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Lentiviral Manipulation of Gene Expression in Human Adult and Embryonic Stem Cells

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ABSTRACT

Human stem cells could revolutionize the field of medicine by providing a diverse range of cell types for tissue replacement therapies and drug discovery. To achieve this goal, genetic tools need to be optimized and developed for controlling and manipulating stem cells *ex vivo*. Here we describe a lentiviral delivery system capable of high infection rates in human mesenchymal and embryonic stem cells. The lentiviral backbone was modified to express mono- and bi-cistronic transgenes and was also used to deliver short hairpin ribonucleic acid for specific silencing of gene expression in human stem cells. We show that lentiviral transduction can be used to alter gene expression without altering the genes' ability to differentiate *in vitro*. These vectors will enable rapid analysis of gene function in stem cells and permit the generation of knock-in/knock-out models of human disease in the rapidly developing field of gene therapy.

INTRODUCTION

THE THERAPEUTIC USE of stem cells has the potential to treat a wide range of clinical conditions where existing conventional therapies have proved inadequate. In animal models, it has been shown that stem cells can generate clinically useful cell types, such as insulin-producing cells for the treatment of diabetes[1] and dopamine-producing cells to cure Parkinson's disorders.[2] However, our limited understanding of the mechanisms controlling stem cell self-renewal and fate determination hampers the translation of these results into clinical therapies, prohibiting the production of pure populations of defined cell types in clinically relevant quantities. Additional barriers preventing the clinical use of stem cells, such as the immune rejection of grafted cells, also need to be overcome.[3] The limited progress in these areas is due in part to the lack of efficient genetic tools allowing the versatile manipulation of human stem cell gene expression.

Lentiviral-based vectors can efficiently transduce a wide range of cell types, including cells that are post-mitotic and non-dividing.[4] This technology can successfully manipulate murine embryonic stem cells, in which transgenes expressed from lentiviruses are not silenced during differentiation.[5,6] Progress was recently made in translating this technology to human stem cells. Several studies have demonstrated long-term expression of single transgenes in human stem cells using simple lentiviral constructs.[7,8] In addition, progress in the field of ribonucleic acid (RNA) interference has also demonstrated that lentivirally delivered short hairpin RNA (shRNA) is an effective tool for inhibiting gene expression in stem cells.[9] These advances have raised the potential of developing vectors that are capable of complex manipulation of stem cell gene expression.

Here we describe the development of a series of lentiviral vectors permitting complex manipulation of gene expression in human adult and embryonic stem cells. The vectors permit the delivery of mono- and bi-cistronic transgenes, providing long-term expression that is maintained throughout differentiation. By modifying the lentiviral backbone to deliver functional shRNA, we are able to demonstrate the inhibition of specific exogenous and endogenous gene expression. We

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also show that, by combining both technologies, it is possible to specifically knock-down and knock-in gene targets using a single vector. This technology could be used to generate complex knock-in/knock-out models of human disease and to control the stem cell fate of medically important cell types and tissues.

MATERIALS AND METHODS

Cell isolation, culture, and differentiation

Human mesenchymal stem cells (MSCs) were isolated, cultured, and differentiated into osteocytes and adipocytes as previously described[10] and showed the expected cell surface marker profile. MSCs were handled in accordance with local ethical guidelines. Human embryonic stem cell (hESC) lines H9 and HSF-6 were cultured under conditions as previously described, and cells were shown to express the appropriate cell surface markers. HEK293t cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% Fetal Calf Serum and 100 U/mL penicillin and streptomycin (Gibco BRL, Gaithersburg, MD).

Lentiviral vector plasmid construction

All lentiviral vectors were based upon the pHR-CMV-LacZ lentiviral backbone (a gift of D. Trono), the pHR-CSGW vector (a gift from Adrian Thrasher, modified by Yasuhiro Ikeda), and the pSIN-Puro vector (a gift from Greg Towers). pHR-CMV-MCS-IRES-GFP was created by inserting the complete encephalomyocarditis virus IRES removed from the pTRIDENT vector[11] into pHR-CMV-LacZ between the XhoI and KpnI (blunted) sites. Green fluorescent protein (GFP) was removed from pHR-CMV-GFP (gift from JB Rascle) using BamHI(blunted)-XbaI digestion, and inserted into pHR-CMV-IRES cut with KpnI (blunted)-XbaI. The multiple cloning site was inserted by annealing primers MCS_F 5′-GGATCCGCGGTATACTA GTCGA CCCGGGTGGCCACTCGAG-3′ and MCS_B 5′-CTCGA GTGGCCACCCGGGTCGACTAGTATACCGCGGATCC-3′ digesting with BamHI and XhoI, and ligating into pHR-CMV-IRES-eGFP cut with BamHI and XhoI.

The pSIN-RIG(−) vector was created by removing the BamHI-NotI(blunted) fragment from plasmid pDsRedN1 (Clontech, Mountain View, CA) and ligating into the BamHI-XhoI (blunted) backbone of pHR-CMV-MCS-IRES-GFP. The BamHI-XbaI (blunted) fragment was then ligated into the BamHI-XhoI (blunted) backbone of pHR-CSGW. pHR-SIN-RIG(+) was created by introducing a 50-bp sequence (5′-atcgactctctgagtacatgcattaccacctgtgagtactatgtagtca-3′) into the XhoI of pHR-CMV-MCS-IRES-GFP and subcloning as above into the pHR-CSGW backbone.

The pMIN-CMV vector was created by digesting pHR-CMV-MCS-IRES-GFP with XbaI and BamHI, blunting, and religation. The other pMIN vectors were created by digesting pHR-CMV-MCS-IRES-GFP with XbaI and ClaI, blunting and inserting using SacI/XhoI fragment (blunted) of pCG3-EF1a (a gift of JB Rascle), the spleen focus forming virus (SFFV) promoter (removed from pCSGW using EcoRI/BamHi blunted), or the b-actin promoter ClaI/BamHI (removed from pbactin-GFP, a gift from JB Rascle). To create the pMIN-eGFP vectors, eGFP was cloned from pCSGW using BamHI (blunted)-NotI (blunted) into the XbaI site (blunted).

