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Rapid Generation of Stable Transgenic Embryonic Stem Cell Lines Using Modular Lentivectors

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ABSTRACT

Generation of stable transgenic embryonic stem (ES) cell lines by classic transfection is still a difficult task, requiring time-consuming clonal selection, and hampered by clonal artifacts and gene silencing. Here we describe a novel system that allows construction of lentivectors and generation of stable ES cell lines with >99% transgene expression within a very short time frame. Rapid insertion of promoters and genes of interest is obtained through a modular recombinational cloning system. Vectors contain central polypurine tract from HIV-1 element and woodchuck hepatitis virus post-transcriptional regulatory element as well as antibiotic resistance to achieve optimal and homogenous transgene expression. We show that the system 1) is functional in mouse and human ES cells, 2) allows the generation of ES cells expressing genes of interest under the control of ubiquitous or tissue-specific promoters, and 3) allows ES cells expressing two constructs through selection with different antibiotics to be obtained. The technology described herein should become a useful tool in stem cell research. STEM CELLS *2006;24:615–623*

INTRODUCTION

Transgenic embryonic stem (ES) cell lines are important tools for studying development and cell differentiation, yet the generation of such lines remains technically difficult and time-consuming. Gene delivery in ES cells can be achieved by chemical/mechanical transfection or by viral transduction [1]. Establishment of stable transgenic ES cell lines has so far mainly been achieved through electroporation [2, 3]. However, this technique is time-consuming, requiring clonal selection, analysis of antibiotic-resistant clones for transgene expression, and the use of several transgene-expressing clones for follow-up experiments to exclude clonal artifacts.

Retroviral vectors have been used by several groups to achieve stable transgene expression in ES cells [4–7]. However, a major limitation of retroviral vectors is gene silencing that occurs during propagation [8] and differentiation [9] of transduced cells. More recently, lentivectors have been successfully used to transduce ES cells [10–12]. Current generations of lentivectors are self-inactivating [13] and, therefore, compatible with a high biosafety level. Although silencing in ES cells has also been reported with lentivectors [14], it appears to occur to a lesser extent than with traditional retroviral vectors [10, 15]. Thus, lentivectors are promising tools for engineering genetically modified ES cell lines. However, there are several limitations for a widespread use of lentivectors in ES cell research, including 1) the cloning flexibility provided by presently available lentivectors is poor and 2) the transduction efficiencies of ES cells with lentivectors are only in the range of 20%–80% [1]. Thus, selection strategies to obtain homogenously transgene-expressing cell lines are necessary. So far, the most promising tools have been bicistronic lentivectors [16, 17], which allow the coexpression of a gene of interest and a selection marker under the control of the same promoter. However, this approach has two major limitations: 1) transgene expression levels are poorly predictable [18] and 2) the establishment of stable ES cell lines using tissue-specific promoters is not possible, because the selection marker will not be expressed in undifferentiated cells.

The use of tissue-specific promoters driving the expression of reporter genes is of particular interest to mark subsets of ES cell progeny. This approach can be used to monitor ES cell differentiation [19], and it is a powerful tool for purifying cells of a particular lineage [20]. However, due to limitations mentioned above, generation of such cell lines remains a technical challenge.

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In this article we describe a novel lentiviral system for generating stable ES cell lines that overcomes many of the limitations observed with previously available lentivectors. We demonstrate rapid generation of stable ES cell lines expressing transgenes at various levels using different ubiquitous promoters. We also show monitoring of ES cell differentiation by cell type-specific expression of reporter genes.

Materials and Methods

Reagents

Reagents and their sources were as follows: R4–R2 DNA cassette and pLenti6/BLOCK-iT-DEST, pDONR221, and pDONRP4-P1R vectors (Invitrogen, Carlsbad, CA, http://www.invitrogen.com); pWPT-GFP vector was kindly provided by Drs. P. Salmon and D. Trono (Department of Microbiology, Faculty of Medicine, Geneva University, Switzerland); pEGFP-N1 plasmid [BD Biosciences (San Diego, http://www.bdbiosciences.com)/Clontech (Palo Alto, CA, http://www.clontech.com)]; the pGEM®-T Easy plasmid (Promega, Madison, WI, http://www.promega.com); Tα1 α-tubulin and the Synapsin1 promoters were kindly provided by Freda Miller (Hospital for Sick Children, University of Toronto, Canada) [21] and by James Uney (University Research Centre for Neuroendocrinology and Medical Research Council, Centre for Synaptic Plasticity, University of Bristol, Bristol, U.K.) [22], respectively; and the monomeric red fluorescent protein 1 (referred to as RedFP in this report) was kindly provided by Roger Tsien (Howard Hughes Medical Institute, University of California at San Diego, San Diego) [23]. The murine CGR8 ES cell line was from the European Collection of Cell Culture; the human H1 ES cell line was from Wicell Research Institute Inc.; the murine D3 ES cell line was provided by Reinhard Korn (AnTeq AG, Basel, Switzerland); the stromal bone marrow MS5 cell line was provided by Katsuhiko Itoh (Department of Clinical Medical Biology, Kyoto University, Kyoto, Japan) [24]; cell culture media, fetal bovine serum, serum replacement, penicillin, streptomycin, N2 supplement, nonessential amino acids, sodium pyruvate, and neomycin were from Gibco (Grand Island, NY, http://www.invitrogen.com); basic human fibroblast growth factor, blasticidin, and Gateway clonase enzymes were from Invitrogen.

