

# Transduction of human embryonic stem cells by ecotropic retroviral vectors

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## ABSTRACT

**The steadily increasing availability of human embryonic stem (hES) cell lines has created strong interest in applying available tools for gene transfer in murine cells to human systems. Here we present a method for the transduction of hES cells with ecotropic retroviral vectors. hES cells were transiently transfected with a construct carrying the murine retrovirus receptor mCAT1. Subsequently, the cells were exposed to replication-deficient Moloney murine leukemia virus (MoMuLV) derivatives or pseudotyped lentiviral vectors. With oncoretroviral vectors, this procedure yields overall transduction efficiencies of up to 20% and permits selection of permanently transduced clones with high frequency. Selected clones maintained expression of pluripotency-associated markers and exhibited multi-germ layer differentiation both *in vitro* and *in vivo*. hES cell-derived somatic cells including neural progeny maintained high levels of transgene expression. Lentiviral vectors pseudotyped with the MoMuLV envelope could be introduced in the same manner with efficiencies of up to 33%. Transgene expression of lentivirally transduced hES cells remained permanent after differentiation even without selection pressure. Bypassing the regulatory issues associated with the use of amphotropic retroviral systems and exploiting the large pool of existing murine vectors, this method provides a safe and versatile tool for gene transfer and lineage analysis in hES cells and their progeny.**

## INTRODUCTION

Human embryonic stem (hES) cells are derived from the inner cell mass of the blastocyst (1,2). These cells have the potential to proliferate indefinitely in culture and to differentiate into all somatic cell types. These unique properties open

attractive perspectives for regenerative medicine and basic research. The biomedical exploitation of hES cells will largely depend on the availability of technologies for permanent and highly controlled genetic modification.

Retroviral transduction represents a fast and efficient method for transgene expression. Over the years, a large repertoire of well characterized murine retroviral vectors have been successfully applied in animal models, covering a broad range of applications from cell lineage analysis to gene therapy (3–5). The rapid development of the hES cell field has generated a need to apply this technology to human cells. To be broadly applicable in routine laboratory work, such a strategy should ideally permit the direct application of existing retroviral vectors to human cells without additional cloning steps or the stringent safety precautions typically associated with the use of amphotropic viruses (6–10) (NIH Safety Guidelines; available from the ‘NIH Office of Biotechnology Activities’ <http://www4.od.nih.gov/oba/>).

Retroviral vectors derived from the Moloney murine leukemia virus (MoMuLV) have been used widely in gene therapy with multiple already established vectors tested in humans and mice (4,11–17). Due to their permanent and exclusive integration into dividing cells, murine oncoretroviral vectors have become a work horse tool for the lineage analysis of stem and progenitor cells both *in vitro* and *in vivo* (18–20). The ecotropic nature of these vectors (host specificity for mice and rats) has, so far, restricted their application in human cells. Attempts to use amphotropic (host specificity including humans) oncoretroviral vectors for gene transfer into hES cells or human hematopoietic stem cells were further hampered by low transduction efficiency, which may in part be due to the restricted expression of the corresponding retroviral receptor in stem cells (21). To enable transduction with ecotropic murine vectors, we devised a two-step protocol. In a first step the murine retrovirus receptor mCAT1 (22) is transiently expressed in hES cells using the nucleofection technology. In a second step, ecotropic MoMuLV-based vectors are used to transduce the nucleofected hES cells. Our data show that this paradigm permits the permanent transduction and clonal selection of hES cells without affecting their proliferative potential and their pluripotent properties *in vitro* and *in vivo*. Neural progeny derived from the transduced hES cells

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shows sustained transgene expression during proliferation and upon differentiation. Overall transduction efficiency and stability of transgene expression can be further enhanced by the use of ecotropic pseudotyped lentiviral vectors without necessitating further safety precautions typically associated with lentiviruses (6–8,23) (NIH Safety Guidelines; available from the 'NIH Office of Biotechnology Activities' <http://www4.od.nih.gov/oba/>).

## MATERIALS AND METHODS

### Cell culture

hES cells [line H9.2 (24)] were maintained on irradiated mouse embryonic fibroblasts (MEFs) at 5% CO<sub>2</sub> in medium containing Knockout-DMEM (KO-DMEM, Gibco), 20% serum replacement, 1% non-essential amino acids, 1 mM L-glutamine, 0.1 mM  $\beta$ -mercaptoethanol and 4 ng/ml FGF2 (all Invitrogen, Karlsruhe, Germany). The cells were passaged in a 4 day cycle by incubating with 1 mg/ml collagenase IV (Invitrogen, Karlsruhe, Germany) at 37°C for 45 min, maintaining the cells in small clumps. To avoid contamination by feeder cells in the transfection and transduction analysis, the hES cells were cultured on matrigel (R&D Systems, Wiesbaden, Germany) in MEF-conditioned medium for one passage. Colonies were harvested by incubating with collagenase and plated onto matrigel-coated 6-well dishes.

### Construction of the plasmid pCAG-mCAT1-HA

The pCAG-mCAT1-HA plasmid (7.4 kb) was constructed by inserting a BamHI–NotI fragment from the pDNA3-mCAT1-HA vector (22) containing the coding sequence of mCAT1-HA into corresponding cloning sites of the vector pTriEx-1 (gift from Frank Edenhofer). The resulting pCAG-mCAT1-HA plasmid contained the cDNA of the murine retrovirus receptor mCAT1 including a linked haemagglutinin (HA)-tag under transcriptional control of the chicken beta-actin promoter with the CMV early immediate enhancer (CMV-IE enhancer).

### Vector virus production and concentration

Retroviral vectors were obtained from a GP-E-86 producer cell line (25) stably transfected with the HW3-EGFP-IRES-neo plasmid (13). The retroviral vector producing cells were cultured in DMEM (Invitrogen, Karlsruhe, Germany) supplemented with 10% FCS until they reached 80% confluency. Unconcentrated retroviral vectors were produced by changing the medium to feeder-conditioned hES-medium 24 h before infection. Supernatant was harvested, filtered through a 0.45  $\mu$ m mesh and directly used for transduction of mCAT1-expressing hES cells or NIH3T3 mouse fibroblasts. Prior to infection, 8  $\mu$ g/ml polybrene (Sigma, Deisenhofen, Germany) was added to the virus supernatant. To establish concentrated retroviral vectors, virus-producing GP + E-86 cells were cultured in DMEM + 10% FCS for 24 h. Vector virus containing supernatant was harvested and centrifuged at 7000 r.p.m. (6 g) in a Sorvall S-34 rotor for 12–16 h at 4°C. The supernatant was discarded and the pellet was re-suspended in 1/15 of the original volume of feeder-conditioned hES-medium. After filtration through a 0.45  $\mu$ m mesh, 8  $\mu$ g/ml polybrene (Sigma, Deisenhofen,

Germany) were added and the virus was directly used to transduce mCAT1-expressing hES cells or NIH3T3 cells.

