Published in final edited form as:

Stem Cells Dev. 2007 February ; 16(1): 167–176.

Transgenes Delivered by Lentiviral Vector Are Suppressed in Human Embryonic Stem Cells in a Promoter-Dependent Manner

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Abstract

Lentiviruses have been increasingly used for genetic modification of human cells including embryonic stem (ES) cells. Using four ubiquitous promoters—cytomegalovirus (CMV), cytomegalovirus immediate-early enhancer/chicken β -actin hybrid (CAG), phosphoglycerate kinase (PGK), and human elongation factor-1 α (EF1 α)—in a lentiviral vector to drive the expression of the enhanced green fluorescent protein (EGFP) gene in human ES cells and mouse ES cells, we determined the extent of EGFP suppression by assessing the percentage of cells that were transduced with the EGFP gene but did not fluoresce green. A much higher level of transgene suppression was observed in human ES cells as compared to mouse ES cells. The suppression was also highly promoter dependent, leading to inactivation of more than 95% of the EGFP genes under the CMV or CAG promoter while only 55% under the PGK promoter. No promoter-dependent suppression was observed in transient transfection of human ES cells. Thus, the common phenomenon of poor transgene expression in human ES cells may be caused mainly by suppression of the transgene right after transduction and integration. Cautions should be taken to choose the optimal promoter when lentiviruses are used for genetic modification of human ES cells.

INTRODUCTION

HUMAN EMBRYONIC STEM (ES) CELL LINES offer a valuable source for potential cell therapy and a powerful tool for basic research (1,2). The potential of these cell lines can be further enhanced by genetic modifications, which may promote controlled differentiation of stem cells along a specific developmental pathway (3), facilitate purification of a certain type of cells in a mixed population of ES-derived cells (4), or correct genetic defects (5). In spite of all the potential this approach presents, the standard chemical or mechanical methods for transgene delivery exhibited very low efficiency in human ES cells, with the most effective approaches yielding only approximately 1 stably transfected cell per 10⁵ cells (6,7).

Viral transduction usually leads to much higher efficiency than nonviral methods (8). Various viral systems have been developed to accommodate different research purposes. Adenovirus is used for transient expression because it remains epichorosomal in the host cell (9). Adeno-associated virus integrates into the host genome so that it mediates stable transgene expression (10). Although lentiviral vectors have a less defined integration site comparing to adeno-associated virus, they have been shown to lead to much higher stable transducing efficiency

and have become the most promising strategy so far for gene delivery in human ES cells (11). Lentiviruses offer the following advantages over other systems: (1) They have high transduction efficiency. Up to 70% of transduction efficiency has been achieved in human ES cells using a high titer of self-inactivating lentiviruses (12). (2) The transgenes are integrated permanently into the host genome so that gene expression is stable and inheritable (13). (3) Less vector-associated immunogenicity has been observed for lentivirus-mediated transduction compared with other viral vectors (14). By further purifying the transduced cells with flow cytometry or antibiotic selection, human ES cell lines overexpressing green fluorescent protein (GFP) have been established with more than 99% homogeneity (12,15, 16). Besides gene overexpression, gene knockdown by RNA interference has also been achieved in human ES cells using lentiviral vectors (17).

A major concern that may limit the use of viral vectors as gene delivery vehicles is that viral transgenes may be inactivated in the host cells. One well-studied gene inactivation process is called gene silencing, which happens both during propagation (18) and differentiation (19) of ES cells and leads to the inactivation of initially active transgenes. In this study, we report that inactivation also happens immediately after lentiviral transduction and integration of the transgenes into the host genome. We term this process “suppression,” to distinguish it from gene silencing, because the majority of these lentiviral-delivered transgenes are never expressed in human ES cells. Suppression leads to a significantly lower lentiviral transgene expression efficiency in human ES cells than in mouse ES cells. We also compared the lentiviral transgene expression efficiency in human ES cells using four most commonly used ubiquitous promoters. Our results revealed that the genes were suppressed in human ES cells in a promoter-dependent manner.

