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RESEARCH ARTICLE

Enhanced transgene expression in primitive hematopoietic progenitor cells and embryonic stem cells efficiently transduced by optimized retroviral hybrid vectors

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Oncoretroviral vectors have been successfully used in gene therapy trials, yet low transduction rates and loss of transgene expression are still major obstacles for their application. To overcome these problems we modified the widely used Moloney murine leukemia virus-derived retroviral vector pMX by replacing the 3′LTR with the spleen focus-forming virus LTR and inserting the woodchuck hepatitis B virus post-translational regulatory element. To compare requirements crucial for efficient transgene expression, we generated the hybrid retroviral vectors pMOWS and pOWS that harbor the complete murine embryonic stem cell virus (MESV)-leader sequence or a shortened MESV-leader not comprising primer binding site (PBS) and splice donor (SD). Applying these retroviral vectors significantly augmented transgene expression in hematopoietic cell lines and progenitor cells. For transduction of murine embryonic stem (ES) cells the retroviral vector pMOWS that harbors the MESV-PBS and -SD was superior resulting in 65% green fluorescent protein (GFP) expressing ES cells. Surprisingly, in murine and human primitive hematopoietic progenitor cells (HPC), the highest efficiency of up to 66% GFP expressing cells was achieved with pOWS, a retroviral vector that retains the negative regulatory element coinciding with the MoMuLV-PBS. In summary our hybrid retroviral vectors facilitate significantly improved transgene expression in multipotent cells and thus possess great potential for reconstituting genes in primary cells of disease models, as well as for gene therapy.
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Keywords: *primitive hematopoietic progenitor cells; embryonic stem cells; hybrid retroviral vector; MESV-leader sequence*

Introduction

To dissect the molecular cause of human diseases and to develop novel therapeutic strategies retroviral reconstitution of genes in pluripotent cells is of great importance. Although the Moloney murine leukemia virus (MoMuLV)-based retroviral vector MFG has been used in the first successful gene therapy trials,[1] progress in vector design and retroviral packaging systems would be beneficial to improve gene transfer rates and sustained transgene expression.[2]

An advanced form of the MFG-derived retroviral vector is pMX that was generated by inserting the MFG 5′LTR and leader sequence into the MoMuLV retroviral vector pBABE-X.[3] This retroviral vector is frequently used to generate high titers in transient transfection systems and has been successfully applied to introduce genes into hematopoietic cells.4 However, it was reported that the expression of genes transduced by MoMuLV-derived retroviral vectors is affected by down-modulation in primitive HPC.[5,6] presumably by similar mechanisms as in ES cells. In ES cells retroviral silencing is mediated by cell-type specific *trans*-acting transcriptional repressors and by *cis*-acting *de novo* methylation of the integrated provirus.[7] The murine embryonic stem cell virus (MESV) was identified as a retrovirus that can overcome the block of retroviral transcription in ES cells.[8] The most important difference compared with MoMuLV is the elimination of a negative regulatory element (NRE) overlapping with the MoMuLV primer binding site (PBS) that mediates recruitment of the binding factor A or NRE-binding factor,[9,10] a potent transcriptional repressor present in ES cells. In addition, sequence modifications in the MESV-LTR enhancing the affinity for positive factors and decreasing the affinity for negative regulators promote MESV provirus expression in ES cells. Comparable alterations have been observed in the LTR of the murine stem cell virus (MSCV).

To specifically enhance retroviral transgene expression in hematopoietic progenitor cells, Baum *et al*[5] combined elements of MESV with the spleen focus-forming virus (SFFV) 3′LTR to generate the Friend mink cell focus-forming virus/MESV (FMEV)-type hybrid retroviral vector pSF. The SFFV-LTR has been shown to be more potent in hematopoietic cells and differs from the MoMuLV-LTR

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by containing binding sites for Sp1 and the polyomavirus enhancer binding protein/core binding factor (PEBP/CBF) yet lacking a CAAT/enhancer binding protein (C/EBP) binding site.[11] Upon transduction in hematopoietic target cells, the FMEV-type retroviral vector pSF forms MESV/SFFV hybrid LTRs that support increased expression levels compared with conventional MoMuLV-based retroviral vectors.

Further strategies to improve transgene expression by retroviral vectors include the use of an efficient leader sequence,[12,13] insertion of RNA export elements[14] and additional splice signals.[15] The leader sequence harbors, besides the packaging signal necessary for incorporation of genomic RNA into viral particles, the splice donor (SD) upstream of the PBS and the splice acceptor located further downstream. In the retroviral vector MFG sequence, alterations in the leader result in enhanced splicing and as a consequence in augmented transgene expression.[16] RNA transport elements presumably function by enhancing nuclear export of unspliced RNA and increasing polyadenylation.[17] Likewise, the woodchuck hepatitis B virus post-transcriptional regulatory element (WPRE)[14] has been shown not only to promote retroviral transcription, but possibly also translation.[15]

Most commonly, high titer supernatants are being generated by selection of stable producer lines from transfected murine packaging cell lines. However, due to the large number of endogenous retroviral sequences in murine cells this approach can lead to the production of replication-competent virus. In addition, virus produced by murine cells is prone to complement inactivation in human serum.[2] To circumvent these problems retroviral packaging cell lines based on the human embryonic kidney 293T cell line were developed[18] that facilitate rapid production of transducing supernatants in a transient transfection approach.