Lentiviral shRNA construction

pSIN-CSGW and pSIN-Puro were modified to contain a short hairpin sequence by cloning the human U6 promoter from human BC3 cells into the PstI and NsiII sites in pGEM and adding SalI and XbaI sites for hairpin insertion to create pGEM-U6 using PCR primers U6 forward 5′-GGGCTGCAGAAGGTCGGGCAGGAAGAGGGCCTATT TCCC-3′ and U6 reverse 5′-GTTCCAATATGCATAAAA AATCTAGAGAAGCGTCGACGGTGTTTCGTCCTTTC CACAAG-3′. A sequence was added to include EcoRI and BamHI sites by annealing the oligos U6LinkerF 5′-CGA GCTCAGGATCCTGAATTCGATGCA-3′ and U6LinkerR 5′-TCGAATTCAGGAT CCTGAGCTCGTGCA-3′ and inserting this into the Nsi I sites. Short hairpin oligos were designed as detailed in the figures to include a 5′ blunt end, a TTCG loop, a five T U6 termination sequence, and a 3′ XbaI site (e.g., for DsRed [target TAATGCAGAAGAAGACC ATGGG], 5′-TAATGCAGAAGAAGACCATGGGTTCGC CCATGGTCTTCTTCTGCATTACTTTTT-3′ 5′-CTAGA AAAAGTAATGCAGAAGAAGACCATGGGCGAACCC ATGGTCTTCTTCTGCATT-3′).

pGEM-U6L was digested using SalI, blunted using Mung Bean Nuclease, and then digested using XbaI. Annealed short hairpin sequences were then ligated into this vector to make pGEM-U6-sh. pGEM-U6-sh was digested using EcoRI, and the 300-bp fragment containing the U6 promoter and short hairpin was ligated into an EcoRI digest of pSIN-eGFP or pSIN-Puro to make pSINeGFP-sh and pSINPuro-sh. The activity of the insert was orientation independent (data not shown).

Lentiviral vector production

Lentiviral production was established as previously described.[12] One μg of lentiviral plasmids p8.91 and pMD.G (both gifts from D. Trono) and pSIN or pHR plasmids (1.5 μg) were co-transfected into HEK293t cells seeded at 8×10^6 cells in a 10-cm dish 24 h before transfection. Media was changed on the 293t cells 1 h before transfection. The supernatant containing lentiviral vector particles was harvested 60 h after transfection. Viral titer was assessed in terms of 293t infectious units and was determined using serial dilution of lentiviral supernatant and infection of 2×10^5 293t cells in a 6-well format. After infection, quantitative polymerase chain reaction (qPCR) was used to determine the number of integrated viral copies by qPCR.

qPCR quantification of viral titers

qPCR was performed using an ABI TaqManPrism 7000 machine. Primers used for detection of lentiviral packaging complementary DNA are as follows: forward 5′-ACTT FGAAAGCGAAAGGGAAACCA-3′; reverse 5′-GTGCGC GCTTCAGCAA-3′; concentration 300 pM forward/300 pM reverse. Human glyceraldehyde phosphate dehydrogenase (GAPDH): forward 5′-GGAGTCAACGGATTTGG TCGTA-3′; reverse 5′-GGCAACAATATCCACTTTACC AGAGT-3′; probe 5′-CGCCTGGTCACCAGGGCTGC-3′; pM forward/700 pM reverse. All reactions were performed using SYBR green (Affymetrix, Santa Clara, CA) at the concentrations indicated. These conditions were shown through optimization to be sensitive and specific and with no primer-dimer formation. Standard TaqMan cycling conditions were used throughout.

Lentiviral infection protocol

Infection of MSCs was accomplished as follows. Lentiviral vectors were diluted into 1 mL of DMEM (for infection in a 6-well plate) or 5 mL DMEM (for infection in a 10-cm dish). Polybrene was added at a concentration of 8 μg/mL. The viral vector/polybrene mix was left at room temperature for 15 min and then added to the MSCs. After 4 h, the medium on the MSCs was changed. Viral expression was measurable 72 h after infection. Unless otherwise stated, the multiplicity of infection (MOI) for expression vectors was 10, and the MOI for shRNA vectors was 20. Transduction efficiencies of more than 90% were achieved.

Single-cell hESC suspension cultures (5×10^4 cell/mL) were generated using trypsin treatment. Virus supernatants were added at an MOI of 20 unless otherwise stated, with 8 mg/mL polybrene, incubated in suspension for 1 h, and then cells were plated on matrigel using feeder free conditions. eGFP expression was quantified after 7 days using microscopy and fluorescence-activated cell sorting (FACS) analysis. Transduction efficiencies of 50% to 60% were achieved.

Cell analysis

FACS analysis was performed on a Beckton-Dickinson FACS Calibur machine. The SSEA-4 antibody (Developmental Hybridoma Studies Bank [DSHB], Iowa City, IA) was used for FACS analysis as recommended by the manufacturer. Alkaline phosphatase and Oil Red O staining, indicating osteocytic and adipocytic differentiation, respectively, were performed as previously described.[10] Photographs were taken at a magnification of 100× unless otherwise stated, using a Nikon Coolpix 995 digital camera.

Differentiated cell marker staining was performed on hESC embryoid body outgrowths grown on gelatin-coated glass coverslips. Primary antibodies used were mouse monoclonal anti-chicken myosin heavy chain (CAT# MF 20, DSHB), mouse monoclonal anti-rat Class III β-tubulin (TUJ1) (CAT# MMS-435P Covance, DSHB), and mouse monoclonal anti-α-fetoprotein (AFP) clone C3 (CAT# A8452, Sigma, Poole, UK). Secondary goat anti-mouse –PE (DakoCytomation) was used to detect primary antibody.

Western blotting and SSEA-4 expression analysis

SDS-PAGE analysis for survivin expression was performed using electrophoresing samples lysed in Radio-ImmunoPrecipitation Assay buffer for 2 h at 70 V on a 15% polyacrylamide SDS gel. Gels were semi-dry transferred onto nitrocellulose membranes and blocked for 1 h in milk. After overnight incubation with primary mouse anti-survivin antibody (Cell Signaling Technology, Danvers, MA), membranes were thoroughly washed in phosphate-buffered saline and 0.05% Tween-20 and incubated for 1 h with secondary goat anti-mouse horseradish peroxidase (Cal Biochem). Membranes were then washed and developed using ECL Plus (Amersham Biosciences, Buckinghamshire, UK) as described by the manufacturer.