MATERIALS AND METHODS

Cell cultures

Two human ES cell lines [H9 and H1, provided by WiCell Institute, Madison, WI (1)] were tested in this study. They were used between passages 30 and 40. Cells were cultured either on irradiated mouse embryonic fibroblasts (MEFs) or in MEF conditioned medium on Matrigel (20,21). The cell culture medium consists of Dulbecco's modified Eagle medium (DMEM)-F12, 20% knockout serum replacer (Invitrogen, Carlsbad, CA), 100 μ M minimal essential medium (MEM) nonessential amino acids, 1 mM L-glutamine, 0.1 mM 2-mercaptoethanol, 4 ng/ml basic fibroblast growth factor (bFGF; R&D systems, Minneapolis, MN). For passage, cells were dissociated with 1 mg/ml of dispase II for 3–5 min until the edges of the colonies curled up. The colonies were then detached and broken into clumps of about 50- to 100- μ m diameter and plated on either MEF or Matrigel-coated six-well plates in the medium.

The mouse ES cell line D3 (ATCC, Manassas, VA) was cultured either on MEF or gelatin-coated six-well plates in the presence of 1,400 U/ml of leukemia inhibitory factor (LIF; GIBCO/BRL, Grand Island, NY) in ES cell medium consisting of knockout (KO) DMEM supplemented with 15% fetal bovine serum (FBS), 100 μ M MEM nonessential amino acids, 2 mM L-glutamine, and 0.1 mM 2-mercaptoethanol (22). Cells were dissociated to single cells using 0.05% trypsin and 0.53 mM EDTA (Invitrogen, Carlsbad, CA) for passage.

Lentiviral vector constructs

The self-inactivating lentiviral vector (23) backbone used here was based on the FUGW vector described by Lois et al. (24). Various promoters were amplified by PCR and subcloned into the pEGFP-N1 vector (Clontech, Mountain View, CA). The promoters together with the enhanced green fluorescent protein (EGFP) gene were then cloned into the lentivector using *Xba*I/*Not*I. All the constructs were verified by sequencing.

Four commonly used ubiquitous promoters were tested to drive the downstream EGFP gene expression. They are cytomegalovirus (CMV) promoter, CMV immediateearly enhancer/chicken β -actin hybrid (CAG) promoter, phosphoglycerate kinase (PGK) promoter, and elongation factor-1 α (EF1 α) promoter. The forward (fwd) and reverse (rev) primers used for amplifying the promoters were: CMV fwd, GCTATCTAGATCAATATTGGCCA-TT; CMV rev, GTTAGAATTCTGTGGAGAGAAA-GG; CAG fwd, ATTGACTAGTTATTAATAGT; CAG rev, GAGGAATTCTTTGCCAAAAT; PGK fwd, GCT-ATCTAGACTTTTCCCAAGGCAG; PGK rev, GTTA-GAATTCAGGTCGAAAGGCCCG; EF1 α fwd, GCTC-TCTAGATGTCGACGATAAGCTTTG; and EF1 α rev, GGTCCGATCCTAAATGTCGAAATTCCTC.

A woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) was inserted 3' to the EGFP gene to enhance gene expression (25). To minimize the chance of multiple integrations of the transgene upon transduction, we did not include the central polypurine tract (cPPT) sequence in our vector system (26).

Lentiviral preparation, cell transduction, and transfection

Lentiviral particles were produced by co-transfecting HEK 293FT cells (Invitrogen, Carlsbad, CA) with the lentiviral vector pCMV Δ 8.91 and vesicular stomatitis virusG protein (VSV-G) plasmid using the calcium phosphate method described by Karolewski et al. (27). Cell culture medium was collected 48 h later and filtered through 0.45- μ m pore-sized MILLEX-HA filter (Millipore, Billerica, MA). The viral particles were further concentrated 100-fold by ultracentrifugation ($50,000 \times g$, 2 h at 4°C). The pellet was resuspended in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA). The concentrated lentivectors were titered by transducing NIH-3T3 cells and counting the number of green cells. They were then diluted with PBS containing 1% BSA to the same titering (5×10^6 transducing units per milliliter) before use.

For transduction of ES cells, cells were passaged and plated on MEF in six-well plates for 24 h before transduction. Cells were transduced with various lentiviral vectors at a multiplicity of infection (MOI) of 3.

For transient transfection, human ES cells were transfected using Fugene6 (Roche, Switzerland) in a six-well plate following the procedure provided by the manufacturer. The ratio used was 3 μ l of Fugene6 to 1 μ g of DNA for each well.