Vector Constructions

A PCR product flanked by R4–R2 recombination sites and containing the ccdB and chloramphenicol resistance coding sequences was ligated into SpeI-SacII-cleaved pLenti6/BLOCK-iT-DEST lentivector. The woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) from pWPT-GFP was ligated 3′ of the R4–R2 cassette into the EcoRI-cleaved lentiviral construct. The cPPT element from pWPT-GFP was ligated 5′ of the R4–R2 cassette into NheI-SpeI-cleaved lentiviral construct. We named the resulting lentiviral construct $2K7_{bsd}$. To generate the $2K7_{neo}$ lentivector, the blasticidin resistance coding sequence and the bacterial EM7 promoter were replaced by the neomycin resistance coding sequence. To generate entry vectors, the different promoters and genes of interest were cloned into pDONRP4–P1R and pDONR221, respectively, using the Gateway BP clonase enzyme mix. The resulting entry vectors were then recombined into $2K7_{bsd}$ or $2K7_{neo}$ lentivectors using the Gateway LR plus clonase enzyme mix.

Cell Cultures

The CGR8 and D3 ES cells were maintained in BHK-21 medium supplemented with 10% fetal calf serum, L-glutamine, nonessential amino acids, sodium pyruvate, penicillin and streptomycin, and leukemia inhibitory factor. The H1 ES cells were maintained in Dulbecco's modified Eagle's medium (DMEM)/F-12 medium supplemented with 20% serum replacement, L-glutamine, nonessential amino acids, and 4 ng/ml human basic fibroblast growth factor. CGR8 ES cells were cultured on gelatin-coated dishes. D3 ES cells were cultured on irradiated mouse embryonic fibroblasts (MEFs) or STO mouse fibroblasts. H1 ES cells were cultured on irradiated mouse embryonic fibroblasts.

ES Cell Differentiation

Neuronal differentiation was carried out as described [25]. Briefly, irradiated MS5 cells (1.75×10^5 per well) were seeded in 6-well plates. The next day, CGR8 cells (0.6×10^3 to 3×10^3 cells per well) were plated on the MS5 layer in complete DMEM supplemented with nonessential amino acids, 2-mercaptoethanol, and 15% knockout serum. Five days later, cells were then trypsinized and seeded onto polyornithin-coated 6-well plates in complete DMEM supplemented with N2 supplement and human basic fibroblast growth factor. Embryoid bodies were generated by the hanging drop method as described [26].

Lentivector Production and Transductions

The lentivector particles were produced by transient transfection in 293T cells as previously described [27]. The lentivector-containing supernatant was collected after 72 h, filtered through 0.45-μm pore-sized polyethersulfone membrane, and concentrated 120-fold by ultracentrifugation (50,000*g*, for 90 minutes at 4°C). The pellet was resuspended in complete cell culture medium and subsequently added to the target cells. Titers of the concentrated lentivector were estimated by HeLa cell transduction and ranged from 5×10^7 to 10^8 transducing units per milliliter. The multiplicity of infection ranged from 1.2×10^4 to 2.5×10^4 for transduction of murine D3 and CGR8 ES cells and from 15 to 30 for transduction of human H1 ES cells. CGR8 ES cells (10^4 cells per well) were seeded onto gelatin-coated 6-well plates 1 day prior to transduction. Two days later, cells were split in 85-mm gelatin-coated culture dishes. D3 ES cells (2×10^4) were transduced in suspension on gelatin-coated 6-well plates and 1 day later were split onto the blasticidin-resistant STO feeder layer. H1 ES cell aggregates (5×10^5 cells) were transduced in suspension onto the MEF feeder layer or the blasticidin-resistant STO feeder layer. Three days after transduction, blasticidin or neomycin was added to the culture medium of ES cells. Blasticidin selection was maintained for 6 days on murine ES cells or 21 days on human H1 ES cells, and neomycin selection was maintained for 10 days for murine CGR8 ES cells. The antibiotics were used as follows: CGR8 cells, 7.5 μg/ml blasticidin or 400 μg/ml neomycin; D3 cells, 10 μg/ml blasticidin; and H1 cells, 10 μg/ml blasticidin.

Self-inactivation of Lentivectors in ES Cells

We verified whether transduced cell lines were indeed unable to generate infectious lentiviral particles. A stable CGR8 murine ES cell line transduced with $2K7_{bsd}$ EF1-αS/GFP and nontransduced CGR8 cells were seeded at a density of 5×10^5 cells per 85-mm dish and cultured for 72 hours up to 70% confluency without changing the medium. The supernatant was then collected and filtered through a 0.45-μm pore-sized polyethersulfone membrane, and 1 or 2 ml were incubated with 10^4 HeLa cells. Experiments were performed in triplicates. After 72 hours of incubation, no enhanced green fluorescent protein (eGFP) expression could be detected in HeLa cells incubated in either supernatant, demonstrating that 2K7-transduced ES cell lines do not release infectious lentiviral particles into the medium.

Immunofluorescence Microscopy

Immunofluorescence was carried out according to standard techniques. In brief, ES cells were grown on glass coverslips coated with either an MS5 feeder layer or polyornithin in 6-well plates. Cells were fixed with 2% paraformaldehyde for 30 minutes, washed with Hanks' balanced salt solution (HBSS), and permeabilized with 0.5% (vol/vol) Triton X-100 for 30 minutes. Cells were then exposed to primary antibodies overnight at 4°C. After two washes in HBSS containing 1% serum (blocking buffer), cells were stained with secondary antibodies at room temperature for 1 hour (1:1000 dilution in blocking buffer). Cell nuclei were stained with 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) for 10 minutes. Visualization analysis took place on a Zeiss axioplan microscope equipped for epifluorescence. The dilutions for the primary antibodies in blocking buffer were as follows: mouse monoclonal antinestin antibody (1/2500, Chemicon, Temecula, CA, http://www.chemicon.com), rabbit polyclonal anti-synapsin antibody (1/1000, Chemicon), and rabbit polyclonal anti-class III β-tubulin antibody (1/1000, Covance, Princeton, NJ, http://www.covance.com). For secondary detection, Alexa Fluor 488 or 555 conjugates were used (1/1000, Molecular Probes Inc., Eugene, OR, http://probes.invitrogen.com). Rat primary hippocampal neurons were used as positive controls for class III β-tubulin and synapsin immunostaining. Glial cells from mouse hypothalamic median eminence were used as positive controls for nestin immunostaining. For negative controls, immunostaining was performed without first antibody.