RESULTS

Lentiviral infection of human stem cells

Lentiviral vectors can efficiently transduce a wide range of cell types, including human stem cells.[7,8,13] We investigated whether a self-inactivating lentiviral vector (pSIN), which was originally developed for use with hematopoietic stem cells,[14] could be used for efficient transduction of hESCs and primary bone marrow–derived MSCs. All vectors described in this study were tested on hESC lines HSF-6 and H9 (National Institutes of Health codes UC06 and WA09, respectively). Both cell lines gave similar results, and therefore only representative experiments from H9 are presented here.

The level of transgene expression is dependent on the type of promoter used and is variable between different cell types. We therefore tested a range of promoters in MSCs and hESCs to identify a promoter that is universally highly expressed in both cell types. We constructed a series of minimal constructs in which the cytomegalovirus (CMV), the SFFV, elongation factor 1 alpha (EF1α), or the human β-actin promoter drove GFP expression (Fig. 1A). Virus generated from these vectors was pseudotyped with the vesicular stomatitis virus G-protein.[15] GFP expression levels were quantified using FACS analysis after infection of cells at an MOI of ten 293T infectious units per cell. Results are expressed as percentage of cells that are enhanced GFP (eGFP) positive, which should be interpreted as the percentage of cells in which the promoter gave a strong enough expression to cause cells to fluoresce above the background level. After infection of MSCs and hESCs, qPCR was used to ensure that the average viral copy number per cell for each of the promoter constructs was equivalent for each cell line (data not shown).

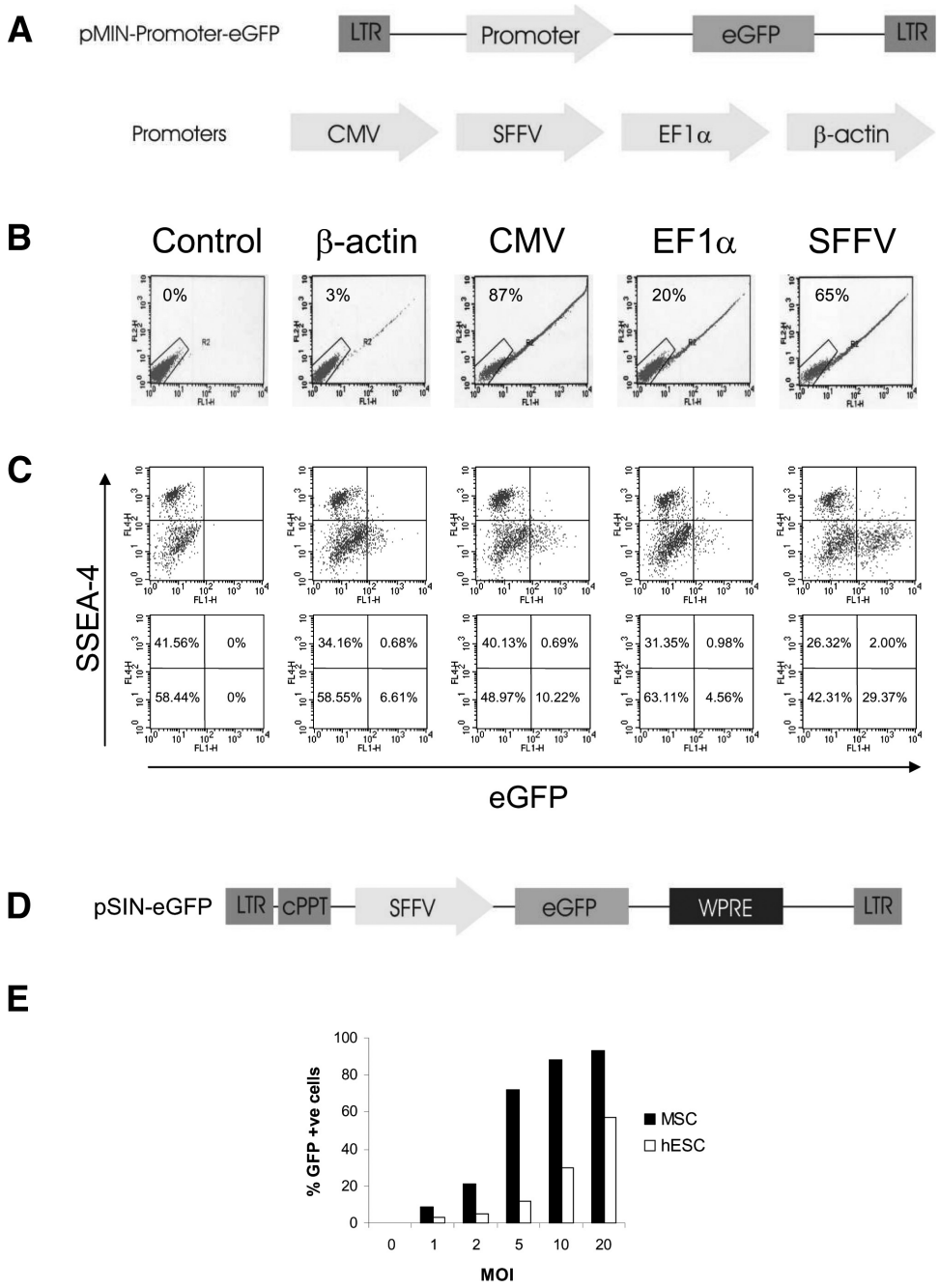

FIG. 1. Lentiviral infection of human stem cells: (**A**) The pMIN vectors containing only the minimal elements required for expression and 1 of the 4 promoters shown (LTR, long terminal repeat; eGFP, enhanced green fluorescent protein) (**B**) FACS analysis of eGFP expression (FL-1) in MSC infected with pMIN promoter constructs. (**C**) FACS analysis of eGFP expression (FL-1) and SSEA-4 expression (FL-2) of hESC infected with pMIN promoter constructs. Control panel represents mock infected cells. (**D**) Map of lentiviral constructs used (LTR, long terminal repeat; cPPT, central polypurine tract; SFFV, spleen focus forming virus; eGFP, enhanced green fluorescent protein; WPRE, woodchuck hepatitis virus post-transcriptional regulatory element) (**E**) Effect of MOI on % eGFP expressing hESC and MSC transduced with pSIN-eGFP.