Fluorescence-activated cell sorting

ES cells were dissociated to single cells by digesting with trypsin-EDTA for 5 min. They were then washed once with PBS and resuspended in BD FACSflow sheath fluid (BD Bioscience, Rockville, MD). Cells were then passed through a BD Falcon 70- μ m cell strainer (BD Bioscience) to remove the incompletely digested clusters. Fluorescence-activated cell sorting (FACS) analysis was performed on the BD FACSCalibur system (BD Biosciences). The threshold of forward scatter (FSC) and side scatter (SSC) were set so that only undifferentiated ES cells were counted. Undifferentiated human ES cells cultured on Matrigel and undifferentiated mouse ES cells cultured on gelatin-coated dishes served as controls for both autofluorescence and FSC/SSC range.

Real-time quantitative PCR

Quantitative PCR (qPCR) was performed using Bio-Rad MyiQ single-color real-time PCR. Genomic DNA was extracted and the gene copy numbers were determined by using the β -actin (ACTB) gene as an external standard. Two genes, Ca²⁺-dependent activator protein for

secretion-2 (CADPS2) and hypoxanthine phosphoribosyltransferase-1 (HPRT1), were used as controls to verify the accuracy of the qPCR method. The forward and reverse primers used for each gene were: EGFP fwd, AGAACGGCATCAAGGTGAAC, rev, TGCTCAGGT-AGTGGTTGTC (28); ACTB fwd, ACTCTTCCAGCC-TCCTTC, rev, ATCTCCTTCTGCATCCTGTC; HPRT1 fwd, CCTGGCGTCGTGATTAGTGAT, rev, AGACG-TTCAGTCCTGTCCATAA; CADPS2 fwd, AATTTGC-CCACCACTTAGAGC, rev, ACTGTTGCCATTGACT-ACCAAAC.

RESULTS

Suppression of lentivirus-delivered EGFP gene expression in human ES cells is promoter dependent

After viral transduction, H9 human ES cells typically began to express EGFP within 48 h and a plateau was reached after 96 h (Fig. 1A, left and middle columns). Quantitative analyses by FACS (Fig. 1A, right column) indicated that, although the same MOI was used for all four lentiviruses, the percentage of fluorescent ES cells was highly promoter dependent (Fig. 1B). Among the four promoters, PGK yielded the highest percentage of fluorescent cells (38.1%), followed by EF1 α (18.6%), whereas the percentages for CAG (3.3%) and CMV (1.1%) were much lower. We also noticed that the EGFP signal intensity varied with different promoters. Although the PGK promoter gave rise to the highest percentage of fluorescent cells, cells transduced with the EF1 α and CAG viruses exhibited brighter signals. This observation was confirmed by FACS as the average fluorescence intensity of the fluorescent cells was higher for CAG- and EF1 α -transduced cells than for PGK-transduced cells (i.e., the peaks of fluorescent cells were more rightshifted; Fig. 1A, right column).

The difference in the percentage of fluorescent cells could be due to the difference in transduction efficiency and/or the difference in the degree of gene inactivation. Because the above four lentiviral vectors were constructed using the same lentiviral backbone and the same MOI was used for transduction, it is unlikely that the transduction efficiency would be significantly different among vectors. To test this, we first conducted regular PCR to examine the presence of EGFP genes in the transduced ES cells. A single colony was transferred from the original plate to a MatriGel-coated plate 96 h after transduction. For CMV and CAG, colonies without any fluorescent cells were intentionally picked (as most colonies did not have any fluorescent cells, these colonies were considered “typical”). For PGK and EF1 α , colonies with representative percentages of fluorescent cells were picked. Cells were allowed to grow on the Matrigel plate for an additional 5 days before they were harvested for genomic DNA extraction and PCR analyses. EGFP bands of similar intensity were observed for the colonies transduced by lentiviruses containing CAG, PGK, and EF1 α promoters (Fig. 2A). The EGFP band for CMV had slightly lower intensity (about 30% lower as assessed by densitometry), which, however, was not significant enough to account for the difference observed in percentage of cells expressing EGFP.

We then carried out qPCR to determine quantitatively the copy number of the EGFP gene in the transduced cells. A control experiment was first carried out to validate the method, in which the copy numbers of the HPRT1 gene (on X chromosome) and the CADPS2 gene (on chromosome 7) in human H1 ES cells were determined using the ACTB gene as an external standard. The H1 ES cell has an XY karyotype (1) and should possess one copy of the HPRT1 and two copies of the CADPS2 gene. Results in Supplementary Fig. 1 (see <http://www.waisman.wisc.edu/~xia/scdsupfig/supplementaryfigures.htm>) showed that the qPCR protocol we used could accurately determine the gene copy numbers with an error within 20–30%. When the same method was used to determine the copy number of the EGFP gene in the transduced cells, results showed that the average copy number of EGFP gene per cell

was about 0.9 and no significant difference was observed for CAG, PGK, and EF1 α . The copy number for CMV is about half compared to the other three promoters (Fig. 2B).