### Transient transfection of hES cells with a mCAT1 expression construct

Cells were transfected with a construct carrying a HA-tagged murine retroviral receptor mCAT1 (22) under control of the CMV promoter or the CMV enhanced chicken beta-actin promoter (CAG). For transfection, hES cells were cultured on matrigel (R&D Systems, Wiesbaden, Germany) in feeder-conditioned medium for one passage. Transfection was performed using three different protocols: Lipofection was performed with Fugene (Roche Diagnostics, Mannheim, Germany) according to the manufacturers' instructions. Electroporation was performed as described elsewhere (26). To prepare cells for nucleofection, cells were digested with accutase II (PAA Lab., Cölbe, Germany) for 10 min to obtain small clumps of ~5–10 cells.  $2\text{--}3 \times 10^6$  cells were nucleofected with 2.5  $\mu$ g DNA, using Amaxa's solution 8352 and nucleofection program B16. A range of 750 000–1 000 000 nucleofected cells were plated on a matrigel-coated 3.5 cm dish in feeder-conditioned media. Transfection efficiencies were measured by anti-HA immunofluorescence 24 h post transfection. Mean percentages were calculated from at least three independent experiments.

### Transduction of hES cells with oncoretroviral vectors

Transduction of hES cells was performed in 3.5 cm dishes with 1 ml of vector virus containing supernatant or 0.75 ml of concentrated vector virus in the presence of 8  $\mu$ g/ml polybrene (Sigma, Deisenhofen, Germany) for 8 h. At the time of infection the cells grew in small colonies of 10–20 cells with an overall confluency of <30%. Transduction efficiencies were determined by enhanced green fluorescent protein (EGFP) immunofluorescence 48 h post infection. Mean percentages were calculated from five independent experiments.

### Transduction of hES cells with pseudotyped lentiviral vectors

Lentiviral vectors were produced by co-transfection of 293T cells with the lentiviral transfer vector pWPI (20  $\mu$ g), a lentiviral expression construct carrying the EGFP-gene under control of the EF1-promoter (gift from Didier Trono) or pLentiPGK-EGFP-SV40-blasticidine (20  $\mu$ g), a lentiviral expression construct based on the pLenti6/V5 expression system (Invitrogen) where the CMV promoter was replaced by a the phosphoglycerate kinase (PGK) promoter element (gift from Harald Neumann), the packaging plasmid pCMV-dR8.91 (15  $\mu$ g; gift from Didier Trono) and the ecotropic envelope plasmid pEcoEnv (5  $\mu$ g) using the calcium phosphate transfection method. Medium was changed 16 h after transfection and the vector virus containing supernatant was collected 36 h later. Vector virus was concentrated by low speed centrifugation as described above and re-suspended in 1 ml of MEF-conditioned media. MCAT1-transfected undifferentiated hES cells or NIH3T3 cells were exposed to vector viruses for 8 h as described above and transduction efficiencies were monitored 48 h after transduction by EGFP expression.

### Southern blot analysis

Genomic DNA was extracted from cells by lysis with SDS and proteinase K digestion followed by isopropanol precipitation. DNA was digested overnight using EcoRI and ClaI (both NEB) restriction enzymes and separated on an agarose gel. A unique ClaI site within the retroviral transgene and EcoRI sites within the host genome enabled the analysis of integration events. DNA was immobilized on a positively charged nylon membrane (Roche Diagnostics) and probed with a <sup>32</sup>P-labelled EGFP fragment. After exposure to X-ray film bands were quantified using the AIDA software (Raytest).

### In vitro differentiation

To study multi-germ layer differentiation *in vitro*, transduced cells were cultured as embryoid bodies (EBs) in non-adhesive plastic dishes in KO-DMEM (Gibco), 20% serum-replacement, 1% non-essential amino acids and 1 mM L-glutamine (all Invitrogen, Karlsruhe, Germany) for 4–8 weeks; 5% FCS was added in some experiments to promote differentiation. EBs were then plated onto polyornithine-coated glass cover slides and fixed 4 days later.

Neural differentiation was performed as previously described (27) with slight modifications. Briefly, 4-day-old EBs generated in the absence of FCS were transferred to polyornithine-coated tissue culture dishes and propagated in ITSFn medium (DMEM/F12 (Invitrogen, Karlsruhe, Germany), 25 µg/ml insulin, 100 µg/ml transferrin, 5 ng/ml sodium-selenite (Sigma, Deisenhofen, Germany), 2.5 µg/ml fibronectin (R&D Systems, Wiesbaden, Germany)) containing 20 ng/ml FGF2. Within 10 days, neural tube-like structures developed in the EB outgrowth. These structures were mechanically isolated and propagated as free-floating neurospheres in N2 medium containing 10 ng/ml FGF2 for 2–8 weeks. Spheres were passaged weekly by trituration into smaller fragments. Spheres or single cells derived thereof were plated on poly-L-lysine/laminin-coated tissue culture dishes and further propagated in Neurobasal medium (Invitrogen, Karlsruhe, Germany) supplemented with B27 (Invitrogen, Karlsruhe) and 10 ng/ml BDNF (R&D Systems, Wiesbaden, Germany) for a period of 3–12 weeks.

### Teratoma formation

Teratoma formation was induced by injecting into SCID-beige mice  $3 \times 10^6$  hES cells at passage 20 after transduction and selection as described (28). After 9 weeks, animals were sacrificed. Teratomas were fixed overnight in 4% paraformaldehyde (PFA) and subjected to histological examination using hematoxylin and eosin (H&E) staining.

### Bromodeoxyuridine incorporation and detection (BrdU-assay)

hES cells cultured for one passage on matrigel were transfected with the mCAT1-HA construct and subsequently subjected to viral transduction as described above. Twenty or forty hours after transduction the cells were labelled with 10 µM bromodeoxyuridine (BrdU, Sigma, Deisenhofen, Germany) for 5 h. The cells were fixed with 4% PFA, permeabilized in 70% ethanol for 10 min, transferred into 6 N HCL and 1% Triton X-100 (15 min, room temperature), followed by 0.1 M sodium borate (in PBS, 0.1% Triton X-100 for 30 min)

and subsequently incubated with a monoclonal antibody to BrdU (1:33, Becton-Dickinson, Heidelberg, Germany). Signal was visualized using a rhodamine-conjugated antibody to mouse IgG (1:100, Jackson ImmunoResearch Laboratories, West Grove, PA). For quantification, cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI, Sigma, Deisenhofen, Germany).

### Immunocytochemistry

Monolayer cultures and disaggregated EBs were fixed with 4% PFA in PBS for 20 min at room temperature. Cells were permeabilized with 0.25% Triton X-100 (Sigma, Deisenhofen, Germany) in PBS (PAA Lab., Cölbe, Germany) for 20 min. For detection of the pluripotency markers Tra-1-60 and Tra-1-81 no Triton X-100 was used. Blocking was performed with 10% FCS (Invitrogen, Karlsruhe, Germany) in PBS for 1 h. Samples were incubated with primary antibodies at 4°C over night, washed twice, incubated with secondary antibody for 45 min, counterstained with DAPI and mounted with Vectashild mounting solution (Vector Laboratories). Secondary antibodies were goat-anti-mouse IgG conjugated to Cy3 (1:500; Dianova) and goat-anti-rabbit IgG conjugated to FITC (1:200; Jackson Immunoresearch). Omission of the primary or secondary antibodies was used to control for unspecific staining. Four-week-old EBs were digested in trypsin/EDTA (Invitrogen, Karlsruhe, Germany) for 25 min, partially dissociated in smaller fragments, plated on polyornithine-coated chamber-slides (Nunc GmbH, Wiesbaden, Germany) and fixed after 24 h. Indirect immunofluorescence analysis was performed using antibodies to Tra-1-60 (1:500) and Tra-1-81 (1:500; both from Chemicon, Hofheim, Germany), alpha-fetoprotein (AFP; 1:200), cytokeratin/Lu-5 (1:1000), epithelial membrane antigen (EMA; 1:50) desmin (1:300) and smooth muscle actin (1:800; all DAKOCytomation, Hamburg, Germany).