MSCs showed an expression pattern similar to that observed for other adherent cell lines (CMV > SFFV > EF1α > β-actin) (Fig. 1B). However, hESCs showed a different pattern (SFFV > CMV > β-actin > EF1α). By staining the cells for stage-specific embryonic antigen (SSEA)-4 (cell surface marker highly expressed on undifferentiated hESCs[10]), we were able to determine the level of GFP expression within the differentiated and undifferentiated populations (Fig. 1C). SSEA-4 low cells consisted of hESCs and the murine feeder cells used for supporting hESC growth.

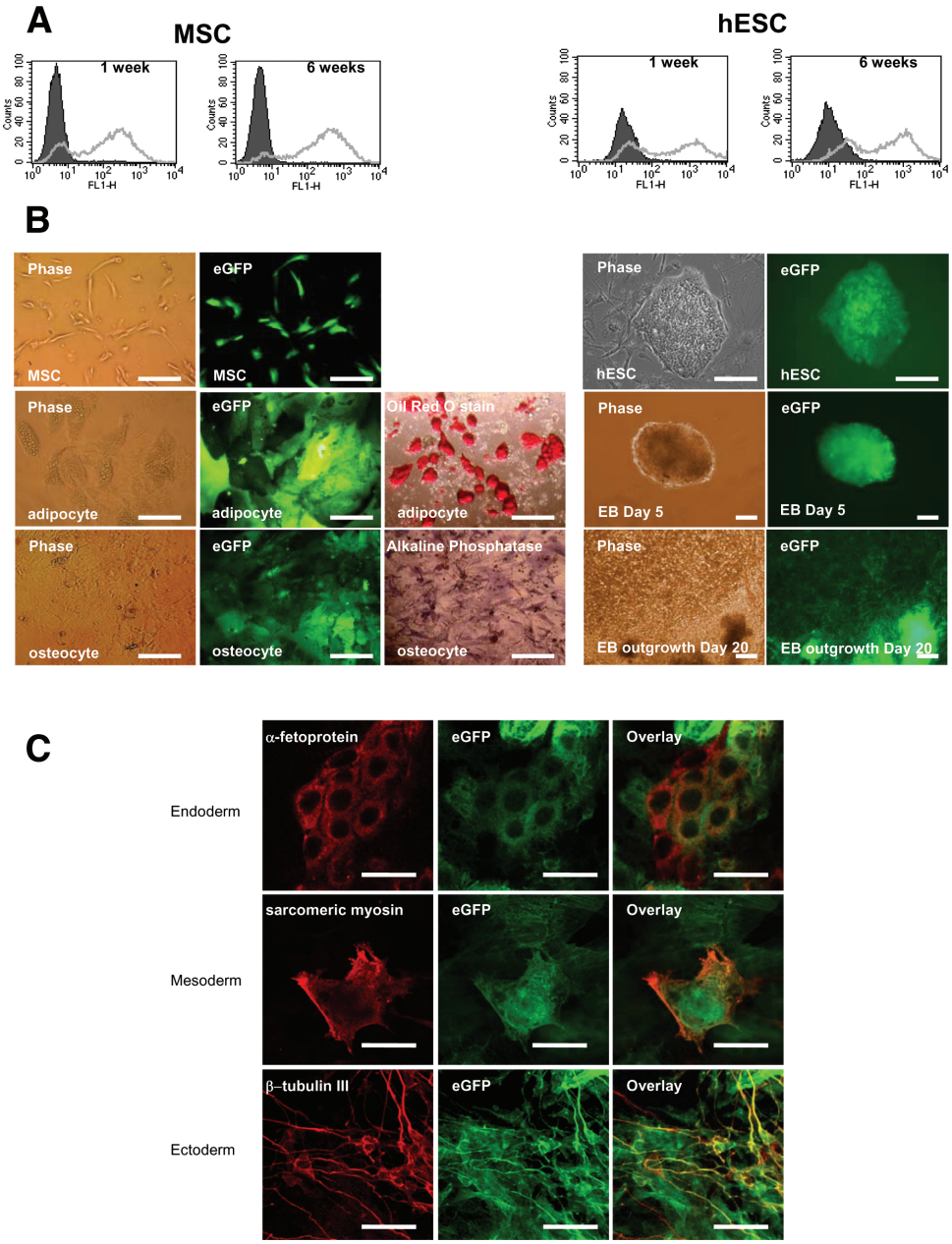

FIG. 2. Lentiviral transgene expression in stem cells is stable throughout differentiation: Cells were transduced with pSIN-eGFP. (**A**) FACS profiles of MSC and hESC showing stable expression of eGFP 6 weeks after infection with pSIN-eGFP. (**B**) Morphology of MSC transduced at a MOI 10 and differentiated into adipocytes and osteocytes. hESC transduced at a MOI 20 and differentiated to EB and EB outgrowths. Scale bar represents 200 μm. (**C**) Colocationation of eGFP with α-fetoprotein, myosin heavy chain, and β-tubulin III by cofocal microscopy of EB outgrowths. Scale bar represents 50 μm.

However, because of the method of infection, more than 95% of transduced cells were hESCs. For all the promoters, the greatest shift in fluorescence intensity was associated with the differentiated cells (SSEA-4 low). Only a small percentage of cells were expressing GFP strongly enough to be detectable in the undifferentiated population, with the SFFV promoter giving the highest levels of GFP expression (2% cells GFP positive). The EF1α promoter, previously shown to be a strong promoter in undifferentiated hESCs,[7] gave the second highest level of expression (1% GFP-positive cells in the undifferentiated population). We therefore decided to use the SFFV promoter in the further studies because of its high expression in MSCs and hESCs.

Previous studies have shown that the incorporation of a central polypurine tract (cPPT)[16] and woodchuck hepatitis virus post-transcriptional regulatory element (WPRE)[17] can increase transgene expression levels. By incorporating these elements into the lentiviral backbone (Fig. 1D), we were

able to enhance the level of GFP expression driven by the SFFV promoter in MSCs and hESCs (Fig. 1E). Expression of eGFP could be detected at high levels in MSCs and hESCs transduced with pSIN-eGFP. When MSCs were infected at an MOI of 10 and hESCs at an MOI of 20, expression could be detected in 88% of MSCs and 57% of hESCs using FACS analysis, and using microscopy, the transduced cells retained a normal morphology. This expression was stable for longer than 6 weeks (Fig. 2A). Transduced hESCs maintained expression of pluripotency-associated markers SSEA-4 and Tra-1-60 when grown in the undifferentiated state (data not shown). Increasing the MOI further did not enhance the infection in MSCs, although the frequency of transduced hESCs could be increased to 86% by an MOI of 50 (data not shown). By using selective mechanical dissociation of eGFP-positive hESC colonies from cultures transduced at an MOI of 10, we could select cultures in which more than 90% of cells were eGFP positive.

