Defining Report

Genetic Engineering of Human Embryonic Stem Cells with Lentiviral Vectors

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ABSTRACT

Human embryonic stem (hES) cells present a valuable source of cells with a vast therapeutic potential. However, the low efficiency of directed differentiation of hES cells remains a major obstacle in their uses for regenerative medicine. While differentiation may be controlled by the genetic manipulation, effective and efficient gene transfer into hES cells has been an elusive goal. Here, we show stable and efficient genetic manipulations of hES cells using lentiviral vectors. This method resulted in the establishment of stable gene expression without loss of pluripotency in hES cells. In addition, lentiviral vectors were effective in conveying the expression of an U6 promoter-driven small interfering RNA (siRNA), which was effective in silencing its specific target. Taken together, our results suggest that lentiviral gene delivery holds great promise for hES cell research and application.

INTRODUCTION

Human embryonic stem (hES) cells represent, at least in principle, an unlimited source of cells for repairing or replacing poorly functioning tissues and organs. Although hES cells possess the capacity to differentiate in vitro to form neural, hematopoietic, endothelial, cardiac, pancreatic cells, and trophoblasts, the low efficiency of directed differentiation of hES cells remains a major obstacle in the practice of regenerative medicine (1–6). Genetic manipulation of hES cells represents an obvious and promising approach for promoting controlled differentiation along a specified developmental pathway. Nevertheless, gene transfer into hES cells has proven difficult, and the safe and effective methods for genetic manipulating of the function and the fate of embryonic stem cells remains an urgent issue in the therapeutic application of stem cell research.

Lentiviral vectors (LVs) offer great promise as gene delivery systems for future gene- and cell-based thera-
pies because these vectors can infect and thereby transduce both dividing and nondividing cells with very high efficiency (7). Unlike adenovirus and adeno-associated viral vectors (8), LV-mediated transduction results in permanent integration of new genetic material into host cell genome with little vector-associated immunogenicity (9). We developed an improved, self-inactivating (SIN) LV system based on pTY-based vectors by extensive deletion of viral elements (7). These vectors have been successfully tested to ensure that their replication deficiency cannot be restored by recombination with the replication-competent virus. Lentiviral vectors can accommodate up to an 8-kb insert and routinely produce high-titer virions, at levels of 10^9–10^11 viral particles (7,10,11,12).

Here, we report the results of a systematic study designed to evaluate the safety, efficiency, and efficacy of LV transduction of hES cells to alter their commitment and differentiation. We also explored the use of lentiviral vectors to effect small interfering RNA (siRNA) gene silencing in hES cells.

**MATERIALS AND METHODS**

**Lentiviral vectors**

Lentiviral plasmids were constructed as previously described (see Fig. 1, below, and refs. 7 and 11). Briefly, pTYF-EFeGFP was generated by inserting a cPPT element (or Flap), amplified from HIV-1NL4-3, into pTYF-EeGFP (7,13). pTYFEmrhGFP was generated by replacing eGFP (15) with a recombinant human green fluorescent protein gene (rhGFP), which was reported to have reduced cytotoxicity (Stratagene, La Jolla, CA). The bicistronic LV plasmid pTYFerhGFP-Pac was generated by ligation of KpnI-digested and T4 DNA polymerase-treated blunt-end pTYFerhGFP with a blunt-end fragment containing poliovirus internal ribosomal entry site (PI) and puromycin resistance gene (pac). To generate the U6 promoter-driven, puromycin-resistant LV siRNA construct (pNEPhU6rPac), we first generated a U6 promoter plus nlacZ reporter gene LV construct pNEZhU6rPac. pNEZhU6rPac was constructed by inserting the human U6 promoter fragment containing two unique BamHI/SpeI cloning sites into the NotI site of pTYFEmlacZ. The U6 promoter was inserted in the opposite orientation from the EF-nlacZ reporter gene, pNEPhU6rPac was then constructed by replacing the nlacZ gene with a puromycin resistance gene. We targeted seven different sites in the eGFP mRNA (the DNA sequence information is available in the supplement online). The lentiviral vectors were generated as previously described (16,17,18). Specific information can be found in supplemental data available from the publisher by request.

**Human ES cell culture and transfection**

Human ES cells (13) used for this study were the WiCell H1 line (passages 32–50). These were plated on Matrigel in six-well plates and split 24–48 h before transduction (14). The cells were transduced with the LV-eGFP vector at different multiplicities of infection (MOI) in the presence of 6 μg/ml Polybrene, and LV gene transduction was determined at different time points by fluorescent microscopy.