As shown above, the difference in transduction efficiency could not account for the difference we have observed in the percentage of fluorescent cells. An alternative explanation for the variation in the percentage of fluorescence-positive cells is that the expression of many EGFP genes was suppressed in the host cell. The extent of EGFP expression suppression was estimated by calculating the ratio between the percentage of nonfluorescent cells and the EGFP copy number per cell, assuming that the number of fluorescent cells equals to the number of the active EGFP genes. As shown in Fig. 2C, more than 95% of the EGFP genes driven by the CMV or CAG promoter were inactive in human ES cells. About 75% of the EGFP genes driven by the EF1 α promoter were inactive. EGFP genes driven by the PGK promoter showed the least extent of suppression (about 55% were inactive).

Similar results were reproduced using another human ES cell line H1 (Supplementary Figs. 2 and 3; see Website for figures).

Promoter-dependent suppression of lentivirusdelivered EGFP gene expression is specific to human ES cells

We then tested if the promoter-dependent suppression takes place in ES cells other than human species. Mouse ES cells were transduced much more efficiently with lentiviruses than human ES cells. Typically, the EGFP signal was observed within 36 h and reached a plateau within 96 h after transduction. FACS analyses showed that the percentages of fluorescent cells were higher than those for human ES cells for all the four lentiviruses (64.6%, 43.8%, 36.2%, and 10.0% for PGK, EF1 α , CAG, and CMV, respectively) (Fig. 3A,B). The pattern of EGFP expression was similar to that in human ES cells, i.e., the PGK promoter had the highest percentage of fluorescent cells whereas EF1 α and CAG virus-transduced cells were generally brighter than PGK virus-transduced ones (Fig. 3A).

Regular PCR analyses on single colony-expanded cells demonstrated the presence of EGFP genes in all transduced clones (Fig. 4A). The copy numbers of integrated EGFP genes determined by qPCR varied among promoters (Fig. 4B). The percentages of suppressed EGFP genes in mouse ES cells were very similar for CAG, PGK, and EF1 α promoters (all ~55%). A slightly higher percentage (about 60%) was observed for the CMV promoter (Fig. 4C).

Suppression of lentivirus-delivered EGFP gene expression in human ES cells is dependent on the integration of the transgenes into the host genome

Transgenes delivered by lentiviruses are integrated into the host genomes and are transcribed as if they are regular host genes (13). To test whether the suppressing effect is dependent on viral integration into the host genome, we transiently transfected human ES cells with the same EGFP lentiviral transfer vectors carrying the above promoters (no virus was produced in this procedure). After transient transfection, vectors would remain as supercoiled circular DNA without integration into the host genome. Because the transfection efficiency of human ES cells is generally too low to be quantified by FACS, the number of fluorescent cells was counted manually and the percentage of fluorescent cells was expressed as the number of fluorescent cells per mm² (Fig. 5B). No significant difference was observed for the four promoters tested (Fig. 5A,B), suggesting that either there was no suppression of the transgenes or transgenes were suppressed to the same extent for different promoters. All transfected cells fluoresced at about the same intensity as quantified by ImageJ (software developed by the National Institutes of Health; data not shown). These results indicate that the promoter-dependent transgene expression suppression in human ES cells is dependent on integration of the transgenes into the host genome.

DISCUSSION

Poor viral transgene expression in human ES cells is a well-known phenomenon. This can result from poor transduction efficiency and gene inactivation. In this study, we have demonstrated that the low transgene expression in human ES cells delivered by lentivirus is largely caused by active suppression of transgenes by the host cells, i.e., blockage of gene expression soon after the transgenes are delivered and incorporated. Unlike in mouse ES cells, the degree of transgene suppression in human ES cells is highly promoter dependent. This finding raises a critical issue in human stem cell biology and potentially in gene therapy field as to how to achieve high efficiency of transgene expression in human ES cells.

