-vector system offers certain advantages in many cases. The reported single vector that expresses both transgene and tTS as a single transcript has to be leaky, at least transiently [43].

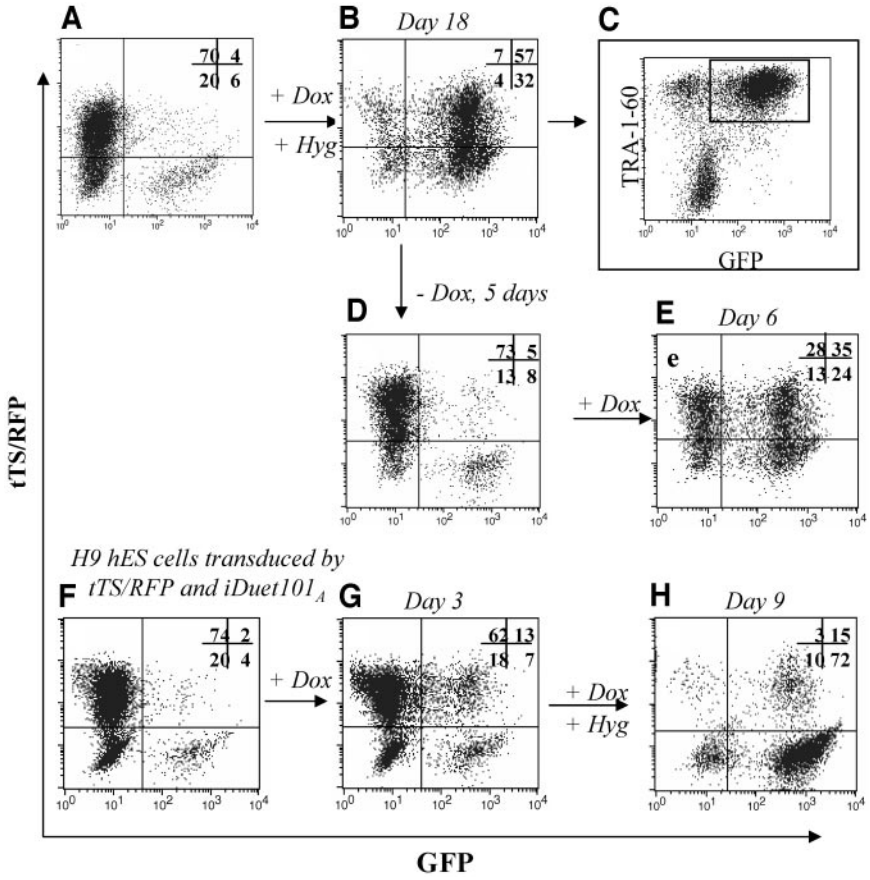

Figure 6. Regulated transgene expression in H1 and H9 human embryonic stem (hES) cells transduced by tTS and iDuet101$_A$ vectors. The same tTS/RFP-transduced H1 hES cells (Fig. 5) were used for testing the iDuet101$_A$ vector that contains the EF1α promoter and GFP reporter **(A–E)**. Although background level of GFP$^+$ cells was slightly higher in the absence of Dox **(A)**, the percentage and intensity of GFP$^+$ cells was also greater **(B)** compared with that by iDuet011$_C$ (Fig. 5). Note that nearly all the GFP$^+$ cells expressed a high level of Tra-1-60 **(C)**, a marker for undifferentiated hES cells. In fact, cells positive for Tra-1-60 cells were gated in all the panels (except **[C]**) to exclude feeder cells and analyze only the undifferentiated hES cells in culture. Similar to iDuet011$_C$, transgene expression from the iDuet101$_A$vector was shut down by Dox withdrawal **(D)** and reinduced again by adding Dox **(E)**. Similar results were achieved using H9 hES cells **(F–H)**, although somehow tTS/RFP expression was lost faster. Results with I-6 cells are shown in supplemental online Fig. 3. Abbreviations: hES, human embryonic stem; Hyg, hygromycin; RFP, red fluorescent protein; tTS, transcriptional suppression domain.

Otherwise the tTS would not be expressed, and the suppression on the transcription of transgene and itself (linked by internal ribosome entry site [IRES]) could not be established. The elegant negative feedback strategy used in this single-vector system required a fast turnover of tTS and longer time to reach equilibrium. To use a separate vector to constitutively express tTS in our two-vector system will ensure the presence of tTS before the transgene expression. In addition, putting the tTS regulator, IRES, a transgene, a selection marker, and other regulator elements into a single vector may not be possible, since the LV payload is limited. Thus, our two-vector tet-SUPER system should offer certain advantages, especially for transgene expression, as we intended for this study. For example, the dual-promoter design in our vector system (i.e., the hygromycin selection cassette) would allow us to select transduced cells easily after Dox induction, regardless the nature of the transgene or its promoter (ubiquitous or stage-specific). A disadvantage of the two-vector system, however, is that we need to transduce a novel target cell twice. Using a high titer of both vectors in a simultaneous transduction may increase the probability of aberrant integrations and genome instability.