Neurospheres were plated *in toto* or after trituration on poly-L-lysine and laminin-coated 3.5 cm dishes and differentiated in Neurobasal medium (Invitrogen, Karlsruhe, Germany) supplemented with B27 (Invitrogen, Karlsruhe, Germany) and 10 ng/ml BDNF (R&D Systems, Wiesbaden, Germany) for 1–6 weeks before fixing. Indirect immunofluorescence analysis was performed using antibodies to nestin (1:200; Chemicon, Hofheim, Germany), beta-III-tubulin (1:1500; BabCo, Covance, USA), MAP2ab (1:200; Chemicon, Hofheim, Germany) and GFAP (1:100; ICN Biomedicals, Irvine, USA). EGFP was detected using an anti-EGFP antibody (1:4000; Abcam, Cambridge, USA).

## RESULTS

### Transient transfection of hES cells with the murine retroviral receptor mCAT1

An expression construct encoding a HA-tagged variant of the murine retrovirus receptor mCAT1 under control of the CMV promoter (22) or the CMV-enhanced chicken beta-actin promoter (CAG) were used to optimize the transfection conditions for hES cells. Lipofection and conventional electroporation yielded only moderate transfection rates. Twenty-four hours after transfection, HA-immunofluorescence was detected in  $19.3 \pm 4.6\%$  of the lipofected and  $34.2 \pm 6.7\%$  of the

electroporated cells (3 independent experiments). To further improve transfection efficiency we explored nucleofection, a recently developed method for enhanced electroporation (Amaxa GmbH, Cologne, Germany). Under optimized conditions we achieved transient mCAT1-HA expression in  $52.1 \pm 8.3\%$  of the undifferentiated hES cells (10 independent experiments; Figure 1A and B). Survival rates with Amaxa's manufacture program B-16 averaged around 70% and were thus comparable to those observed in conventional electroporation [for further information on the nucleofection of hES cells, see Siemen *et al.* (2005) (29)].  $94.4 \pm 2.8\%$  of the mCAT1-HA expressing cells showed nuclear Oct-4 immunofluorescence. Compared to Oct-4 expression of non-transfected cells ( $97 \pm 1.3\%$ ), the nucleofection procedure appeared to have no significant effect on the pluripotent state of the cells.

### Transduction of mCAT1 expressing hES cells by ecotropic MoMuLV-vectors

In this approach, transduction of mCAT1-transfected hES cells was performed with an ecotropic MoMuLV-derived vector encoding the EGFP under control of the MoMuLV-5'LTR IRES-linked to a neomycin resistance gene (neoR; Figure 1C) (13,30). Viral particles were obtained from the supernatant of stably transfected GP+E-86 cells (25), yielding titers of up to  $4.3 \times 10^7$  transducing units per ml as determined by infection of NIH3T3 mouse fibroblasts. Starting 24 h after transfection with the mCAT1 construct, the hES cells were exposed to 1 ml of cell-free viral supernatant for 8 h. Forty-eight hours post transduction up to 5% of the total cell population showed EGFP-expression. Normalized to the proportion of mCAT1-expressing cells determined in previous experiments, this corresponds to a calculated transduction efficiency of  $\sim 10\%$  of the mCAT1-expressing cells. Since the hES cells were maintained on feeder cells (MEFs), we expect a large proportion of retroviral particles to be adsorbed by this murine cell population. Thus, feeder-free cultivation systems may yield even higher transduction efficiencies. No EGFP-positive cells were observed in untransfected control populations.  $93 \pm 4\%$  of EGFP-positive cells showed nuclear staining with an antibody to the Oct4-protein (compared to  $97 \pm 2.4\%$  of the non-infected cells), confirming that the infected cells largely maintain their pluripotent state.

### Concentration of viral supernatant using slow speed centrifugation

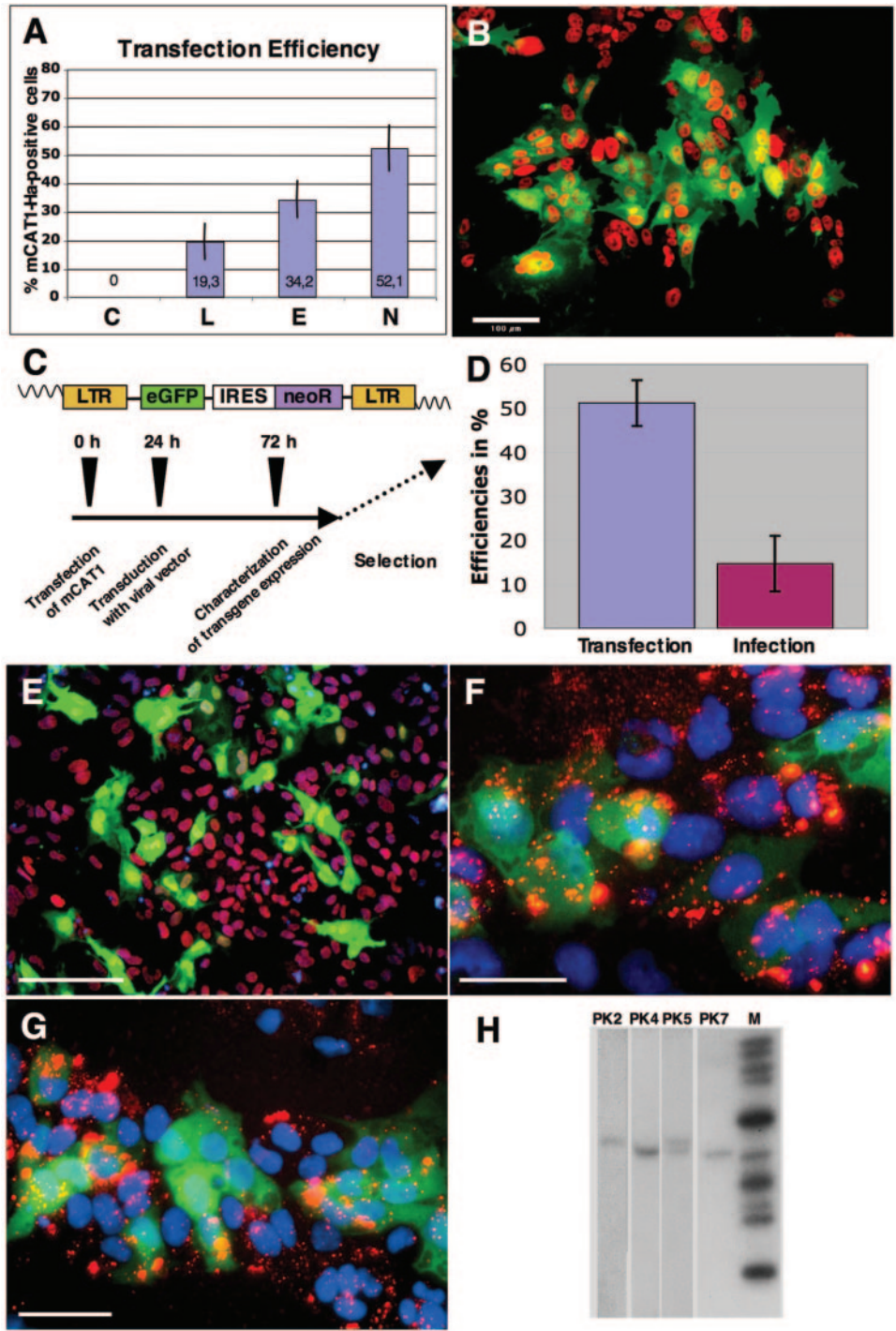
As transduction efficiencies of MoMuLV-derived vectors highly depend on viral titers we searched for efficient methods to concentrate virus-containing supernatant. Ultracentrifugation and subsequent re-suspension of the virus-containing pellet in 1/10 of the original volume yielded only an  $\sim 2$ -fold increase in viral titer, indicating that ultracentrifugation is not an adequate method to concentrate ecotropic MoMuLV-vectors. Therefore we chose a different protocol based on low speed centrifugation over a prolonged period of 12–16 h at 6 g (17,31). By re-suspending the viral pellet in 1/10 to 1/20 of the original volume, viral titers could be increased 10- to 18-fold resulting in viral titers (MOI) of up to  $8.4 \times 10^8$  infectious particles per ml (as determined on NIH3T3 mouse fibroblasts).