Quantitative Analysis of Cells Expressing Fluorescent Proteins

Feeder cell-independent eGFP-transduced cells (CGR8) were analyzed by flow cytometry using a FACScan (BD Biosciences). Quantification of expression of fluorescent proteins in feeder cell-dependent D3 murine ES cells (which were cultured in the presence of RedFP-positive feeder cells) were quantified by direct cell counting using a fluorescence microscope.

For flow cytometry analysis, H1 ES cells were rinsed with phosphate-buffered saline, incubated in trypsin-EDTA for 20 minutes, and passed through a 60-μm cell strainer (Falcon). Cells were labeled on ice with TRA-1–85 antibody (Developmental Studies Hybridoma Bank, Iowa City, IA, http://www.uiowa.edu/~dshbwww) at a 1:100 dilution, rinsed, and incubated with a goat anti-mouse-PE secondary antibody (DAKO, Glostrup, Denmark, http://www.dako.com) at a 1:80 dilution. Cells were analyzed with a FACScan (BD Biosciences).

For the studies investigating the activity of the Tα1 α-tubulin/RedFP and Synapsin1/eGFP constructs during neuronal differentiation of CGR8 cells, fluorescence intensity of eGFP and RedFP in a given cell was quantified using the Metamorph software.

RESULTS

Construction of the 2K7 Lentivector

We intended to construct a lentivector with the following properties: 1) easy insertion of promoters and genes of interest; 2) possibility to select transduced cells through antibiotics; and 3) high level transgene expression. To construct such a vector, we modified a commercially available self-inactivating lentiviral vector (pLenti6/BLOCK-iT-DEST, Invitrogen). We replaced its recombination site with a double recombination cassette (R4–R2 Gateway cassette), which allows the recombination of a promoter and a gene of interest from two separate entry vectors. To enhance transgene expression, the WPRE was inserted 3′ to the recombination site [28]. To increase the number of integrated transgenes upon transduction, the central polypurine tract from HIV-1 (cPPT) was inserted 5′ to the recombination site [29]. We named the vector obtained through this procedure 2K7. As the pLenti6/BLOCK-iT-DEST vector contained a blasticidin resistance, the first version of 2K7 also contained this resistance and is therefore specified as $2K7_{bsd}$. To allow double transduction and selection, we also constructed a version of the vector where the blasticidin resistance was replaced by a neomycin resistance. This vector will be referred to as $2K7_{neo}$. A general scheme of the 2K7 vector is shown in Fig. 1.

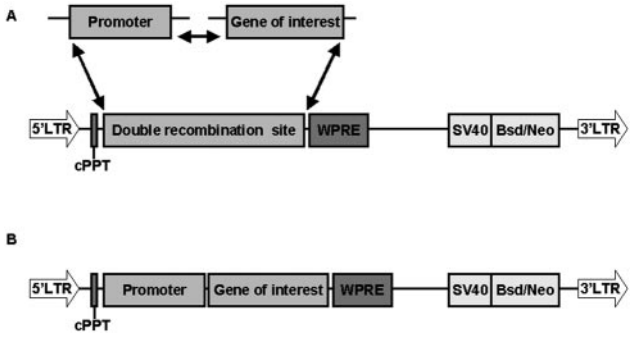

Figure 1. Schematic of the 2K7 lentivector and the insertion of promoters and genes of interest. The recombination site of pLenti6 BLOCK-iT-DEST was replaced with a cassette flanked by the R4 and R2 recombination sites, thus enabling the spatially controlled insertion of both a promoter and a gene of interest from two separate entry vectors. Arrows indicate recombination events between the different elements, resulting in the expression construct. 3′ to the recombination site, a SV40 promoter drives the expression of an antibiotic resistance. The WPRE and cPPT element were inserted into the vector backbone to increase transgene expression and copy number of the integrated transgene, respectively. Finally, a version of the vector containing a neomycin instead of the blasticidin resistance was generated. This allows selection for the expression of two different constructs in the same cell. **(A):** Schematic representation of two entry vectors and the recipient 2K7 lentivector; arrows show recombination events that allow directional cloning. **(B):** Resulting expression lentiviral construct.