During this study, we also revealed an unexpected feature of LVs containing dual promoters (and dual genes), such as the Duet vectors described here. We and others have used the ubiquitous PGK promoter as the second promoter (to drive a selection gene), in addition to the EF1α or UBC promoter normally reserved for the gene of interest (transgene) upstream to the PGK promoter [10, 44, 45]. When we inserted the tetO-binding site at the position C (between the transgene and the PGK promoter; Fig. 1), we observed that the transgene expression level is even higher than that from the parental Duet011 vector in cells that did not express tTS at all (Fig. 2A, left columns). One explanation is that PGK promoter used here is bidirectional, and a transcript going upstream would be an antisense inhibitor to the transgene transcript from the UBC promoter going downstream [44]. The insertion of the 7-copy tetO-binding site (and the palindrome structure) may block this transcript from the PGK promoter, similar to the case in iDuet011$_B$, where the tetO-binding site, as a part of transcript, blocked the GFP expression (Fig. 2A). Thus, the GFP expression from the UBC promoter is relatively greater by the iDuet011$_C$ vector than that from its parental Duet011 vector (lacking the tetO-binding site). This speculation is further supported by a recent report describing an improved version of LV [45]. It was found that if the distal (5′) region of the downstream PGK promoter (314 bp) is deleted, the expression of the upstream GFP gene is enhanced [45]. The insertion of the tetO site at the position C for the tet inducible system is beneficial, reducing unwanted effects of the PGK selection cassette on inhibition of the upstream transgene. However, the potential disadvantage with the tetO$_7$ site at the C position (after transgene) is that it may be farther away from the promoter if the size of a transgene could be much larger than that of GFP (~720 bp), resulting in the reduced efficiency of the upstream promoter suppressing.

There are several limitations in the tet-SUPER LV system we describe here and in previous publications [25, 43], which could be overcome. First, leaky expression in the absence of Dox is much lower (compared with the tet-on system) but still present. This could be due to the following: (a) the trace amount of tetracycline or its derivatives present in FBS when the bovine received similar antibiotics in its lifetime; and/or (b) the transgene being transcribed from a cellular promoter after the LV integrated into a transcriptionally active chromatin region. The