### Enhanced transduction of mCAT1 expressing hES cells by concentrated MoMuLV vectors

To explore whether transduction efficiencies could be increased by elevated viral titers, vector virus-containing supernatant was concentrated over night, re-suspended in 1/10 of original volume and filtered through a  $0.45 \mu\text{m}$  mesh. Concentrated viral vectors were directly used to transduce mCAT1-transfected hES cells cultured on matrigel in feeder conditioned media, and transduction efficiencies were measured 48 h post transduction using immunofluorescence. Using this method  $16.4 \pm 5.9\%$  of the total cell population showed EGFP-expression (Figure 1D). Normalized to the proportion of mCAT1-expressing cells determined in previous experiments, this corresponds to a calculated transduction efficiency of  $\sim 30\%$  of the mCAT1-expressing cells. No EGFP-positive cells were observed in untransfected control populations (data not shown). Furthermore, no significant increase in toxicity of the concentrated viral vector was observed as judged from total cell counts and the number of detached cells at 24–72 h after transduction. Forty-eight hours after transduction,  $89.6 \pm 6.8\%$  of the transduced cells and  $96.2 \pm 2\%$  of the non-transduced cells continued to express Oct-4 (Figure 1E). In addition, transduced and non-transduced cells maintained expression of the pluripotency markers Tra-1-60 and Tra-1-81 at comparable levels. Forty-eight hours after transduction, these markers were expressed in  $86 \pm 4.6\%/88 \pm 8.2\%$  of the EGFP-positive cells and  $88 \pm 4\%/91 \pm 6.2\%$  of the non-transduced cells, respectively (Figure 1F and G;  $n \geq 3$  independent experiments). These data indicate that the two-step transduction procedure has no significant influence on the pluripotent state of the cells. Performing two rounds of transduction with freshly concentrated virus could not increase transduction efficiencies in our hands. This could be due to rapid downregulation of the mCAT1 receptor over time. Indeed, 48 h after transfection only  $10.2 \pm 8.1\%$  of the cells still showed mCAT1-HA expression. Furthermore, we noticed a significant increase in cell death upon repetitive infection. Since the calculation of transduction efficiencies could, in principle, be biased by changes in cell proliferation upon transduction, we performed additional BrdU incorporation studies. Twenty or forty hours after transduction,  $31 \pm 6\%/27 \pm 3\%$  of the EGFP-expressing cells and  $34 \pm 5\%/26 \pm 6\%$  of the non-transduced cells showed BrdU immunoreactivity, respectively (data not shown), indicating that extrapolation of the transduction rate is not biased by changes in cell proliferation.

### Permanently transduced clones maintain transgene expression and pluripotency

We next asked whether this method is suitable for selecting permanently transduced clones from low titer infections. Unconcentrated viral vectors were used for these experiments to enable the identification of individual clones in a mass culture setting. 200 000 mCAT1-transfected cells were plated in a 6-well dish containing neomycin-resistant murine embryonic feeder cells (MEFs). Twenty-four hours later the cells were transduced with unconcentrated viral supernatant for 8 h. At this time about 100 000 of the plated 200 000 cells could be detected using an antibody to human nuclei. Forty-eight hours post infection G418 was added to the medium.



**Figure 1.** Transduction of hES cells with murine retroviral vectors. (A) Transfection efficiencies of undifferentiated hES cells using optimized protocols for lipofection (L), electroporation (E) and nucleofection (N) techniques. (B) mCAT1-HA expression (green) in nucleofected hES cells cultured on matrigel. Twenty-four hours after plating, the cells show a flattened morphology typical for colonies propagated on matrigel. Nuclear expression of Oct-4 (red) reflects their undifferentiated state. (C) Schematic illustration of the two-step protocol used for transduction of hES cells with ecotropic retroviral vectors. First cells are nucleofected with a construct encoding the murine retrovirus receptor mCAT1. Twenty-four hours later they are transduced with a murine retroviral vector. Transduced cultures are either analyzed for transgene expression after 48 h or subjected to selection of permanently transduced clones. The integrated provirus expresses the EGFP transgene from the viral LTR linked to a neomycin resistance gene (neoR) by an internal ribosome entry site (IRES). (D) Forty-eight hours after infection,  $16.4 \pm 5.9\%$  of the total cell population showed EGFP-expression. Normalized to the proportion of mCAT1-expressing cells determined in (A), this corresponds to a calculated transduction efficiency of  $\sim 30\%$  of the mCAT1-expressing cells (mean values from  $n = 5$  independent experiments). (E–G) Transduced EGFP-positive cells continue to express the pluripotency-associated markers Oct-4 (E), Tra-1-60 (F) and Tra-1-81 (G) (all red; counterstain DAPI). (H) Five passages after transduction, four clones were subjected to Southern analysis. A single integration of the EGFP transgene could be detected in clones PK2, PK4 and PK7. Clone PK5 displays two bands, which could be the result of a double integration, a mixed clone population or a mutation of the vector provirus. Restriction analysis was performed with EcoRI (for genomic DNA) and ClaI (unique site within the retroviral vector). Scale bars: B,E: 100  $\mu\text{m}$ ; F: 30  $\mu\text{m}$ ; G: 50  $\mu\text{m}$ .