Constitutive Expression of eGFP in Mouse and Human ES Cells

We first transduced the feeder cell-independent CGR8 murine ES cell line with the $2K7_{bsd}$ vector. We used three different lentiviral constructs. All three contained eGFP as the gene of interest but under the control of three different ubiquitous promoters: a 590-basepair (bp) human cytomegalovirus (CMV) immediate early promoter, a 1264-bp human EF1-α promoter, and a 260-bp, intron-less fragment of the human EF1-α promoter [30]. Throughout the text, these promoters will be referred to as CMV, EF1-α, and EF1-αS, respectively. Three days after transduction, approximately 30–50% of CGR8 cells were eGFP-positive. Transduced cells were subsequently subjected to blasticidin (7.5 μg/ml) selection for 6 days. eGFP expression after blasticidin selection was analyzed by fluorescence microscopy and by flow cytometry (Figs. 2A–2E). All three promoters led to eGFP expression, but expression levels were markedly different. The CMV promoter led to relatively low levels, the EF1-αS promoter to intermediate levels, and the EF1-α promoter to high levels of eGFP expression. Quantitative analysis by flow cytometry demonstrated that, under the control of the long and the short forms of the EF1-α promoter, eGFP was expressed in over 99% of CGR8 cells but, under the control of the CMV promoter, only in approximately 95% of cells. Once blasticidin selection was terminated, expression levels for all three promoters were well maintained over time in culture, even in the absence of the antibiotic. For one of the cell lines (EF1-αS/eGFP), we performed quantitative analysis: flow cytometry showed that over 99% of CGR8 were still eGFP-positive with similar mean fluorescence intensity after 30 days in culture (Figs. 2F, 2G). Thus, as described before for other lentivectors [10], integration of the 2K7 vector in undifferentiated mouse ES cells is stable and maintained over a prolonged period. These results also suggest that it is not necessary to maintain blasticidin selection beyond 6 days after transduction to maintain a pure population of transduced cells.

Although transgene silencing was not detectable in undifferentiated cells, it might occur during ES cell differentiation. Thus, we wondered whether eGFP fluorescence was maintained during neuronal differentiation of the transduced CGR8 cell lines described above. We induced neuronal differentiation by culturing EF1-α/eGFP-transduced CGR8 cells on a layer of MS5 cells followed by replating and culture on polyornithin-coated dishes for 4 days (as described in Materials and Methods). As shown in Figures 3A and 3C, all cells remained eGFP-positive during neuronal differentiation. Differentiated neurons were identified by immunostaining with an anti-β3-tubulin antibody (Fig. 3B). The overlay of eGFP fluorescence and anti-β3-tubulin immunostaining (Fig. 3D) demonstrates that a substantial fraction of cells were differentiated towards neurons. Note that there is no apparent difference in eGFP fluorescence intensity between β3-tubulin-positive and -negative cells. Similar results were obtained with CMV/eGFP-transduced and EF1-αS/eGFP-transduced CGR8 cells (data not shown).

We next studied the transduction of feeder cell-dependent ES cell lines. Two different approaches were chosen for the

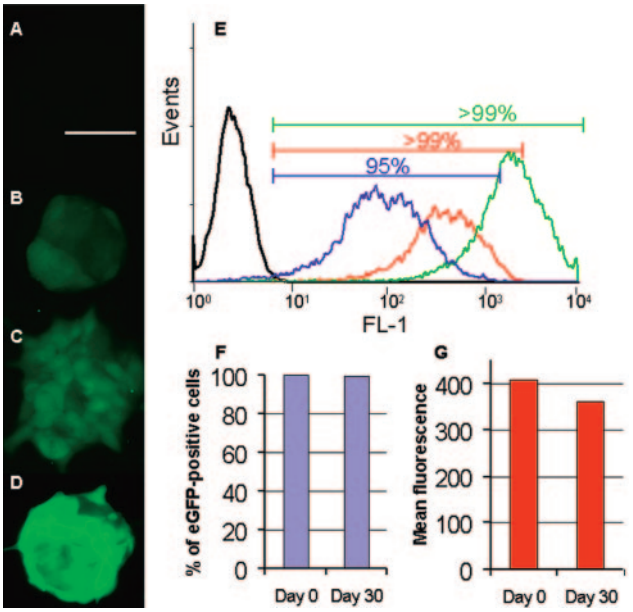

Figure 2. eGFP expression under the control of different ubiquitous promoters in mouse embryonic stem (ES) cells. The feeder cell-independent CGR8 mouse ES cell line was transduced with different $2K7_{bsd}$ lentiviral constructs to express eGFP under the control of different ubiquitous promoters. Three days after transduction, undifferentiated cells were submitted to blasticidin selection (7.5 μg/ml) for 6 days. Fluorescence images of nontransduced **(A)** and stably transduced undifferentiated CGR8 cells expressing eGFP under the control of CMV **(B)**, EF1-αS **(C)**, and EF1-α **(D)** promoters. Exposure times were 2000 ms for **(A–C)** and 300 ms for **(D)**. **(E):** flow cytometry analysis of eGFP expression under the control of CMV (blue), EF1-αS (red), and EF1-α (green) promoter; untransduced cells are shown in black. **(F, G):** Bar graph showing, respectively, percentage of eGFP-positive cells and mean fluorescence intensity of EF1-αS/eGFP-transduced CGR8 cells, after accomplished blasticidin selection (day 0) and after 30 days in culture without blasticidin (day 30). Scale bar: 50 μm.