**Fluorescence-activated cell sorting analysis**

Fluorescence-activated cell sorting (FACS) analysis was performed as previously described (12). Undifferentiated hES cells without eGFP transduction served as a reference for autofluorescence.

**Cytochemistry and immunocytochemistry**

Chemicon’s hES characterization kit was used to characterize eGFP-positive hES cells, following the manufacturer’s manual. Fluorescence images of these hES colonies were photographed under a fluorescence microscope.

**RT-PCR**

The presence of Oct4 and Nanog in the hES cells was detected by RT-PCR, using primers that amplify a 217-bp fragment of the Oct4 gene (forward, 5'-GACAACAAT-GAGAACCT TCAGGAGA-3'; reverse, 5'-TTCTG-GGCCGGTTCAGAAACA-3') and a 354-bp fragment of Nanog gene (forward, 5'-TGCTATTCTCGGCCAGTTG-3'). After 35 cycles of RT-PCR, the products were detected by ethidium bromide staining.

**Embryoid body formation and induction of cardiomyocyte differentiation**

The transduced hES cell cultures were harvested using 200 U/ml of collagenase IV (Invitrogen), and in ultra-low-attachment six-well plates (Corning Inc.Acton, MA), the cell clumps were allowed to aggregate into embryoid bodies (EBs) in suspension for 6 days using EB formation medium consisting of 78% Dulbecco’s modified Eagle medium (DMEM) with high glucose, 1% l-glutamine, 1% β-mercaptoethanol, 1% nonessential amino acids (NEAA), 10% fetal bovine serum (FBS; Invitrogen), then EBs were transferred onto gelatin-coated plates at 2 EBs/cm² and cultured for 15 days in cardiomyocyte differentiation medium containing 80% KO-DMEM, 1 mmol/L l-glutamine, 0.1 mmol/L β-mercaptoethanol, 1% nonessential amino acids stock, and 20% FBS (Invitrogen). The cultures were then examined for the presence of beating cells.
**Teratoma formation**

To examine the formation of teratomas, puromycin-selected eGFP hES cells ($1 \times 10^6$/mouse) were injected with a sterile 25-gauge needle into the thigh muscle of 8-week-old male CB-17 severe combined immunodeficiency (SCID) mice (The Jackson Laboratory, Bar Harbor, ME). Six to eight weeks after transplantation, animals were sacrificed for histological examinations. The tissues of tumors developed at the injection site of the transduced hES cells were fixed with 10% formalin for Hematoxylin & Eosin (H&E) staining. Part of the sam-

![Diagram](image-url)

**FIG. 1.** An improved lentiviral vector system for eGFP and bicistronic rhGFP-puromycin resistance gene expression. (A) Schematic diagram of the NHP/TYF LV system. The helper construct pNHP, which encodes HIV-1 Gag-Pol and the Tat and Rev regulatory proteins, and pHEF-VSV-G, which encodes the pseudotype VSV envelope protein, are illustrated. The two transducing vectors used in this study are pTYF-EFeGFP, which encodes enhanced GFP, and pTYF-EFrhGFP-PI-Pac, which encodes both rhGFP and puromycin resistance protein (pac), using a poliovirus internal ribosomal entry site (PI), which is driven by the human elongation factor (EF) 1α promoter. (B) Kinetics of LV transgene expression in hES cells. The time-course of LV-eGFP expression at MOI 20 after transduction is illustrated at top, and a dose-curve of eGFP expression using various MOI of LV-eGFP at 96 h after transduction is shown at bottom. Magnification, 100×.
samples was transferred to 30% sucrose after formalin fixation for 24 h. Sucrose-saturated tissues were then embedded with OCT compound and stored at −80°C. The cryostats were sectioned at a 6-μm thickness and observed directly under fluorescence microscope.

RESULTS

High-efficiency transduction of hES cells by LVs

We developed an efficiency- and safety-improved LV transgene system, pTYF vector (Fig. 1A), which has extensive deletions, including a deletion in the viral long terminal repeats (LTRs), and thus does not carry any active viral promoter after infection; the vector is considered to be a self-inactivating or SIN vector (7,10). Cotransfection into 293T cells with two helper plasmids, pNHP encoding viral capsid and enzymes and pHEF-VSVG encoding viral envelope (Fig. 1A) resulted in the generation of replication-defective vectors with titers higher than 10^9 infection units/ml after concentration (7). For this study, we generated two LVs (pTYF-EF-eGFP and pTYF-EF-rhGFP-PI-Pac; Fig. 1A) with titers more than 10^9 infection units/ml.