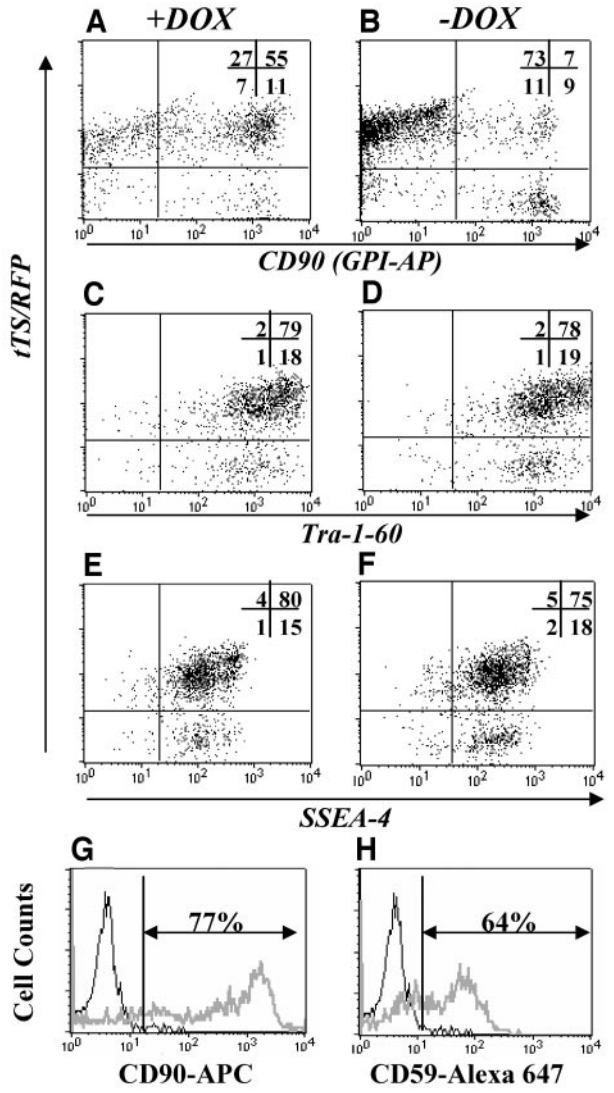

Figure 7. Inducible expression of the *PIG-A* transgene in human embryonic stem (hES) cells after iDuet101$_A$/*PIG-A* transduction. The AR1-c1 (PIG-A-negative, GFP$^+$) H1 hES cells were first transduced by the tTS/RFP regulator vector, sorted, and expanded. Then the hES cells were transduced by the iDuet101$_A$/*PIG-A* virus and induced by Dox. After one passage (5 days), the cell surface marker expression of undifferentiated hES cells (based on constitutive GFP expression) was analyzed by flow cytometry. The expressions of GPI-APs such as CD90 **(A, B)**, undifferentiated hES cell marker Tra-1–60 **(C, D)**, and SSEA-4 **(E, F)** in tTS/RFP$^+$ and tTS/RFP$^-$ hES cells are shown. The percentage of hES cells in each quadrant is denoted. **(G–H):** Analysis of GPI-AP expression in a different experiment (6 days after Dox induction). The cell surface expressions of two GPI-APs, CD90 and CD59, in the tTS/RFP$^+$ cells were plotted as histograms (gray lines). Black lines show the levels of background staining (<1%). Abbreviations: DOX, doxycycline; GPI-AP, glycosyl-phosphatidylinositol-anchored protein; RFP, red fluorescent protein; tTS, transcriptional suppression domain.

former can be overcome by use of prescreened FBS, although it is unlikely to be the case with hES cells, since serum-free media were often used. The latter is consistent with previous data from us and other investigators using LVs. A few percent of cells with transgene expression were often observed even when a promoterless LV was used at high MOI (data not shown). This could be overcome by placing an insulator sequence in the LTR to shield the interference from nearby cellular promoters [46]. Alternatively, the cells harboring leaky expression can be pre-eliminated by sorting or selection based on the transgene expression.

Second, a major source of uncontrolled transgene expression in a fraction of cells is due to the lack of tTS expression. This is particularly a problem in hES cells, where we need extended time to select and expand the transduced populations. Variable levels of cells after prolonged cell cultures tended to loss the tTS/RFP expression as we observed in hES cells (Figs. 5, 6), even after we sorted the tTS/RFP-transduced cells to a high extent of purity (supplemental online Fig. 2). We do not know whether spontaneous loss of tTS/RFP transgene expression is solely due to unfavorable DsRed2/RFP expression and/or tTS expression in hES cells. One way to overcome this is to replace the DsRed2 with other genes encoding less toxic and bright RFPs, or by a drug (such puromycin) selection gene that allows constant selection.

Third, we noticed that the tet-SUPER-mediated transgene expression was not restored fully in the tTS-expressing cells even when saturating amounts of Dox was added. We often observed that ~50% of cells were induced to express transgene in tTS-expressing hES cells in the presence of Dox, as compared with that in parental cells lacking tTS cells. This indicated that tTS-mediated transcription suppression was more sustained and "locked" in some transduced cells, and even in a tetO-independent manner (supplemental online Fig. 2A, top row). It appears that transgene expression is more reversible in the subsequent cycle of suppression and induction by withdrawal and adding Dox, if the inducible cells were previously sorted (Fig. 4). The exact mechanism of this "sustained" or locked suppression mediated by tTS is unclear. One possible approach to improve is to reduce the level of tTS in target cells. In our studies, we tended to use sorted cells that expressed high levels of tTS/RFP in subsequent induction of transgene vectors. A recent article indicated that a very low level of tTS is sufficient for full suppression [43], so in the future, we will investigate whether reducing the level or using a short-lived tTS protein will help us to achieve better and complete restoration of transgene expression.

Despite the limitations, we have demonstrated a universal LV system harboring a transgene whose expression is highly sensitive to Dox inducer and reversible after stable gene transfer. This is the first time that inducible transgene expression in hES cells has been reported. During the revision of this report, a paper was published to report an inducible expression in one monkey ES cell line that can be stably transfected by a plasmid vector containing an improved tet-off system [47]. Together, reversible genetic modification of hES cells offers an advanced tool to study gene functions in multiple stages of human developmental processes and cell differentiation to specific lineages. Additional studies are needed to demonstrate whether the regulated gene expression can be achieved in various progeny cells in vitro and in vivo.

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DISCLOSURES

The authors indicate no potential conflicts of interest.

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