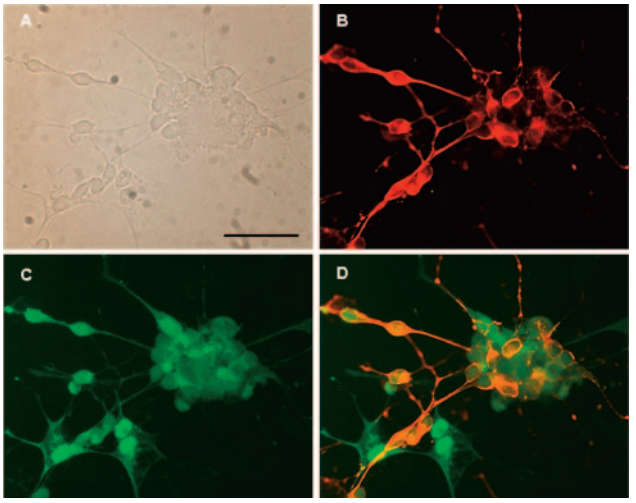

Figure 3. eGFP expression is maintained during neuronal differentiation of murine embryonic stem (ES) cells. EF1-α/eGFP-transduced CGR8 cells were differentiated towards neurons by coculture on the mouse bone marrow MS5 cell line for 5 days, followed by replating and culture on polyornithin-coated dishes for 4 days. Cells were then immunostained with anti-β3-tubulin antibodies. **(A):** Phase contrast; **(B):** β3-tubulin staining (red); **(C):** eGFP fluorescence (green); **(D):** merge. Note that both, β3-tubulin-positive and β3-tubulin-negative cells express eGFP. Scale bar: 50 μm.

mouse D3 cells and the human H1 cells. D3 cells were transduced with the EF1-α/eGFP vector in the absence of feeder cells and subsequently cultured and subjected to blasticidin selection on a STO feeder cell line (Figs. 4A, 4B). The STO cells had been, in a first step, transduced with a lentivector expressing a red fluorescent protein, RedFP (mRFP1, [23]) and a blasticidin resistance. Thus, feeder cells were red, and transduced D3 ES cells were green. We then evaluated the percentage of eGFP-positive D3 cells by counting eGFP-positive cells (see Materials and Methods). Over 99% of D3 cells were eGFP-positive (data not shown).

We next investigated the use of the 2K7 vector for the transduction of human ES cells based on protocols similar as described above. Blasticidin-resistant STO feeder cells and H1 cells were cocultured and incubated for 24 hours with the EF1-αS/eGFP lentivector. Days 3–24 after transduction, cells were subjected to blasticidin selection. This selection did not lead to a depletion of feeder cells. Within the H1 colonies, areas of necrosis appeared between 2 and 5 days after initiation of antibiotic selection, followed by an enrichment in eGFP-positive H1 cells. H1 ES cells were subsequently passaged on freshly irradiated blasticidin-resistant STO feeder cells (transduced with empty $2K7_{bsd}$ vector, that is no expression of a fluorescent protein). Colonies of H1 ES cells containing almost exclusively eGFP-positive cells could be observed after 2 weeks of selection (Figs. 4C, 4D). Flow cytometry analysis was performed after 3 weeks of selection. Cells were immunostained with the anti-human TRA1–85 antibody [31] to distinguish human ES cells from the feeder cells, and over 95% of TRA1–85-positive cells were also eGFP-positive (Fig. 4F). In addition, we developed a protocol that allows selection on primary MEFs, which are more commonly used as feeder cells for human ES cells. As MEF and human ES cells are cotransduced, they also become eGFP-positive and blasticidin-resistant. As opposed to the results shown in Figures 4C and 4D, cells were not passaged, and feeder cells therefore remained fluorescent (Figs. 4G, 4H).

Thus, feeder cell-dependent human and mouse ES cells can be efficiently transduced and blasticidin-selected to obtain transgene-expressing cells almost exclusively.

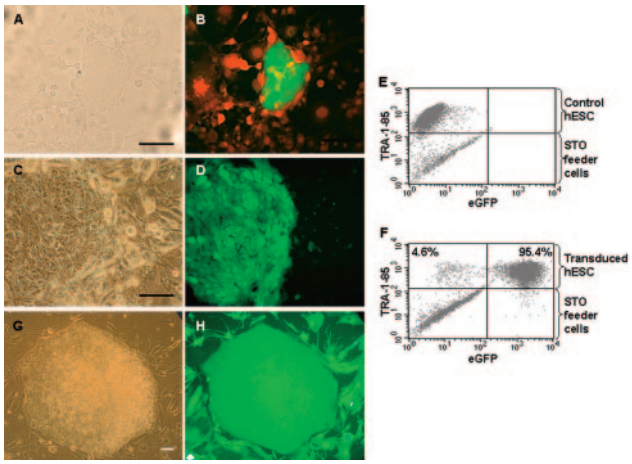

Figure 4. Transduction of feeder cell-dependent mouse and human embryonic stem (ES) cells by the 2K7 vector. Undifferentiated mouse D3 and human H1 ES cells were transduced with a $2K7_{bsd}$ lentiviral construct to express eGFP under the control of the EF1-α promoter. D3 ES cells were transduced and subsequently submitted to blasticidin selection for 6 days on STO feeder cells expressing a red fluorescent protein (RedFP) and blasticidin resistance. H1 human ES cells were transduced with a $2K7_{bsd}$ vector containing eGFP under the control of the EF1-αS promoter and were cultured and submitted to blasticidin selection on a blasticidin-resistant STO feeder layer for 3 weeks. Alternatively, H1 human ES cells were transduced on an MEF feeder layer and subsequently submitted to blasticidin selection. **(A, B):** Phase contrast and fluorescence imaging of transduced D3 ES cells (green) on STO feeder layer (red). **(C, D):** Phase contrast and fluorescence imaging of transduced H1 ES cells (green) on STO feeder cells. **(E):** Flow cytometry analysis with the pan-human TRA-1–85 antibody allows discrimination between human H1 ES cells (upper left quadrant) and STO feeder cells (lower left quadrant). **(F):** After 3 weeks of antibiotic selection, >95% of H1 human ES cells are eGFP-positive (upper right quadrant). **(G, H):** Phase contrast and fluorescence imaging of transduced H1 ES cells (green colony) on MEF feeder cells (also green). Scale bars: 100 μm.