After 8 days of selection, individual colonies could be identified, isolated and further expanded. The number of selectable clones per 6-well dish ranged between 8 and 20. Thus, the overall efficiency of retrieving permanently transduced clones with unconcentrated retroviral vectors was already 1–2/10 000 hES cells. Whereas differences in EGFP-expression were noted between different clones, transgene expression within individual clones was quite uniform. Visible EGFP autofluorescence was observed in ~25% of the clones. Using an anti-EGFP antibody all clones showed detectable EGFP-expression. Eight randomly picked clones were further propagated and studied across several passages. Without antibiotic selection all clones showed downregulation of EGFP after several weeks in culture. In contrast, homogeneous expression of the EGFP-transgene was maintained under G418-selection over more than 60 passages.

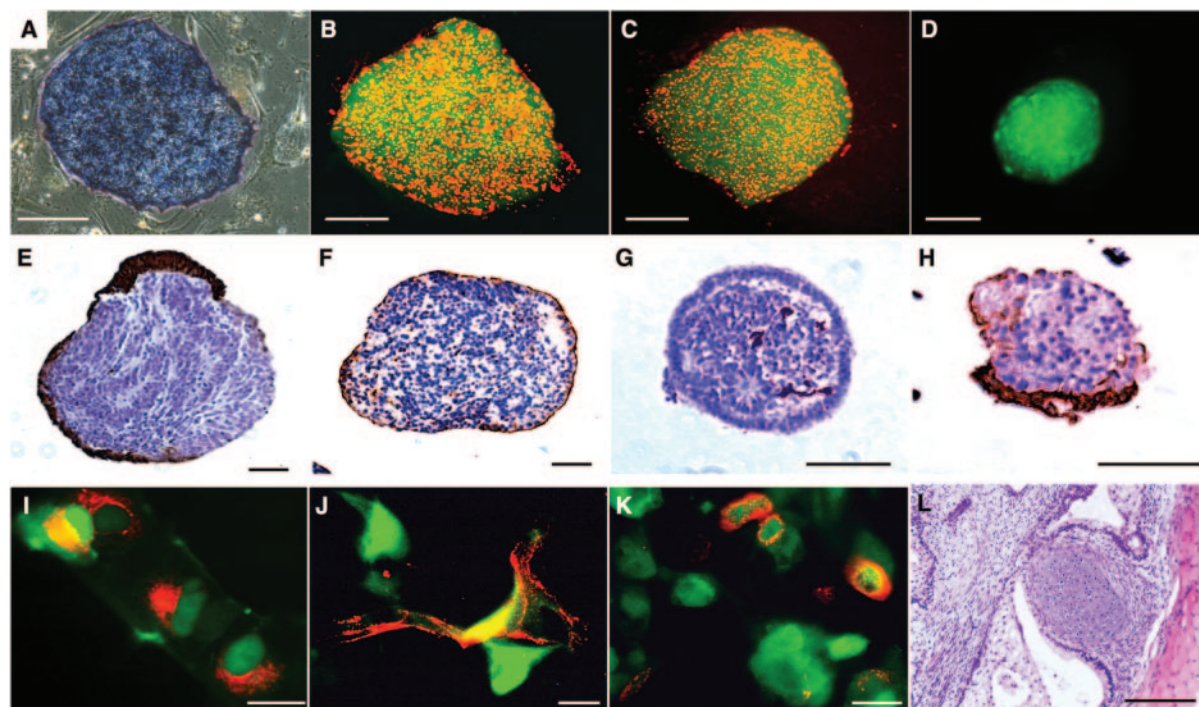
Transduced EGFP-positive clones continued to express markers of pluripotency, including alkaline phosphatase, Tra-1-60, Tra-1-81 (Figure 2A–C) and Oct-4. Upon withdrawal of G418, ~50% of the cells lost visible EGFP fluorescence within five passages. Performing immunostainings with an anti-EGFP antibody, EGFP-expression could still be detected in  $65.2 \pm 12.2\%$  of the cells after 4 weeks without selection pressure. These observations suggest that the transduced undifferentiated cells retain pluripotency and self-renewal capacity during prolonged cultivation, but are bound to lose retroviral transgene expression in the absence of selection pressure. To confirm that transgene expression resulted from stable integration of the provirus into the host

DNA, we conducted Southern blot analyses of genomic DNA prepared from four different clones five passages after transduction. In all four clones we found genomic integration of the EGFP-expressing provirus. Whereas clones PK2, PK4 and PK7 showed a single integration of the viral transgene, clone PK5 displayed two bands, which could be the result of a double integration, a mixed clone population or a mutation of the vector provirus (Figure 1H). These data indicate that the loss of EGFP expression is not due to a loss of the transgene but may be related to transcriptional changes such as silencing of the viral LTR.

### Transgene expression is retained throughout germ layer differentiation *in vitro*

We further investigated the potential of virus-transduced cells to differentiate into derivatives of all three germ layers. When cells were cultured in non-adhesive conditions, EB formation was observed within 24 h. In the absence of G418, a pronounced downregulation of EGFP expression was noticed during the first days of EB formation. When 6-day-old EBs were dissociated and plated, only  $32 \pm 8\%$  of the cells had retained EGFP immunofluorescence. In contrast, EBs propagated in the presence of G418 showed homogeneous and strong transgene expression over a period of at least 6 weeks (Figure 2D).

We then studied whether the transduced EBs retain the potential to differentiate into derivatives of all three germ layers. To that end, EBs were propagated for at least



**Figure 2.** Following retroviral transduction, hES cells maintain transgene expression and pluripotent differentiation into derivatives of all three germ layers. (A–C) Selected clones express the pluripotency-associated markers alkaline phosphatase (A), Tra-1-60 (B, red) and Tra-1-81 (C, red) while sustaining high levels of EGFP-expression (green). (D) Under selective conditions, EGFP-expression is maintained in differentiating EBs for at least 6 weeks. (E–H) Differentiating EBs show expression of cytokeratin (E), epithelial membrane antigen (F), desmin (G) and alpha-fetoprotein (H), reflecting multi-germ layer differentiation. (I–K) Transgene expression in single cells derived from 4-week-old EBs, 4 days after plating. EGFP-positive cells co-express alpha-fetoprotein (I), smooth muscle actin (J) and epithelial membrane antigen (K) (all red). (L) Selected clones give rise to teratomas containing various somatic tissues 9 weeks after injection into immunodeficient SCID-beige mice. Scale bars: A–C: 200  $\mu$ m; D–H: 50  $\mu$ m; I–K: 20  $\mu$ m; L: 200  $\mu$ m.

4 weeks under selective conditions and then plated on polyornithine-coated glass slides or embedded in paraffin for sectioning and immunocytochemistry. Cells of all three germ layers could be detected both in the outgrowth of plated EBs and in sectioned EBs. Cells of epithelial and endodermal differentiation expressing cytokeratin, EMA and AFP were preferentially located in the outer cell layers of the EBs; desmin-immunoreactive cells indicative of mesodermal differentiation were typically located in the center of the EBs (Figure 2E–H). Double labeling with an antibody to EGFP revealed strong transgene expression in cells positive for AFP, smooth muscle actin and EMA (Figure 2I–K). We further conducted a teratoma assay using a selected clone cultured for 30 passages in the presence of G418. Nine weeks after injection of  $3 \times 10^6$  cells, five of eight recipient mice developed teratomas with pronounced differentiation into multiple somatic tissues (Figure 2L).