Transgene expression during differentiation

To determine whether lentiviral infection had any adverse effect on stem cell function, we assessed the potential of MSCs and hESCs to differentiate after infection with pHR-SIN-eGFP. When MSCs were transduced at an MOI of 10, no change in cell doubling time or viability was detected during propagation of the cells (data not shown). In addition, transduced cells retained the ability to differentiate to adipocytic and osteogenic lineages (upon induction), with transgene expression maintained throughout the assay (Fig. 2B). Differentiation of MSC was assessed using oil red O staining of lipid droplets in adipocytes or the expression of alkaline phosphatase as a marker for osteogenic differentiation.[18]

Differentiation potential of hESCs was assessed using embryoid body (EB) formation.[19] When hESCs are grown in suspension culture, in conditions permissive for differentiation, they form tight cell aggregates termed EBs, which consist of differentiated cell types of all 3 germ layers. In agreement with previous reports,[8] transduced hESCs formed typical EBs upon induction, and when transferred to adherent culture conditions, outgrowths from the EBs contained a diverse range of cell morphologies, including neural-like projections and beating cardiomyocytes (Fig. 2C). Concomitant with differentiation, hESCs lost expression of cell surface markers (SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81) characteristic of the pluripotent state (data not shown). We confirmed that transduced hESCs were pluripotent using immunostaining and confocal microscopy, which was used to co-localize GFP expression with markers of endoderm (α-fetoprotein), mesoderm (myosin heavy chain), and ectoderm (β-tubulin III) in EB outgrowths (Fig. 2C). These results show that the SFFV promoter is not silenced during differentiation and hence is a good promoter for use with stem cells.

At high viral titers, where the MOI was greater than 20, MSCs and hESCs retained markers of the undifferentiated state, but these cells started to show aberrant growth characteristics and failed to differentiate normally (data not shown). Because of the adverse effect infection at high MOI, we routinely used MOI less than 20 in all subsequent experiments.

Bi-cistronic transgene expression

Lentiviral vectors currently used with stem cells are only capable of single transgene expression. However, in other vector systems, multiple transgene expression has been achieved by linking the expression of 2 genes using an internal ribosomal entry site (IRES).[20] This system has been shown to require additional cellular cofactors; hence, expression is cell-type dependent. We therefore modified the lentiviral backbone to determine whether bi-cistronic gene expression was functional in stem cells. DsRed and eGFP were cloned into the pSIN backbone separated by the human encephalomyocarditis virus IRES (Fig. 3A).[21]

Although this construct was functional in 293 cells (data not shown), we found that MSCs transduced with this vector failed to express DsRed cloned upstream of the IRES element. This defect could be corrected by separating the upstream gene from the IRES element using a 50 bp linker, allowing comparable levels of eGFP and DsRed expression in MSCs and hESCs (Fig. 3B, C). The requirement for a 50 bp linker to achieve functional bi-cistronic expression was surprising but could be explained by the secondary structure of the IRES element and the close proximity of the 3′ end of the DsRed cistron close to the IRES structure causing premature termination of translation.

hESCs showed a marked toxicity to DsRed, reducing the efficiency with which red-IRES-green–positive colonies could be isolated. Further transfection and infection experiments showed this to be a DsRed and not a lentiviral or IRES-related problem (data not shown). Using DsRed2, which has recently been reported to have minimal toxic effect in hESCs, could potentially alleviate this problem.[22]

Functional RNA interference in stem cells

It has been shown that lentiviral vectors are able to deliver RNA interference (RNAi) to human primary and murine stem cells.[23] Recently, it was also shown that lentiviral vector–mediated RNAi can efficiently suppress gene expression in undifferentiated hESCs, although it was not reported whether this suppression was stable during differentiation.[9] We therefore expanded previous studies by confirming that shRNA delivered by our lentiviral vector was functional in hESCs and MSCs and demonstrating that co-expression of a drug resistance marker could be used to select for transduced cells. We also assessed the extent of gene suppression during differentiation.

To test this, we produced lentiviral constructs encoding a human U6 promoter and a short hairpin targeting eGFP or