We first studied the time course of LV transgene expression in hES cells using eGFP as a reporter gene. After LV (pTYF-EF-eGFP) transduction, a series of fluorescent pictures was taken of individual hES cell colonies at 24, 48, and 96 h time points. The GFP-positive hES cells were detected as early as 24 h. GFP fluorescence intensity increased during time and peaked at 96 h. An intense and nearly homogeneous expression of GFP after 96 h was observed by fluorescent microscopy (Fig. 1B). We also noted that cells of small colonies were more readily amendable to infection than those of large colonies (not shown). We examined the dose-dependency of LV transduction at three different MOIs (5, 10, and 20). It is evident that a high MOI (20) was more efficient than a low MOI (5) for LV-mediated hES cell transduction (Fig. 1B).

Selection of GFP-positive hES cells by a bicistronic LV

To quantify the transduction efficiency and to select the GFP-positive cells, hES cells were transduced with pTYF-EF-eGFP or pTYF-EF-rhGFP-PI-Pac LVs. The expression of GFP was examined by flow cytometry analysis. No fluorescence was detected in hES cells (Fig. 2A). At a MOI of 20, after 96 h, over 70% of the pTYF-EF-eGFP-transduced hES cells expressed GFP, as assessed by fluorescence (Fig. 2B). The transduction efficiency of the bicistronic LV pTYF-EF-rhGFP-PI-Pac in hES cells was similar to pTYF-EF-eGFP transduction without selection (not shown). However, when selected by puromycin resistance for 10 days (2 passages) after the bicistronic LV infection, over 90% of the cells were eGFP-positive (Fig. 2C). These data indicate that the pTYF LV transduction of hES cells is highly efficient and could result in homogenous gene transduction with a bicistronic vectors after selection.

Stable transgene expression and hES characteristics during self-renewal

To investigate whether the transduced undifferentiated hES cells could maintain transgene expression during self-renewal, GFP expression was monitored in prolonged culture. The stably transduced hES cells were cultured on Matrigel-coated six-well plates and split every 5 days. The cells retained the typical morphology of hES colonies, with a distinct boundary between colonies. After 40 passages (>6 months, the first 5 passages with puromycin selection), the expression of GFP was maintained at levels similar to the early passages without significant silencing (Fig. 3A). We next examined the ex-
FIG. 3. Characterization of long-term GFP-positive hES cells. (A) Six-month hES cell culture after LV-pTYF-EF-eGFP transduction. Magnification, 100×. (B) Puromycin-selected rhGFP-expressing hES cells characterized for the expression of typical hES cell markers. Indirect immunofluorescence staining of SSEA-1, SSEA-4, TRA-1-60, and TRA-1-81 in rhGFP-expressing hES cell colonies is shown. The red fluorescence highlights the expression of the indicated markers. The corresponding green GFP images are shown below. (C) Alkaline phosphatase activity in the hES cells. (D) RT-PCR analysis of rhGFP-transduced hES cells. Both Nanog and OCT4 are expressed in rhGFP-transduced hES cells and nontransduced hES cells. The human aortic smooth muscle cells (HASMC) are used as the negative control.

FIG. 4. In vitro differentiation of GFP-expressing hES cells. (A) EB formation (day 4). Magnification, 40×. (B) Myocardial clusters in differentiated GFP-transduced hES cells (EBs at 15 days in cardiomyocyte differentiation medium; 100×). A movie of the beating cell clusters containing cardiomyocytes derived from eGFP-transduced hES cells is available online at http://etcetera or will be supplied on request from the editor at scd-editor@msn.com (magnification, 100×).
pression of molecular markers and alkaline phosphatase (AP) activity to confirm the undifferentiated status of hES cells after LV transduction and long-term propagation. Results shown in Fig. 3B demonstrated that hES cell markers (SSEA-4, TRA-1-60, and TRA-1-81) remained positive, and SSEA-1 remained negative, as shown by fluorescent immunocytochemistry in these hES cell colonies after a long-term propagation. At the same time, strong green fluorescence was still detected. In addition, the hES cell colonies exhibited AP activity as demonstrated by cytochemistry (Fig. 3C). By RT-PCR, we showed that the LV-GFP-expressing hES cells expressed OCT4 and Nanog (Fig. 3D). Taken together, these results demonstrate that the LV-transduced hES cells could maintain their undifferentiated status for a prolonged period of time with self-renewal properties like non-transduced hES cells.