### Transgene expression is retained throughout neural differentiation

To explore whether maintenance of transgene selection pressure is required after lineage commitment, EBs were plated onto polyornithine-coated cell culture dishes and propagated in neural selection medium containing DMEM/F12, insulin (25  $\mu\text{g}/\text{ml}$ ), transferrin (100  $\mu\text{g}/\text{ml}$ ) sodium selenite (5  $\text{ng}/\text{ml}$ ), fibronectin (2.5  $\mu\text{g}/\text{ml}$ ), FGF2 (20  $\text{ng}/\text{ml}$ ) and G418 for a period of 10 days. After 7 days, G418 was discontinued. On day 10, neural islands containing dense neural tube-like structures were mechanically isolated and further propagated on poly-HEMA-coated tissue culture plates in DMEM/F12 supplemented with N2 (N2 media) and 10  $\text{ng}/\text{ml}$  FGF2 as free floating neurospheres as described (27,32). Using a 100  $\mu\text{l}$  pipette tip, neurospheres were dissociated in weekly intervals for a total time period of up to 3 months. Throughout this time the neurospheres displayed strong EGFP expression without evidence of transgene down-regulation (Figure 3A).

When passage six neurospheres were dissociated and plated,  $89 \pm 7\%$  of the plated cells showed immunoreactivity for the EGFP transgene. For long-term differentiation, neurospheres were plated on poly-L-lysine/laminin-coated tissue culture plates and propagated in Neurobasal medium supplemented with B27 and BDNF (10  $\text{ng}/\text{ml}$ ) for a period of 3–8 weeks. A dense outgrowth of neural cells was detected within a week after plating (Figure 3B). After 3–4 weeks, the plated neurospheres had generated an intricate network of neurites. Immunofluorescence analysis revealed strong expression of the neuronal marker antigens beta-III-tubulin and MAP2ab within the neurite outgrowth (Figure 3C–E). In addition to neurons, the outgrowth contained a smaller fraction of GFAP immunoreactive astrocytes (Figure 3F). Overall,  $91 \pm 6\%$  of cells within the neurosphere outgrowth retained EGFP immunofluorescence.

### Transduction of mCAT1 expressing hES cells by pseudotyped lentiviral vectors

Considering the increasing use of lentiviral vectors, we were interested in whether our two-step system could also accommodate ecotropic pseudotyped lentiviral vectors. To address this question we used the murine-specific ecotropic

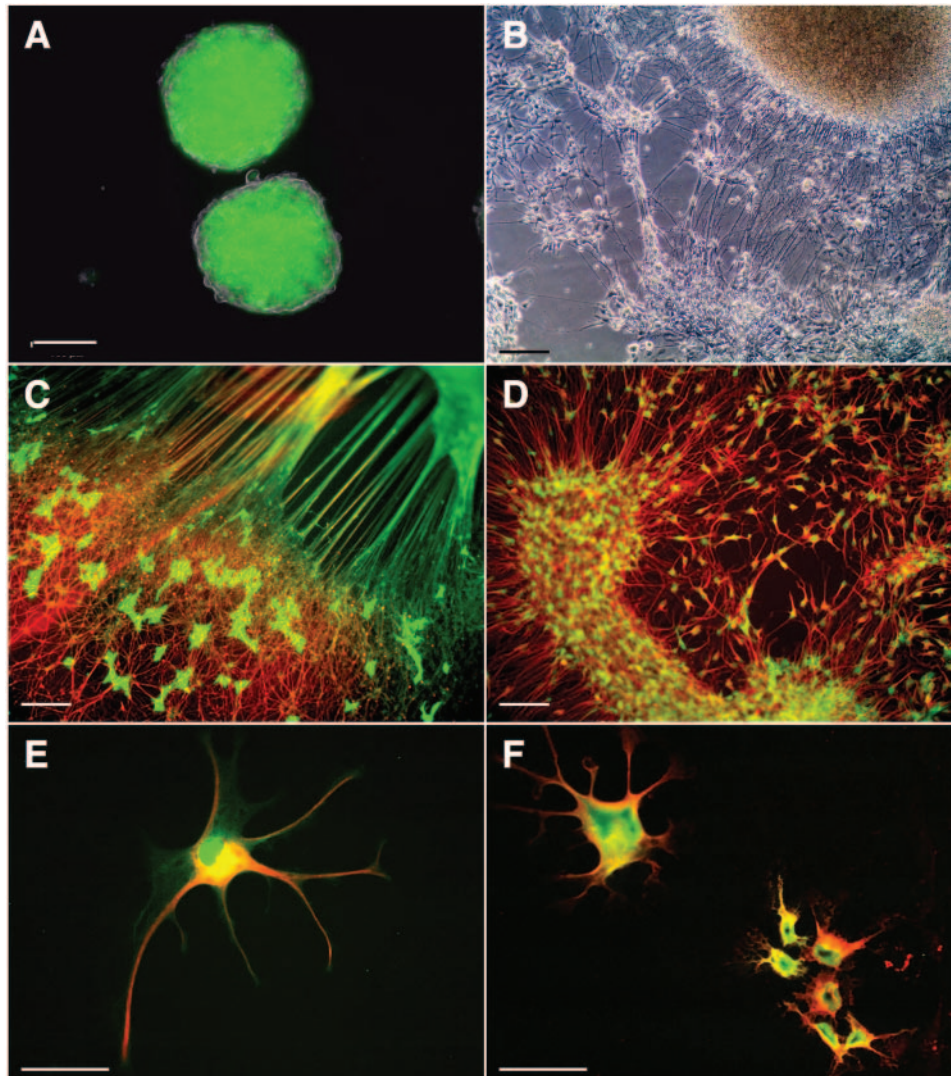
MoMuLV-envelope as lentivirus-packaging glycoprotein. Viral vectors were designed to express the EGFP-transgene either under the control of the PGK promoter with a blasticidine resistance gene under control of a SV40 promoter (pLentiPGK-EGFP-SV40-blasticidine) or under control of the EF1-promoter (pWPI). With pseudotyped vectors we obtained titers of up to  $1.8 \times 10^9$  transducing units per ml as determined by infection of NIH3T3 cells. Twenty-four hours after transfection with mCAT1, hES cells were exposed to 1 ml of cell-free viral supernatant for 8 h. Immunofluorescence analysis performed 48 h post transduction revealed that  $27.2 \pm 6.5\%$  of the pLentiPGK-EGFP-SV40-blasticidine-transduced cells and  $26.1 \pm 7.2\%$  of the pWPI-transduced cells were EGFP-positive (Figure 4A and B). Normalized to the proportion of mCAT1-transfected cells determined in previous experiments, this corresponds to a calculated transduction rate of  $\sim 50\%$  of the mCAT1-positive cells. No EGFP-positive cells were observed in non-transfected control populations.