By quantifying the extent of transgene suppression in human and mouse ES cells, we have demonstrated that suppression of lentivirus-delivered genes is a common phenomenon in ES cells. However, compared to mouse ES cells, human ES cells exhibit a much higher tendency to suppress transgenes delivered by lentiviruses, as high as over 95%. We have omitted the cPPT sequence in our vectors and used a low MOI to minimize the possibility of multiple integrations of transgene, thus making it unlikely that the degree of transgene suppression is overestimated. More importantly, overestimation will only happen when there is more than one copy of active (not suppressed) EGFP genes in the same cell. Because our study revealed that the percentage of suppression is as high as 95% (especially for CMV and CAG promoters), the possibility of any single cell possessing more than one copy of active EGFP genes is extremely low. Such a high degree of transgene suppression presents serious technical hurdles to human ES cell biologists. Indeed, only about 1% of human ES cells transduced with lentiviruses driven by the CAG promoter expressed the transgene GFP (29).

The mechanism of suppression is beyond the scope of this study. However, we have shown that this promoter-dependent suppression in human ES cells is dependent on viral integration into the host genome, as no promoter dependent suppression was observed for transient transfection. Many studies have demonstrated that the gradual loss of retroviral gene expression is caused by one or multiple epigenetic modifications of the viral DNA following its integration (30,31). A similar mechanism might be responsible for lentivirus-delivered transgene suppression observed in our study. Another possibility is that lentivirus-delivered genes have a high tendency to be incorporated into inactive regions of the human ES cell genome, and the choice of different promoters affects this tendency. Studies have shown that lentiviral vectors do not integrate into the host genome randomly, but instead favor gene expression hotspots (32,33). Whether the integration sites differ from cell types is still not clear.

Although a significant percentage of transgenes are inactive in human ES cells after transduction, we also noticed that the active transgenes were stable during propagation. Loss of EGFP expression was not detected in an EF1 α -EGFP lentivirus-transduced human ES clone for at least 10 passages (data not shown), which is consistent with previously reported data (12,15,16).

Our findings in this study provide a general guideline for future attempts of modifying the genome of human ES cells using lentiviruses. None of the four commonly used ubiquitous promoters tested here could achieve a low degree of gene suppression, a high level of protein expression, and sustained transgene expression at the same time. Therefore, researchers should weigh the pros and cons of each promoter and choose promoters accordingly.

If a high percentage of active transgene (i.e., low extent of suppression) in human ES cells is preferred, the PGK promoter should be used. In this study we used a low MOI of lentivirus to facilitate quantification. At a higher MOI, much higher transduction efficiency (>70%) can be achieved without further purification by FACS or antibiotic selection using the EF1 α promoter (12). We expect even higher efficiency for the PGK promoter based on our observation in this

study. In addition, the viruses pseudotyped with VSV-G used in the current study would be taken up by MEFs with higher efficiency than human ES cells, which reduces the effective virus MOI. This can be solved by using lentiviruses pseudotyped with other envelopes that selectively infect human ES cells (34). The use of human ES cell-specific pseudotyped lentivirus will also greatly facilitate the FACS of transduced human ES cells by avoiding the contamination from transduced feeder cells.

On the other hand, if a high level of transgene expression is desirable, the EF1 α promoter would be a better choice. The CMV promoter is obviously an inferior promoter for human ES cells due to its high degree of suppression and poor protein expression. Although the CAG promoter is severely suppressed, which is consistent with a previous report (29), we also found that the transgene was expressed abundantly in nonsuppressed cells. More importantly, it has been reported that the CAG promoter remains active after human ES cells are differentiated into neurons (29). In contrast, neurons differentiated from GFP-expressing human ES cell clones driven by the EF1 α promoter turned off GFP expression completely following long-term in vivo differentiation (35). Considering this, the CAG promoter would be a better choice than EF1 α for sustained expression of transgenes. So far, the β -actin-based promoter is the only ubiquitous promoter that has been reported to have little loss of gene expression during human ES cell propagation and in vitro differentiation (36). Thus, the CAG promoter may be suitable for establishing genetically modified stable ES cell lines for functional analysis of differentiated cells.