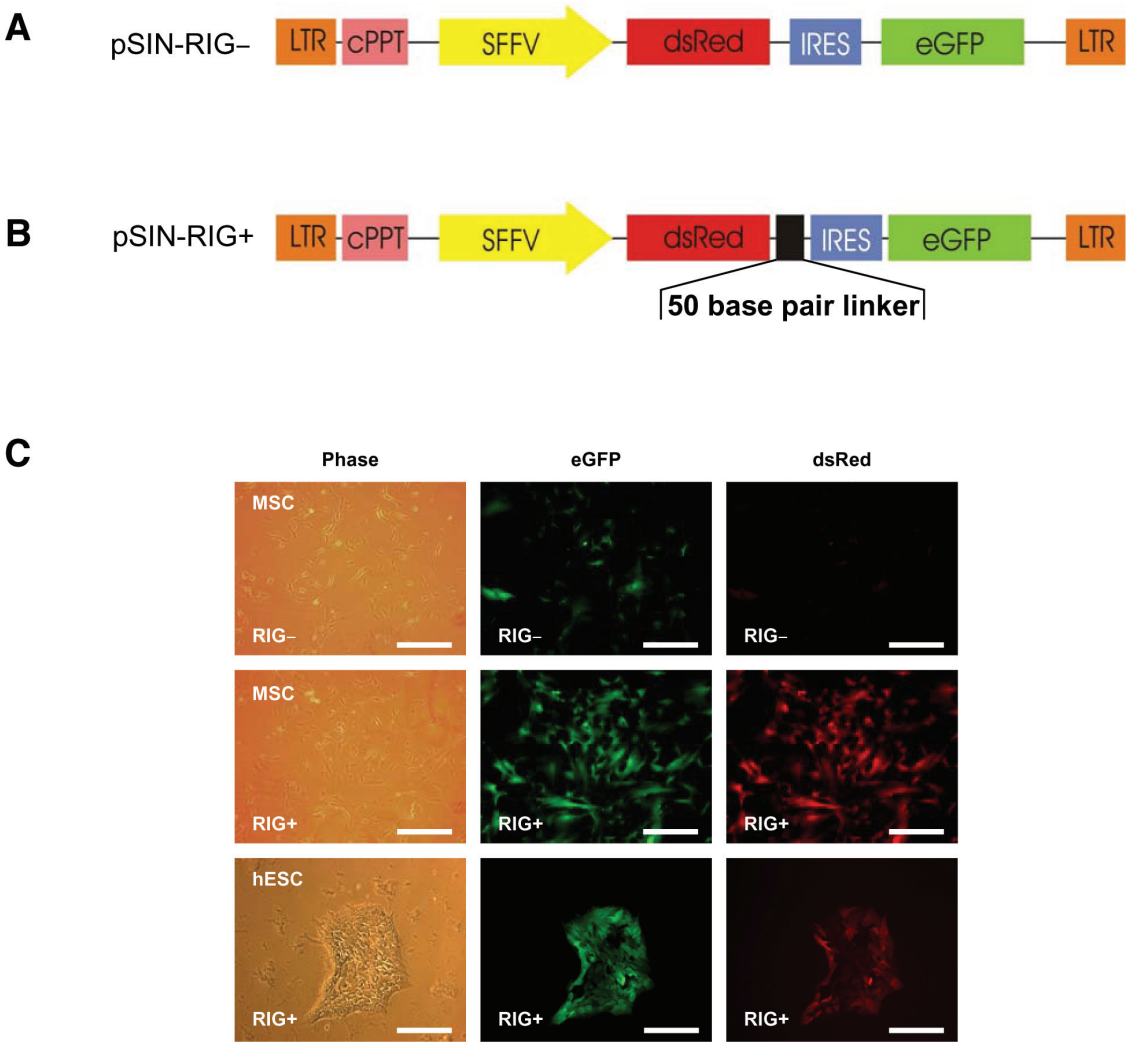

FIG. 3. Functional bi-cistronic transgene expression in MSC and hESC. (**A**) Maps of lentiviral constructs used (LTR, long terminal repeat; cPPT, central polypurine tract; SFFV, spleen focus forming virus; eGFP, enhanced green fluorescent protein; DsRed, red fluorescent protein; IRES, internal ribosomal entry site); (**B**) Site of insertion of 50 bp linker in pSIN-RIG+. (**C**) Infection of MSC and hESC with pSIN-RIG− (no linker) and pSIN-RIG+ (with the linker) demonstrating the requirement for a 50 bp linker for bicistronic expression in stem cells. Scale bars represent 200 μm. (Color images available online at www.liebertpub.com/ten.)

LacZ as a control (Fig. 4A). This construct also contained a puromycin resistance cassette driven from the SFFV promoter. We initially chose to target eGFP expression because changes in fluorescence can be easily quantified at the single cell level, and down-regulation of eGFP expression has been shown not to alter viability or growth of stem cells.[24] The shRNA target sequences chosen had no sequence similarity to any other gene as determined using BLAST analysis. We generated MSC and hESC lines stably expressing GFP by transduction with pSIN-SFFV-eGFP. These cells were subsequently infected with pSINPuro-sh-LacZ or pSIN-Puro-sh-eGFP (MOI 10) and treated with puromycin to select for stably transduced cells. After 10 days of selection, lower eGFP expression was visible in cells transduced with sh-eGFP (Fig. 4B) with a 90% reduction in fluorescence intensity was observed using FACS analysis (Fig. 4C). In contrast, cells infected with the control sh-LacZ construct showed no reduction in eGFP expression. These results confirm that lentiviral vectors co-expressing shRNA and a drug selection marker are functional in human stem cells.

To determine whether RNAi was stable during differentiation, the above cells were induced to differentiate. Transduced MSCs showed normal differentiation to adipocytes and osteocytes, demonstrating that neither double viral infection nor the cellular process of RNAi interfered with stem cell function (Fig. 5A). Silencing was stable throughout differentiation, whereas eGFP expression was unaffected in the control pSINPuro-sh-LacZ cells. Similarly, transduced hESCs showed no deleterious effects from shRNA expression, as assessed using normal EB formation, with stable