Sustained GFP expression in hES cells throughout in vitro differentiation

To test whether LV transgene expression could be sustained during in vitro hES cell differentiation, we examined LV-GFP expression in the following hES cell differentiation stages: (1) EB formation, (2) spontaneous differentiation, and (3) directed cell lineage-specific differentiation. The transduced hES cell maintained the same capacity to form EBs and LV-GFP was highly expressed in the EBs (Fig. 4A). When the EBs were transferred to a gelatin-coated six-well plate for 6 days, differentiated cells expressing GFP spontaneously grew out (data not shown). To examine LV transgene expression in the specific lineage of differentiated cells, we developed a protocol to induce cardiomyocyte growth in the in vitro hES culture. The EBs derived from GFP-transduced hES cells were cultured in cardiomyocyte-inducing medium as described in Materials and Methods. Fluorescence microscopy analysis revealed an intense expression of GFP within newly generated, beating cardiomyocytes (Fig. 4B). Green cell clusters with autonomous excitability were recorded by real-time video (see Xiong-SCD.avi video, on line at http ectera or from the editor at sceditor@msn.com). Thus, our data demonstrated that LV-eGFP-transduced hES cells maintain transgene expression during EB formation as well as after spontaneous and directed cell lineage specific differentiation.

Stable LV transgene expression in hES-derived teratoma

To test whether LV transgene could maintain expression during in vivo differentiation and to verify the pluripotency of LV-transduced hES cells, LV-transduced hES cells were transplanted to non-obese diabetic (NOD)/SCID mice. We used the undifferentiated hES cells as the control. All of the mice (n = 5) that had been engrafted with the LV-transduced hES cells developed teratomas (Fig. 5A, panels 1–3). The tumor was palpable 3 weeks after inoculation and grew to about 1.5 cm in diameter by 6–8 weeks. Histological analysis by H&E staining of the teratomas excised after 6–8 weeks showed tissues representing all three germ layers. As shown in Fig. 5A (panels 4–9), LV-transduced hES cell-derived tissues in teratomas contained ectoderm (e.g., primitive neuroectoderm and hair follicle structure), mesoderm (e.g., muscle and cartilage), and endoderm (e.g., glandular structures). By direct in situ fluorescence analysis, all tissues in teratomas exhibited marked GFP expression (Fig. 5B). These data indicated that LV-transduced hES cells maintained their pluripotency and formed teratomas in vivo.

Gene silencing in hES cells by LV-siRNA

Silencing of endogenous genes in hES cells will be a powerful method for hES cell research and will have great therapeutic potential. To examine the possibility of gene silencing by LV-siRNAs, we transduced selected GFP-positive hES cells with LV siRNA vector targeting GFP mRNA to determine the silencing effect. A human U6 promoter-driven siRNA cassette was cloned in the reverse orientation into a LV adjacent to an EF1α-pac selectable gene (pTYF siRNA-Pac). A series of seven different siRNA constructs, encoding different GFP targeting sequences were initially tested in 293 cells by DNA cotransfection. The construct (#6) showing best effect for gene silencing was chosen (Fig. 6A, representative pictures of constructs #3 and #6). LV-EF-eGFP (without puromycin selection marker)-transduced hES cells that exhibited stable GFP expression were used as target cells. Individual hES cell colonies were continuously monitored under a fluorescence microscope after LV-GFP-siRNA-Pac (with the puromycin selection marker) transduction. We noted that the green fluorescence signal was significantly reduced at 72 h, and reached the maximal inhibition at 120 h after LV-siRNA transduction (Fig. 6B). In addition, we documented that all of remaining hES cell clusters are lost GFP expression after 15 days (3 passages) under puromycin selection (data not shown). These results clearly demonstrated that U6 promoter-driven siRNA, when transduced by LVs, could induce gene-specific RNA interference effect and establish stable gene-silencing in hES cells.