Differences in the extent of suppression should also be considered when modular lentivectors with antibiotic selection markers are used to establish stable human ES cell lines (15). Usually different promoters are used to drive expression of the selection marker and the target transgene. On the basis of our results, cells that are drug resistant but do not express the transgene could arise if the promoter driving the selection marker is suppressed to a lower extent than the one driving the transgene. This situation would give rise to false positive clones. Indeed, we observed that when the PGK promoter was used to drive the neomycin-resistance gene and the CAG promoter used to drive the GFP gene, only 1 out of around 15 neomycin-resistant clones was fluorescent. In this case, the GFP clones can be manually isolated or sorted by FACS to establish true positive clones. However, in most other cases when the transgene products do not fluoresce, the use of selection markers driven by another promoter is virtually futile because only very small portions of the clones are true transgene protein positives. To avoid this problem, we recommend using the same promoter to drive both the selection marker and the transgene. An alternative approach is to use the bicistronic lentivector, which allows the expression of the target gene and the selection marker under the control of one common promoter (37–39).

In summary, our current study has revealed that transgenes such as EGFP delivered through lentiviruses are actively suppressed in human ES cells right after transduction in a promoter-dependent manner. Our findings suggest that this suppression effect contributes significantly to the poor expression of transgenes in human ES cells. Therefore, selection of appropriate promoters and measures to minimize transgene suppression will likely facilitate genetic modification of human ES cells and potentially gene- and cell-based therapy development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study has been supported by the NIH-NINDS (NS045926, NS046587) and partly by a core grant to the Waisman Center from the National Institute of Child Health and Human Development (P30 HD03352).

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FIG. 1.

EGFP expression in human H9 ES cells is promoter dependent. Human ES cells (the H9 line) were transduced with lentiviruses containing EGFP driven by different promoters at a MOI of 3 and analyzed 96 h later. **(A)** The bright-field and GFP channel views of representative transduced clones were shown in the left and middle columns, respectively. The promoters were indicated on the left. The numbers of fluorescent cells were then quantified by FACS and the representative results are shown in the right column. Two peaks were observed in FACS for each clone, with the left peak representing non-fluorescent cells while the right peak representing the fluorescent cell population. **(B)** The percentages of fluorescent cells were

calculated from the FACS analyses. Results shown were the mean of three independent experiments with indicated standard deviations.

FIG. 2.

EGFP suppression in human H9 ES cells is promoter dependent. Genomic DNA of human ES cell clones transduced with various lentiviruses was extracted and used as template for PCR. (A) Regular PCR amplified a 300-bp fragment of the EGFP gene, showing that all the clones contain EGFP genes. The GAPDH gene was also amplified and used as a loading control.

(B) The average copy numbers of the EGFP gene ($\pm$ standard deviation) in the transduced clones were determined by qPCR using the human ACTB gene as an external control ($n = 3$).

(C) The extent of suppression for each lentivector was calculated by dividing the percentage of nonfluorescent cells (results from Fig. 1B) by the average copy number of the EGFP gene. Results were plotted as the mean of three independent experiments with indicated standard deviations.

FIG. 3.

EGFP expression in mouse ES cells under different promoters. Mouse ES cells were transduced with different lentiviruses at MOI 3. **(A)** The bright-field and GFP channel views of representative clones 96 h after transduction were shown in the left and middle columns, respectively. The names of the promoters were indicated on the left. The numbers of fluorescent cells were then quantified by FACS and shown in the right column. **(B)** The percentages of fluorescent cells were quantified and plotted as the mean of three independent experiments with indicated standard deviations.

FIG. 4.

The degree of EGFP suppression in mouse ES cells is similar with different promoters. **(A)** All of the mouse ES clones transduced with different lentivirus were EGFP positive as shown by regular PCR using EGFP-specific primers. The GAPDH gene was amplified to show an equal amount of loading. **(B)** The average copy numbers of EGFP genes in the transduced clones were determined by qPCR using mouse ACTB gene as an external control ($n = 3$). **(C)** The extent of suppression for each lentivector was calculated by dividing the percentage of nonfluorescent cells (shown in Fig. 5B) by the average copy number of the EGFP gene. Results were plotted as the mean of three independent experiments with indicated standard deviations. A *t*-test showed that the differences among CAG, PGK, and EF1 α promoters were not significant ($p > 0.05$).

FIG. 5.

Transient transfection efficiency in human H9 ES cells is independent of promoters. Human ES cells were transiently transfected with EGFP lentiviral transfer vectors containing EGFP driven by different promoters. **(A)** The brightfield and GFP channel views of representative transduced cells were shown in the left and right columns, respectively. The promoters were indicated on the left. **(B)** The numbers of fluorescent cells per mm² of ES clone area were manually counted and plotted. Results shown are the mean of three independent experiments with indicated standard deviations. No significant difference between any two of the four promoters was detected by *t*-test ($p > 0.05$).