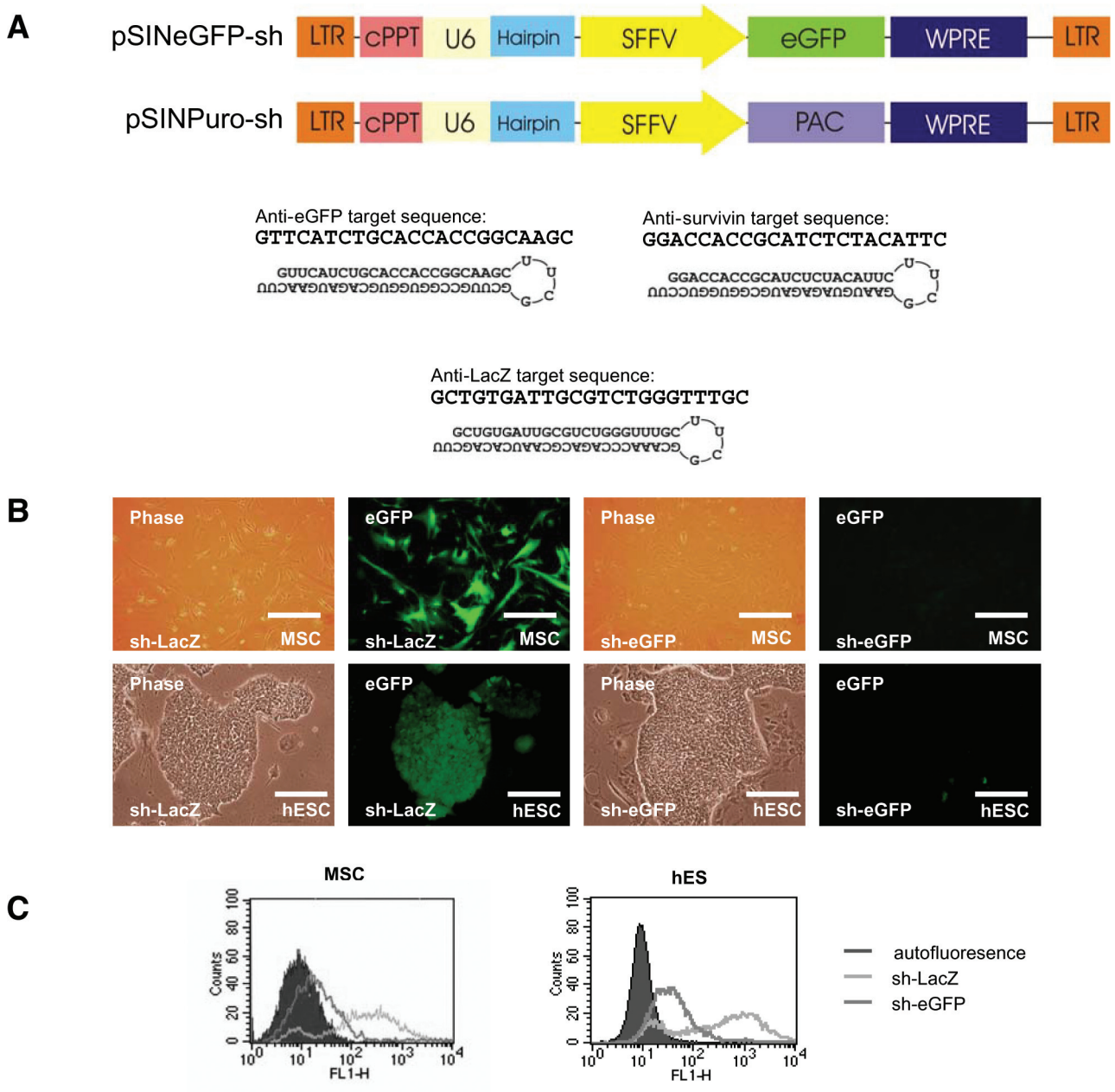

FIG. 4. shRNA functions to silence gene expression in adult stem cells. (**A**) The lentiviral vector construct used and the target sequences against eGFP, LacZ, and survivin. (**B**) knockdown of eGFP expression in MSC (top) and hESC (bottom). Scale bar represents 200 μm. (**C**) FACS analysis of RNA interference in MSCs and hESCs showing 90% reduction in fluorescence intensity. (Color images available online at www.liebertpub.com/ten.)

silencing of eGFP expression throughout culture and differentiation (Fig. 5B).

Single vector knock-in knock-out

Finally, we assessed the ability of a single vector to simultaneously express and inhibit gene function in stem cells. We created a vector pSINeGFP-sh-survivin encoding eGFP and a short hairpin targeting survivin, an endogenous anti-apoptotic gene[25] (Fig. 4A). MSCs infected with pSINeGFP-sh-survivin showed an efficient knockdown of survivin levels with a simultaneous expression of eGFP (Fig. 5C). This vector was also functional in hESCs, where stable reduction in endogenous survivin levels concomitant with expression of eGFP was observed (Fig. 5C).

DISCUSSION

Our inability to manipulate and control stem cell function hampers the translation of stem cell biology to clinical practice. Given the recent use of viral vectors for *ex vivo*