DISCUSSION

Our findings systematically document for the first time that LVs are highly efficient in transducing hES cells in a manner that stably maintains transgene expression. At
FIG. 5. Teratoma formation of eGFP-transduced hES cells in NOD/SCID mouse. The GFP-positive hES cells ($1 \times 10^6$/mouse) were injected into the thigh muscle of NOD/SCID mice. (A) Gross pictures and H&E staining. Gross pictures of representative teratomas (panels 1, 2, and 3). Ectoderm derivatives: hair follicle (panel 4) and primitive neural tissue (panel 7). Mesoderm derivatives: smooth muscle (panel 5) and cartilage (panel 8). Endoderm derivatives: epithelium (panels 6 and 9). (B) Representative pictures of GFP expression in derivatives of three germ layers of teratomas. Cells showed positive for GFP protein expression. Cell nuclei were highlighted by DAPI (blue; magnification, 100×).
the same time, LV-based gene delivery does not alter the two key properties of hES cells: self-renewal and pluripotency. In our study, the self-renewal capacity of LV-transduced GFP-expressing hES cells was demonstrated by long-term culture for at least 6 months (>40 passages) without loss of hES cell characteristics and with persistent GFP expression. The pluripotency of these cells was demonstrated (1) in vitro by EB formation and directed cell differentiation, and (2) in vivo by teratoma formation, while maintaining GFP expression in all progeny. Moreover, we successfully developed a bicistronic lentivector containing a GFP reporter gene and a puromycin resistance gene (pac), allowing us to select for only those hES cells exhibiting LV-mediated transgene expression. In addition to eliminating untransduced hES cells, this approach also synchronizes the directed differentiation of hES cells, thus facilitating future research and development in regenerative medicine. As demonstrated here, the LV-siRNA technology also provides a gene “knock-down” strategy for hES cells, as evidenced by the efficient silencing of a target gene (in this case GFP), thus offering a useful tool to study specific gene function(s) during hES cell differentiation and organ development.

Constitutive expression of key transcription factors has been shown to direct the differentiation of murine ES cells and liver stem cell into specific cell types, such as dopamine-producing neurons and insulin-producing cells (19–21). Controlled gene “knock-in” and “knock-down” are invaluable tools for dissecting the underlying molecular mechanism of cell differentiation during embryogenesis and for generating functional cells needed for cell replacement therapy. However, gene transfer to human ES cells is difficult to achieve by chemical transfection or electroporation methods (22,23). Eiges et al. compared the efficiencies of several chemical-based methods for the transfection of hES cells and showed that transient expression of the target gene occurred in no more than 10% of the cells (22). While electroporation appears to be more effective for introducing foreign DNA into mouse ES cells, such is not the case for human ES cells. Zwaka et al. only obtained 350 positive clones from 1.5 × 10⁷ hES cells using electroporation to deliver linearized plasmid DNA (23). Furthermore, plasmid DNA-based gene transfer requires extended periods for co-selection with selective markers. Of the different adenovirus (Ad) or recombinant adeno-associated virus (rAAV) serotypes tested as vectors for hES cells, only the Ad5 and rAAV(2) serotypes transduce hES cells, but at low efficiencies, 11.2% and 0.012% respectively (24). The recognized advantage of lentiviral vectors is their ability to transduce both dividing and nondividing cells with transduction efficiencies that often approach 100% (7). Transduction with LVs results in permanent integration into host cell genome, with little to no vector-related immunogenicity, a well-known problem with Ad and rAAV vectors (8). We have further improved the efficacy and safety of an HIV-based lentiviral vector system by extensively deleting all of the viral genes, several essential genetic elements, and most of the enhancer/promoter sequences in the LTRs (10,18).

LV-mediated hES cell transduction resulted in transgene expression as early as 24 h after gene transfer and reached its peak intensity by 96 h. Without further selection, we documented that up to 67–87% of the hES cells were positive for LV transgene expression. Other studies have reported either similar (87%) or much lower (7%) transduction efficiency using LVs encoding GFP as a reporter for transgene expression (25,26). The high transduction efficiency observed in this study may be attributed to the optimized elements, including the vector modifications and the strong human elongation factor 1α promoter in our LV constructs (10,27). In addition, the much lower MOI needed for LV-mediated transduction is a great advantage over such vectors as Ad and rAAV. As demonstrated in this report, LV at a MOI of 20 was more efficient than a MOI of 5 (Fig. 1B); however, the serum content in the media did not affect transduction efficiency in our study (data not shown). It should be noted that the feeder layer-free culture, which eliminates the absorption of the viral particles by feeder layer, might also contribute to the high efficiency transduction.