Neuron-Specific Transgene Expression During ES Cell Differentiation

We next investigated whether the 2K7 vector was able to drive tissue-specific transgene expression. For this purpose, we generated stable CGR8 cell lines with lentivectors containing eGFP under the control of neuron-specific promoters: 1) the Tα1 α-tubulin promoter, active during early neuronal differentiation and after neuronal injury [21], and 2) the Synapsin1 promoter, active in more mature neurons and marking the establishment of synapses [22]. Both promoters have been successfully used in viral vectors to drive tissue-specific transgene expression [32, 33]. ES cell differentiation was induced by coculture on MS5 cells (Figs. 5A–5C, 5E, 5F) to obtain a high yield of neurons [25] or by formation of embryoid bodies to obtain a mixed cell population, including neurons (Fig. 5D). The activity of the neuron-specific promoters was monitored by the appearance of green fluorescence, and cells were characterized by immunolabeling using anti-β3-tubulin antibodies or antisynapsin antibodies (neurons) and antinestin antibodies (neuronal precursors).

Under the control of the Tα1 α-tubulin promoter, eGFP expression appeared at day 3 of differentiation on MS5 cells. In most β3-tubulin-positive neurons, the activity of the Tα1 α-tubulin promoter (as evidenced by green fluorescence) is present (Fig. 5A). An overlay of the same field with 4′,6-diamidino-2-phenylindole (DAPI) staining to visualize all cells (Fig. 5B), shows that most β3-tubulin-negative cells are also eGFP-negative (i.e. the Tα1 α-tubulin promoter is not active). However, nestin-positive neuronal precursors (which are mostly β3-tubulin-negative, data not shown) occasionally also displayed activity of the Tα1 α-tubulin promoter (Fig. 5C). This corroborates data from the literature suggesting that the Tα1 α-tubulin promoter activity precedes the one of the β3-tubulin promoter and partially overlaps with the activity of the nestin promoter [20]. We also differentiated the Tα1 α-tubulin/eGFP-transduced cells toward embryoid bodies to study whether Tα1 α-tubulin promoter activity remains neuron-specific even in a heterogeneous cell population; in this model, eGFP expression also correlated with β3-tubulin staining (Fig. 5D).

Under the control of the Synapsin1 promoter, eGFP fluorescence first appeared at day 5 of differentiation on MS5 cells. It was present in a subset of β3-tubulin-positive neurons (Fig. 5E) and correlated with synapsin immunoreactivity (Fig. 5F).

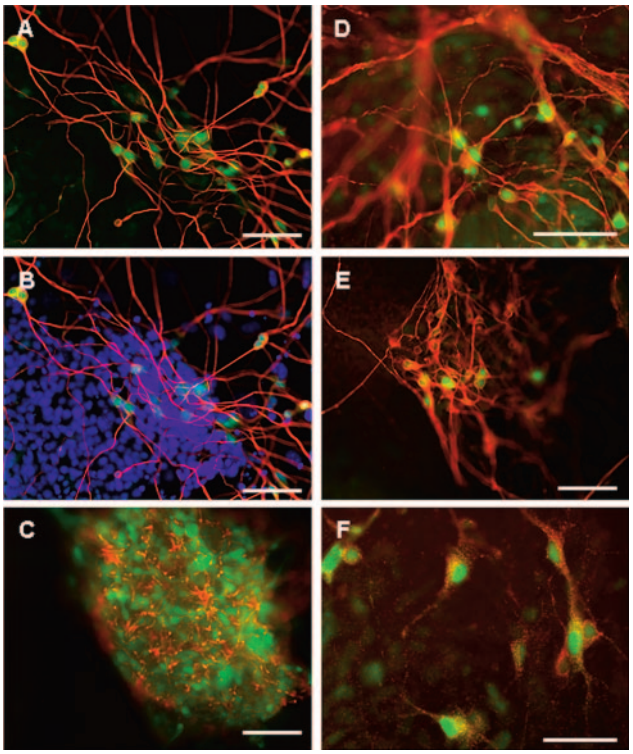

Figure 5. eGFP expression driven by neuron-specific promoters. Murine CGR8 embryonic stem (ES) cells transduced with various promoter/eGFP constructs were differentiated either towards neurons on MS5 cells for 5 **(A–D)** or 7 days **(E)**, or towards embryoid bodies for 12 days after plating **(F)**. Differentiated cells were analyzed for eGFP expression by direct fluorescence (green) and for different neuronal markers by immunofluorescence (red). **(A):** Tα1 α-tubulin/eGFP-transduced cells immunostained with a β3-tubulin antibody (red). **(B):** Same picture as **(A)**, but overlaid with 4′,6-diamidino-2-phenylindole staining (blue) to demonstrate the presence of non-neuronal, eGFP-negative cells. **(C):** Tα1 α-tubulin/eGFP-transduced cells immunostained with a nestin antibody (red); **(D):** embryoid body, derived from Tα1 α-tubulin /eGFP-transduced cells, immunostained with a β3-tubulin antibody. **(E):** Synapsin1/eGFP-transduced cells immunostained with a β3-tubulin antibody; **(F):** Synapsin1/eGFP-transduced cells immunostained with a synapsin antibody (red). Scale bars: **(A–E):** 100 μm; **(F):** 50 μm.