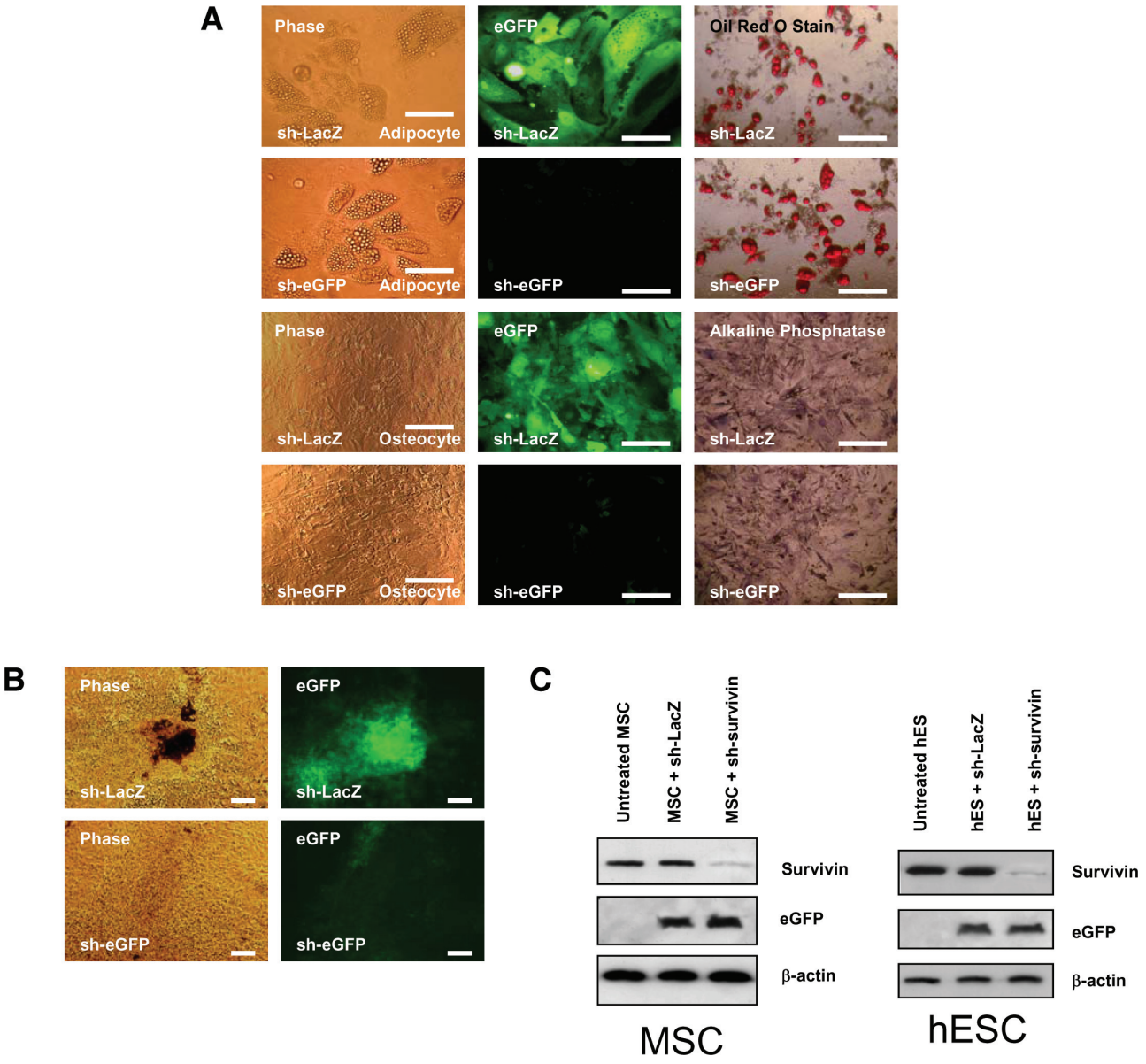

FIG. 5. Stability of RNAi during differentiation and single vector knock-in/knock-out. (**A**) MSC infected with pSIN-eGFP and then with either pSINPuro-sh-LacZ (control) or pSINPuro-sh-eGFP (eGFP target). Cells were induced to differentiate into adipocytes (top) or osteocytes (bottom) showing that the reduction in eGFP expression is stable throughout differentiation; (**B**) inhibition of eGFP expression in hESC during EB outgrowth formation; (**C**) Western blot of MSC and hESC infected with pSINeGFP-sh-survivin showing simultaneous eGFP expression and survivin inhibition. Scale bars represent 200 μm. (Color images available online at www.liebertpub.com/ten.)

manipulation of stem cells in clinical trials,[26] we therefore investigated whether a series of lentiviral vectors could be used to manipulate gene expression in pluripotent hESCs that have the potential to differentiate into all cell types and in adult MSCs that are lineage restricted to mesoderm fates. Although it has been previously reported that lentiviral vectors can transduce both cell types,[7,8,13] we wanted to expand these studies further by demonstrating that modified lentiviral vectors can be used for complex manipulation of stem cell gene expression.

The pSIN vectors used in this study are based on the human immunodeficiency virus-1 genome and are considered to be the safest currently available because of the extensive deletions abolishing self-replication and its ability to recombine with wild-type viruses.[27] In agreement with previous reports, we found that pSIN-based vectors could efficiently transduce MSCs at low MOI, whereas hESCs were more recalcitrant to infection. At low viral input (MOI < 20), we observed no detectable defects in MSC and hESC function. This was assessed using *in vitro* differentiation of MSCs to

osteocytes and adipocytes and by the ability of hESCs to form EBs, which upon prolonged culture, gave rise to a range of cell morphologies that stained positive for cell markers specific for mesoderm, endoderm, and ectoderm fates, as well as producing cells with functional characteristics, such as pulsating cardiomyocytes. This is in agreement with a previous study that showed no defects in hESC differentiation *in vitro* (EB formation) or *in vivo* (teratoma formation).[8] However, at higher viral loads, we observed aberrant cell function in MSCs and hESCs. We presume that these effects are due to genomic damage as a result of multiple viral insertions, highlighting the need for further studies to assess the effect of viral copy number on stem cell function.

Although transduction frequencies of up to 90% have been reported in hESCs using high (MOI > 20) viral input,[6] it is clear from our results that such cells are likely to have a reduced differentiation capacity, despite showing unaltered growth behavior in the undifferentiated state. Surprisingly, these cells maintained expression of pluripotent markers, despite being unable to form EBs, demonstrating the risk of using marker expression as the sole criterion for pluripotency. To retain differentiation potential after viral transduction of hESCs, it was necessary to use low MOI. This led to reduced infection efficiency; however, pure populations of transduced cells could be enriched using selection procedures such as mechanical dissociation of individual colonies or by the use of a drug selection marker. Using such procedures, we have been able to produce pure cultures of hESCs expressing eGFP, using an MOI of as low as 5 (data not shown).

Lentiviral vectors currently developed for use with MSCs and hESCs are relatively simple, typically permitting the expression of single transgenes. However, complex manipulation of stem cell function will probably require expression of multiple genes, which, using current vectors, would require multiple viral insertions, increasing the risk of adverse effects from high viral load. We found that multiple transgenes could be expressed from a single vector using a modified IRES element that avoids the need for cumbersome dual-promoter cassettes. This could be used to express multiple transgenes to manipulate stem cell function or, alternatively, permit the linkage of a selection marker (eGFP or drug selection) to transgene expression.

Gene function studies in human tissues using current approaches are difficult and laborious. The discovery of RNAi, in which the expression of small double-stranded RNA molecules can be used to specifically knock-down expression of targeted genes, has recently revolutionized the field. Knock-down of gene expression using shRNA has been demonstrated in hESCs and MSCs.[19] However, these studies have not addressed whether stable expression of shRNA from a lentiviral vector has any deleterious effects on stem cell function. We modified the lentiviral backbone to express shRNA driven from the U6 promoter. Using this vector, we were able to show specific stable suppression of eGFP expression in MSCs and hESCs with no alteration in the ability of the transduced cells to differentiate. In addition, we were able to show that co-expression of a drug selection marker with the shRNA from the lentiviral backbone allowed rapid selection of transduced cells expressing the shRNA.

The ability to knock-in/knock-down targeted genes offers a real potential to extend gene therapy to conditions that require complex gene manipulation. This notion could be extended to the control of stem cell differentiation, in which lineage commitment often involves the concomitant induction and inhibition of signaling pathways. Using the lentiviral backbone, we demonstrated the feasibility of single-vector knock-in/knock-down manipulation by creating pHR-SINeGFP-sh-survivin, which expresses eGFP from the SFFV promoter and shRNA, targeting the endogenous anti-apoptotic gene survivin. This vector was functional in MSCs and hESCs with altered gene expression stable throughout differentiation. The ability to stably knock- down survivin demonstrates that lentiviral-delivered shRNA is an effective tool for inhibiting endogenous gene expression and that survivin is non-essential for normal growth and differentiation of MSCs and hESCs.

In conclusion, we have shown that lentiviral-based vectors can be used for the complex manipulation of gene expression in human adult and embryonic stem cells. This technology will enable the systematic analysis of gene function in stem cells and could potentially be used to control cell fate. In addition, the application of RNAi in human embryonic stem cells could be used to mimic genetic disorders and could potentially be exploited therapeutically.

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