Overexpression of a target gene and/or a selection marker should greatly facilitate preparation of pure transgene-expressing hES cells, especially by using bicistronic gene strategies to introduce a cell selection marker needed to obtain desired cells in high purity. In this study, our choice of a bicistronic LV construct encoding both GFP and puromycin resistance protein worked especially well in hES cells. By gradually increasing the puromycin dosage from 0.2 μg/ml to 1.5 μg/ml, we modified the puromycin selection protocol for hES cells. After puromycin selection for 10 days, 93% of the cells were LV-GFP positive, as determined by fluorescence-activated cell sorting (FACS) (Fig. 2C). Given the high transduction efficiency and the co-selection marker in our LVs, we have demonstrated a highly efficient gene delivery method for genetic modification of hES cells.

Long-term stable gene transfer into hES cells has not been extensively explored in previously published studies (25,26). Here we showed that LV-transduced hES cells could be maintained in feeder-free condition at undifferentiated state for prolonged periods. After about 40 passages, we detected no significant loss of LV-GFP expression. These results indicate that LVs offer significant advantages for hES cell modifications. Our data also demonstrated that LV-transduced hES cells not only maintained ES cell morphology, but also retained the expression of molecular markers of undifferentiated hES cells, including SSEA-4, TRA-1-60, TRA-1-81, Oct-4,
FIG. 6. LV-siRNA silencing of eGFP expression in hES cells. (A) Schematic illustration of U6 promoter-driven LV-siRNA targeting eGFP. The U6-siRNA was inserted into the LV construct in the reverse orientation in relation to the EF1α-pac selective marker gene. The sequences of #6 siRNA short hairpin precursor and the eGFP targeting site are shown. (B) Effective suppression of eGFP by LV-siRNAs in 293 cells. 293 cells were co-transfected with pHEF-eGFP and a LV-siRNA plasmid at 1:10 molar ratio and the expression of eGFP was monitored and photographed under a fluorescence microscope. Upper left, control positive eGFP; upper right, control negative eGFP; lower left, #3 siRNA targeting eGFP; lower right, #6 siRNA targeting eGFP (magnification, 100×). (C) Silencing eGFP expression by #6 LV-siRNA targeting eGFP in hES cells.
for gene- or cell-based therapies. Thus, we have provided ample evidence indicating that LV-based gene delivery can maintain stable transgene expression without affecting the pluripotency of hES cells.

Our experimental results also indicate that LV transgene expression persisted after EB formation and spontaneous in vitro differentiation. In addition, we showed that LV transgene expression was maintained during directed specific cell lineage differentiation from hES cells to cardiomyocytes. Our observations are consistent with other studies (25;26;28) showing no significant gene silencing detected after lentiviral gene transfer into both mouse and hES cells during spontaneous in vitro differentiation or in vivo teratoma formation. A sustain transgene expression was also observed in lentiviral-mediated hES cell-derived neurons and hematopoietic cells (25,26).

By analyses of in vivo hES cell-derived teratomas, we detected stable LV-mediated GFP transgene expression in tissues including ectoderm, mesoderm, and endoderm. These results provide evidence that LV-transduced hES cells can maintain their pluripotency in vivo. Several groups have reported that a large portion of the gene expression in LV-transduced tissues was silenced in mouse ES cell-delivered teratomas (29,30). Indeed, we have also found that LV-transduced mouse embryonic P19 cells (a teratoma cell line) are very sensitive to transgene silencing (Chang et al., unpublished data). In agreement with our observation, Gropp et al. has found that transgene expression by lentiviral delivery is sustained during hES differentiation in vivo (26). Taken together, LV transgene silencing appears to be a species- or cell-dependent phenomenon.

Besides transgene expression, we have previously shown that LVs have also been shown to be an effective tool for siRNA delivery (16,17). During the preparation of this manuscript, Zaher et al. has reported RNA interference in hES cells delivered by LVs achieved an overall 50% of the transduction using a MOI at 50 (31). In our current study, we targeted GFP-positive hES cells with an U6-driven LV siRNA targeting a highly effective siRNA target site in the GFP mRNA, and demonstrated that 72 h after transduction, the green fluorescence signal became significantly reduced. After ~15 days under puromycin selection, which was constructed in the siRNA vector as a co-expression marker, all of the remaining hES cell clusters displayed markedly reduced GFP expression. Therefore, LV siRNA can effectively and stably silence endogenous genes in hES cells.

In summary, we have demonstrated that hES cells can be efficiently and stably transduced by LVs without losing their pluripotency and differentiation potential. This powerful gene delivery technology will provide useful tools for studying hES biology and future applications for gene- or cell-based therapies.

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