We next wanted to monitor the emergence of neurons within embryoid bodies (EBs). There was a marked autofluorescence of the cell culture medium and of cell debris in the green emission range; thus eGFP fluorescence gave only poor results with live imaging of embryoid bodies (data not shown). We therefore transduced CGR8 cells with a Tα1 α-tubulin/RedFP vector and monitored the in vivo appearance of red fluorescence within the embryoid body. Six days after plating, spots of red fluorescence appeared at the margin of the EB and progressively enlarged over time. The RedFP-positive regions of the EB were characterized by bundles of parallel oriented cells. The red fluorescence remaining localized to these bundles over time in culture. Figure 6 shows phase contrast (6A–6E) and fluorescence (6F–6J) images of the same region of an EB between days 10 and 14 after plating. Thus, the Tα1 α-tubulin/RedFP construct allowed monitoring of the emergence of neurons within the EB and defining of morphologically distinct regions as sites of neurogenesis.

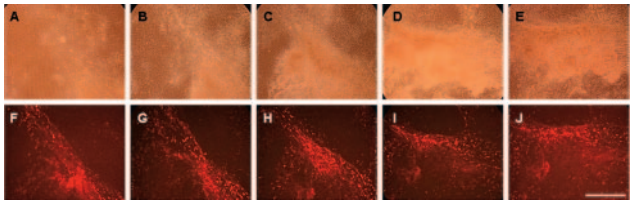

Figure 6. Live monitoring of Tα1 α-tubulin promoter driven expression of red fluorescent protein in embryoid bodies. A CGR8 embryonic stem (ES) cell line expressing a red fluorescent protein (RedFP) driven by the Tα1 α-tubulin promoter was used to generate embryoid bodies (EBs). Several days after plating of the EBs, regions at the margins of the EB show bundles of cells positive for red fluorescent protein. Pictures of the same region of an EB were taken every 24 hours. **(A–E):** Phase contrast imaging. **(F–J):** RedFP (red). Time points (after plating of the EBs): **(A, F):** day 10; **(B, G):** day 11; **(C, H):** day 12; **(D, I):** day 13; and **(E, J):** day 14. Scale bar: 500 μm.

Two-Color Monitoring of Neuronal Differentiation Steps

We next investigated the temporal relationship of Tα1 α-tubulin and Synapsin1 promoter activation during neuronal differentiation of ES cells. For this purpose, we used 2K7 vectors with two different antibiotic resistances, namely $2K7_{bsd}$ and $2K7_{neo}$, which allowed us to engineer ES cells homogeneously expressing two constructs through double antibiotic selection. We generated a CGR8 cell line with RedFP expression controlled by the Tα1 α-tubulin promoter and eGFP expression controlled by the Synapsin1 promoter. After 4 days of coculture with MS5 cells, cell clusters expressing RedFP were visible, whereas almost no eGFP expression was observed (Figs. 7A–7C). After 7 days, abundant clusters of cells expressing both RedFP and eGFP were found (Figs. 7D–7F). To investigate the relative activities of the Tα1 α-tubulin and the Synapsin1 promoters, we quantified RedFP and eGFP fluorescence at days 4 and 7 of coculture on the MS5 feeder layer using the Metamorph software (Fig. 7G). This analysis yielded interesting results as follows: 1) the differentiation state of cells, as judged by the relative expression of RedFP over eGFP expression, was astonishingly homogeneous in a given cluster (a linear regression of data shown in Fig. 7G yielded r values of .88 and .95 for days 4 and 7, respectively), and 2) during neuronal differentiation, ES cells first activate the Tα1 α-tubulin promoter, followed by activation of the Synapsin1 promoter. These experiments demonstrate that two-color monitoring with different promoter/reporter constructs is a powerful tool to follow neuronal differentiation of ES cells.

DISCUSSION

In this report we describe a novel system to rapidly generate transgenic ES cell lines using lentivectors based on recombinational cloning technology. This technology allows the insertion of both promoters and genes of interest in a single recombination reaction. After antibiotic selection, mouse and human ES cell lines expressing transgenes of interest with a high degree of purity and stability are obtained.

Recombinational Cloning into Lentivectors

Lentivectors are large plasmids and offer only limited flexibility for cloning with restriction enzymes. Alternatives to restriction

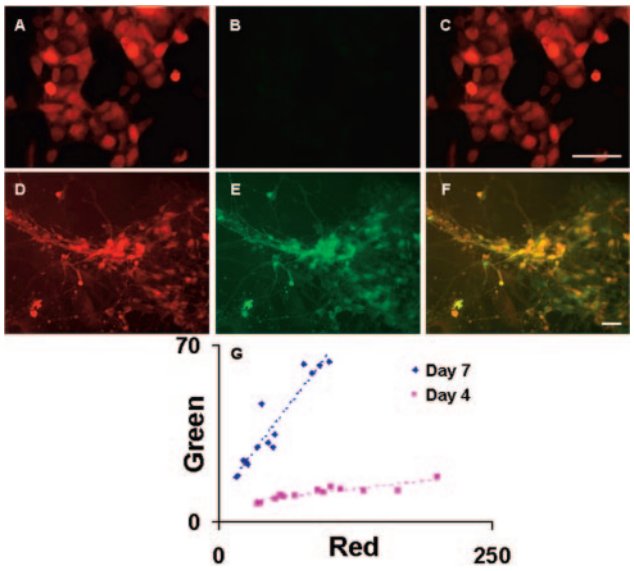

Figure 7. Double transduction of murine embryonic stem (ES) cells to study the activity of two neuronal promoters. CGR8 cells were transduced with two lentiviral constructs to drive the expression of a red fluorescent protein (RedFP) by the Tα1 α-tubulin promoter (neomycin resistance) and eGFP by the Synapsin1 promoter (blasticidin resistance). After double antibiotic selection, cells were cocultured on a MS5 feeder layer for four **(A–C)** or seven **(D–F)** days. **(A)** and **(D):** Red fluorescent protein (red); **(B, E):** eGFP (green); **(C, F):** merge. After 4 days of differentiation, red fluorescence is already present, whereas almost no eGFP is detectable **(A–C)**. After 7 days of differentiation, most cells present red and green fluorescence at different intensities **(D–F)**. **(G):** Analysis of red (*x*-axis) and green (*y*-axis) fluorescence intensities of 13 randomly chosen cells from **(C, F)**. The *x*- and *y*-axis values are arbitrary fluorescence units. The position of the cells on the graph indicates their relative state of maturation. Dotted lines are linear regressions. Scale bars: 50 μm.