### Transgene expression of selected lentivirus-transduced pluripotent cells remains permanent *in vitro* and *in vivo*

We next asked whether transgene expression mediated by lentiviral vectors remains permanent in selected hES cells, thus overcoming the limitation of continuous drug selection in the MoMuLV-transduced cells. To that end, pLentiPGK-EGFP-SV40-blasticidine-transduced cells were plated on matrigel 48 h after transduction and exposed to blasticidine (4  $\mu\text{g}/\text{ml}$ ) for 3 days. Following this selection step, the cells were continuously cultured on feeders (MEFs) for more than 20 passages in the absence of blasticidine. At passage 20 post selection, lentivirus-transduced cells continued to express markers of pluripotency (alkaline phosphatase, Oct-4, Tra1-60 and Tra1-81) along with the EGFP transgene (Figure 4C–F). To study stability of transgene expression upon induction of differentiation, lentivirus-transduced cells were aggregated to form EBs and cultured in the presence of 5% FCS for 8 weeks. Upon plating, these EBs generated an outgrowth of cells expressing EGFP as well as the differentiation-associated markers cytokeratin, AFP and desmin (Figure 4G–I). Maintenance of pluripotency of the lentivirally transduced cells was further confirmed by a teratoma assay. To that end,  $3 \times 10^6$  undifferentiated hES cells from passage 20 post selection were injected into SCID-beige mice. Nine weeks after injection, six out of eight mice had developed teratomas exhibiting prominent EGFP-expression (Figure 4J) and various different tissues including adenoid tissue, epithelium and cartilage (Figure 4K and L).

## DISCUSSION

Our results demonstrate that transient expression of the murine retrovirus-receptor mCAT1 in hES cells permits permanent transduction by ecotropic retroviral vectors. This method should provide significant practical advantages and enable applications, which go beyond those of conventional lenti- and adenoviral transduction. First, the large number of already established murine MoMuLV-derived vectors, commonly available as supernatant from stable virus producing cell lines, can be directly applied to hES cells without



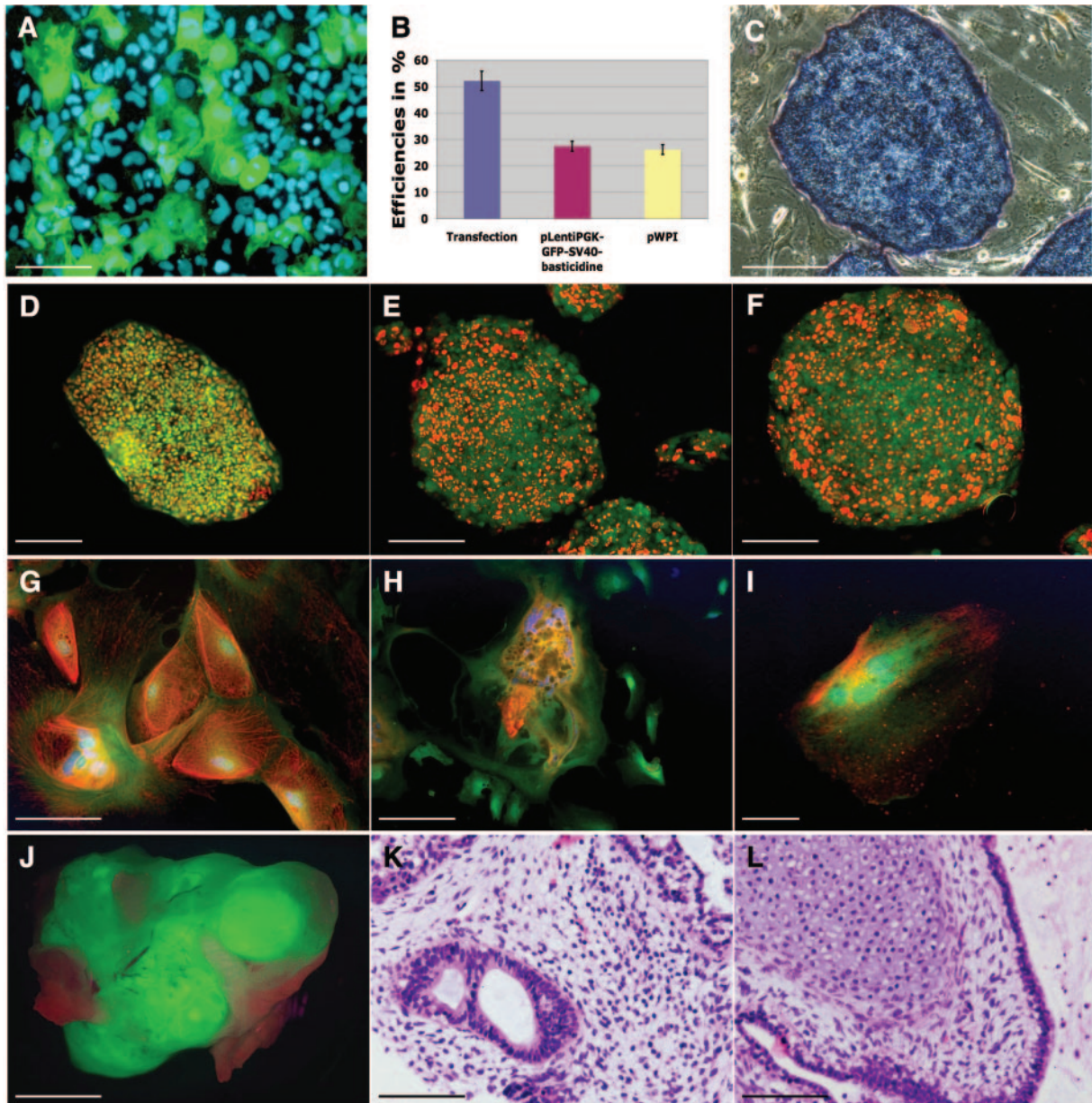
**Figure 3.** Retrovirally-transduced hES cells maintain transgene expression throughout neural differentiation. (A) Following discontinuation of G418 selection, neurospheres derived from transduced hES continue to express EGFP across several passages. Shown are EGFP-expressing neurospheres cultured for 4 weeks (five passages) without G418. (B) Following growth factor withdrawal, plated spheres show extensive neurite extension. (C–F) Plated neurospheres and single cells derived thereof express the neuronal marker antigens beta-III-tubulin (C) and MAP2ab (D and E); astrocytes can be readily detected with an antibody to GFAP (F). Both neurons and glial cells exhibit prominent EGFP expression. Scale bars: A–D: 100  $\mu$ m; E and F: 20  $\mu$ m.

additional cloning steps. Second, as ecotropic vectors are unable to infect wild type human cells (7,33), transduction of hES cells can be performed without the safety concerns typically associated with the use of amphotropic vectors, which may bear the risk of toxic effects for both recipient and experimenter (8–10). The inability to infect normal human cells should even permit the transduction of critical vectors carrying immortalizing genes or active oncogenes under standard safety conditions (33,34). Furthermore, the two-step-protocol offers the possibility to restrict the viral transduction by cell type-specific expression of the retrovirus receptor. In murine systems, avian retrovirus receptors have been used in this manner for cell type-specific gene transfer (35–39), although the avian vectors have limitations with respect to the transgene size. Whereas the CMV and CAG promoter elements employed in this study are expected to yield ubiquitous expression in a broad variety of cell

types, the use of suitable cell type-specific promoters should permit targeted expression of the retrovirus receptor in defined cell lineages (40,41). Along these lines, our two-step approach might be applied for the selection of specific somatic lineages from differentiating hES cell cultures. Finally, oncoretroviruses such as MoMuLV are particularly suited for the study of progenitor cell populations because they only infect dividing cells. This property has made them a tool of choice for cell lineage analysis in many tissues including the CNS (42,43). Thus, in addition to targeted gene transfer, our system might be particularly useful for *in vitro* clonal analyses of retrovirally labelled hES cell-derived somatic stem cells and *in vivo* lineage tracing of transplanted human cells (18,19).

A critical aspect in stem cell transduction is maintenance of stem cell properties. Using bicistronic oncoretroviral vectors carrying an antibiotic resistance gene, we selected





**Figure 4.** Pseudotyped lentiviral vectors enable efficient and permanent transduction of hES cells without selection pressure. (A and B) Ecotropic pseudotyped lentiviral vectors efficiently transduce mCAT1 expressing hES cells. Forty-eight hours after transduction with concentrated virus supernatant,  $27.2 \pm 6.5\%$  and  $26.1 \pm 7.2\%$  of the total cell population transduced with pLentiPGK-EGFP-SV40-blasticidine (A) and pWPI expresses EGFP, respectively. Normalized to the mCAT1 transfection rate, this corresponds to a calculated transduction rate of  $\sim 50\%$  of the mCAT1-transfected cells. (C–F) Selected pLentiPGK-EGFP-SV40-blasticidine-transduced cells continue to express markers of pluripotency. hES cells transduced by lentiviral vectors were selected via a blasticidine drug resistance for three days and continuously cultured on feeders (MEFs) without further drug selection. Twenty passages after transduction the cells show sustained expression of alkaline phosphatase (C, blue), Oct-4 (D), Tra-1-60 (E), and Tra-1-81 (F) (all red) while retaining high levels of transgene expression. (G–J) Transgene expression is retained during germ layer differentiation *in vitro*. Twenty passages after transduction, lentivirally transduced cells were aggregated to form EBs and continuously cultured in the presence of 5% FCS for 8 weeks. In the EB outgrowth, differentiation into derivatives of all three germ layers could be detected. Cells expressing cytokeratin (G), alpha-feto-protein (H) and desmin (I) retain strong EGFP transgene expression. (J–L) Transgene expression is retained after teratoma formation *in vivo*. Upon transduction and selection,  $3 \times 10^6$  cells were injected into immunodeficient SCID-beige mice. Nine weeks after injection, strongly EGFP-positive teratomas had formed (J). Teratomas contained various tissues including adenoid tissue (K), cartilage and epithelium (L). Scale bars: A: 100  $\mu\text{m}$ ; C–F: 250  $\mu\text{m}$ ; G: 100  $\mu\text{m}$ ; H: 250  $\mu\text{m}$ ; I: 50  $\mu\text{m}$ ; J: 2.5 mm; K–L: 100  $\mu\text{m}$ .

permanently transduced hES clones for further characterization. The transduced clones maintained the expression of pluripotency-associated markers and the ability to generate derivatives of all three germ layers *in vitro* and *in vivo*. Thus, the two-step-procedure involving transfection of the mCAT1-receptor and subsequent retroviral transduction

appears not to interfere with the pluripotent properties of ES cells.

A key challenge associated with the use of ecotropic retroviral vectors is the difficulty to obtain high-titer viral stocks. The viral membrane is often very fragile and/or unstable, making it difficult to concentrate viral particles by collection

over time and subsequent ultracentrifugation. In the current study we have used low speed centrifugation over a period of 12–16 h to concentrate the virus. We obtained titers of up to  $8.4 \times 10^8$  transducing units per ml for MMLV-based vectors and up to  $1.8 \times 10^9$  transducing units per ml for lentiviral vectors which permitted efficient infection of mCAT1-expressing hES cells, thus providing a possible solution to bypass restrictions by low titers of ecotropic retroviruses.

Previous studies in mouse ES cells have shown that transgenes expressed from oncoretroviral vectors are rapidly silenced upon differentiation (15,44–46). Consistent with these observations we noticed a down-regulation of EGFP in undifferentiated hES cells cultured over several passages, and a rapid EGFP silencing in differentiating hES cell-derived EBs. Down-regulation could be avoided by using a bicistronic vector containing an IRES-linked resistance gene (neoR) and maintenance of neomycin selection during differentiation. hES cell cultures continuously propagated in the presence of 150 mg/ml G418 maintained their proliferation rate as well as their potential to generate EBs and derivatives of all three germ layers. Interestingly, no further G418 treatment was required once the cells had been shifted into a neural precursor state. hES cell-derived neural precursors proliferated as neurospheres in FGF2 for at least 6 passages and subsequently differentiated for 6 weeks in the absence of growth factors showed no obvious loss of EGFP expression. Thus, our transduction system appears to be suitable for permanent transgene expression in differentiated hES cell-derived progeny, particularly in the neural lineage. It may, therefore, also be applicable for *in vivo* lineage analysis of hES cell-derived somatic cells. It should also be noted that the retroviral construct used in our study carries a classic 5' LTR leader sequence. These vectors are known to show a high frequency of promoter silencing with low expression levels in ES and EC cells (47). Constructs with different 5' LTR or 3' LTR leaders such as the murine stem cell PCMV virus (MSCV), the murine embryonic stem cell virus (MESV) or combinations thereof have been tailored to applications in stem cell technology and might be used to further enhance the efficiency of our transduction system (11,48–51).

A critical parameter in assessing advantages and limitations of viral gene transfer systems for hES cells is transduction efficiency. Using optimized transfection protocols and concentrated oncoretroviral vectors, we achieved transduction rates of up to 20% of the total cell population, equaling an estimated transduction efficiency of up to ~40% of the mCAT1-expressing cells. In comparison, direct transduction of hES cells by lentiviral vectors pseudotyped with vesicular stomatitis virus glycoprotein (VSV-G) yields transduction efficiencies of 38–87% (52,53). These high transduction rates have made lentiviral vectors an attractive tool for gene transfer in stem cells and their progeny (53–67). Lentiviral infection of hES cells has been shown to not affect their pluripotent state and to yield stable transgene expression upon germ layer differentiation (52–54). Recently, knock-down strategies based on siRNA-expressing lentiviral vectors have further kindled interest in this system (68–73). Considering these developments, we became interested in accommodating lentiviral vectors in our ecotropic transduction paradigm. Using lentiviral vectors pseudotyped with the

ecotropic MoMuLV glycoprotein, we obtained transduction efficiencies of up to 33% of the total cell population, equaling an estimated transduction efficiency of up to ~60% of the mCAT1-expressing cells. Lentivirally transduced cells maintained expression of the transgene throughout proliferation and differentiation without further selection pressure. Maintenance of pluripotency was confirmed by the co-expression of pluripotency-associated markers in EGFP-positive transduced hES cells and multi-germ layer differentiation in EGFP-positive EBs derived thereof. Furthermore, lentivirally transduced hES cells transplanted 20 passages after selection formed teratomas with sustained EGFP expression. Thus, our two-step protocol also enables lentivirus-based permanent expression of transgenes in pluripotent hES cells under standard safety precautions for murine ecotropic vectors.

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