cloning are therefore particularly interesting for lentivectors. Recombinational cloning uses enzymes recognizing sequences that are virtually absent from the mammalian genome and from most vectors. In a single-step recombination process, a DNA sequence flanked by recombination sites is replaced by a sequence of interest also flanked by recombination sites [34]. In the context of lentivectors, this has very relevant advantages over restriction cloning as cloning efficiency is invariably high (in the range of 80%–100%), and there are virtually no cloning site incompatibilities.

Another advantage is the possibility to reliably insert two sequences of interest in a defined order into the target vector in a single recombination reaction. For this purpose, we decided to use the Gateway cloning system, which allows multisite recombinational cloning, such as directional cloning of both a promoter and a gene of interest into the target vector [34] with virtually no limitation in their combination. Thus, recombinational cloning technology provides a very high flexibility that cannot be reached with restriction cloning.

Other groups have been using recombinational cloning directly in commercially available lentivectors (Gateway Technology; Invitrogen, [35–39]). However, the vectors used in these studies lack elements that have been shown to be crucial for optimal transgene expression, in particular WPRE and cPPT [28, 29, 40]. We thus incorporated WPRE and cPPT elements in the backbone of the 2K7 vector; this resulted in greatly enhanced transgene expression up to levels comparable to the most advanced lentivectors (data not shown).

Selection of Transduced ES Cells

Upon lentiviral transduction, approximately 20%–80% of ES cells are transduced [1]. Thus, methods of selection are necessary to obtain a pure population of transduced cells. One potential approach is to use bicistronic lentivectors, containing both a gene of interest and a selection marker under the control of the same promoter. However, their use is limited, because protein expression remains poorly predictable [18], and tissue-specific promoters cannot be used, because the selection marker will not be expressed in undifferentiated cells.

In the 2K7 lentivector, the antibiotic resistance is under the control of a ubiquitous promoter, which ensures its expression independently from the promoter used to drive the expression of the gene of interest. In our hands, this approach is extremely useful for work with ES cells, because: 1) more than 99% of transduced murine ES cells and more than 95% of transduced human ES cells expressed the transgene after antibiotic selection, and 2) even if transgene expression was driven by a highly tissue-specific promoter (e.g. Synapsin1), antibiotic selection could be performed in undifferentiated cells.

The ES cell population obtained after antibiotic selection of 2K7-transduced cells is polyclonal. We consider this as an advantage, because artifacts due to clonal selection are avoided. Indeed, both the sites of 2K7 insertion and the number of copies per cell are expected to differ from one cell to another [10]. We have not attempted to measure the average number of vector copies per cell; however, based on our experimental conditions, several copies per cell are likely [10]. Given the polyclonal nature of our transduced lines, possible gene disruptions by transgene insertion should not have an impact on the behavior of the cell population. And indeed, we have not observed altered cell growth or differentiation in transduced lines.

2K7 Lentivector as a Tool for Controlled and Targeted Gene Expression

Using our system, we compared the efficiency of three ubiquitous promoters in undifferentiated CGR8 murine ES cells. Activity levels were low for the CMV promoter, intermediate for the EF1-αS promoter, and high for the EF1-α promoter. Because these promoters can be easily combined with a gene of interest, it is possible to rapidly generate different cell lines expressing a transgene at different levels.

Lentivectors have shown efficient tissue-specific transgene expression upon injection in animals [32, 33]. Here we demonstrate that, using the 2K7 lentivector with tissue-specific promoters, transgene expression specific for cell type and cellular state of differentiation can be obtained in ES cells. The specificity of the promoters was maintained in this system as demonstrated by: 1) the good correlation between immunostaining and reporter gene expression (Fig. 6F) and 2) the corroboration between our observations in ES cells and published data obtained in transgenic animals (see for example the temporal relationship of nestin and β3-tubulin expression with the activity of the Tα1 α-tubulin promoter in Figs. 6B, 6C, and in Ref. 20). This is of particular interest in the field of ES cell research,

because it allows the targeted expression of a transgene of interest in a given cell type or at a given differentiation stage.

Two-Color Live Monitoring of ES Cell Differentiation

Single-color life monitoring of ES cell differentiation has been performed previously. It was considered a powerful tool for studying the promoter activation during neuronal differentiation and also an efficient tool for monitoring strategies to direct ES cell differentiation [3]. The availability of the $2K7_{neo}$ and the $2K7_{bsd}$ vectors allowed us to go further. Double transduction followed by double antibiotic selection permitted two-color monitoring through two different promoter/reporter constructs. In our studies, two different fluorescent proteins allowed us to monitor simultaneously the activity of two different promoters during neuronal differentiation and therefore to track neurogenesis in living cells.

CONCLUSION

In summary, we describe the novel generation of 2K7 lentivectors, which is particularly well suited for work with ES cells. Key features include the possibility to select cell lines through different antibiotic resistances and the rapid insertion of any combination of promoters and genes of interest through recombinational cloning.

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DISCLOSURES

The authors indicate no potential conflicts of interest.

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