Here we report a novel set of retroviral hybrid vectors that promote the production of high titers in a transient transfection approach and show significantly improved transgene expression in hematopoietic cell lines, murine and human hematopoietic progenitors and murine embryonic stem cells. By combining elements present in the potent retroviral vectors pMX and pSF with the WPRE, we observe that inactivation of the NRE is important for efficient retroviral transgene expression in ES cells whereas this effect is less pronounced in primitive HPC.

Results

Our aim was to construct retroviral vectors optimized for (1) the production of high titers upon transient transfection in 293T cell-derived retroviral packaging cell lines and (2) efficient transgene expression in hematopoietic progenitor cells and embryonic stem cells. As parental vector that was altered by inserting or replacing specific elements, the retroviral vector pMX[3] was used, since this vector is extremely efficient in 293T-derived cells. In our hybrid retroviral vectors (Figure 1), the MoMuLV 3′LTR present in pMX was replaced by the SFFV 3′LTR derived from pSFβ1[5] to improve transgene expression in the hematopoietic system. Furthermore, to ensure provirus expression in pluripotent cells, the R/U5 region of the 5′LTR and the leader sequence in pMX were substituted by the respective MESV sequence resulting in the hybrid

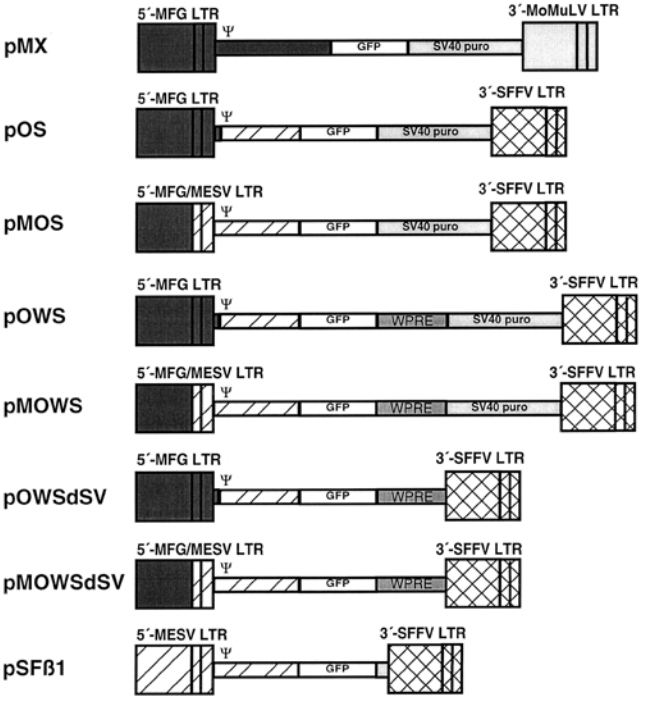

Figure 1 *Schematic representation of retroviral vectors. Both LTR composed of U3, R and U5 region are symbolized by a large box and in between packaging signal (Ψ), the multiple cloning site (MCS) and the SV40-puro^R cassette are indicated. The parental retroviral vector pMX is composed of the MFG derived MoMuLV 5′LTR and leader sequence (dark gray) and the MoMuLV 3′LTR (light gray), whereas pSFβ1 comprises the MESV 5′LTR and -leader sequence (hatched boxes) and the SFFV 3′LTR (crosshatched box). In our hybrid retroviral vectors the 3′LTR present in pMX was replaced by the SFFV 3′LTR. For pMOS the MFG R/U5 region and -leader sequence was replaced by the respective MESV sequence, whereas in pOS a shortened MESV-leader not comprising PBS and SD was introduced. The insertion of WPRE in pOWS and pMOWS is indicated. The retroviral vectors pOWSdSV and pMOWSdSV lack the SV40-puro^R cassette. In each vector GFP was inserted in the MCS as a reporter gene. As negative control, the pMX vector without GFP was used in all experiments except where indicated.*

retroviral vector pMOS. For comparison the hybrid retroviral vector pOS was generated that harbors a shortened MESV-leader sequence and retains the NRE-binding element overlapping with the MoMuLV-PBS, as well as the MoMuLV-SD. To improve post-transcriptional processing and nuclear export of unspliced genomic retroviral RNA, the WPRE was integrated either upstream of the SV40-promoter puromycin resistance cassette (pOWS and pMOWS) or upstream of the 3′LTR (pOWSdSV and pMOWSdSV). The efficiency of the various retroviral vectors was determined by monitoring expression of GFP inserted as a reporter gene.

Elements controlling rapid production of high vector titers by transient transfection

A key element controlling transcription of genomic retroviral RNA in packaging cell lines is the enhancer promoter element located in the U3 region of the 5′LTR. To compare the efficiency of retroviral vectors harboring a MESV 5′LTR (pSF), a MFG 5′LTR (pMX, pOS, pOWS) or a MFG/MESV hybrid 5′LTR (pMOS and pMOWS), the retroviral vectors were transiently transfected into the 293T cell-derived retroviral packaging cell line Phoenix-Eco. Serial dilutions of the supernatants were used for

the transduction of NIH3T3 cells to determine retroviral vector titers generated (Table 1). Whereas transfection of pSF harboring a MESV 5′LTR resulted in only 0.6×10^6 transducing units/ml (tU/ml) the rate was increased approximately three-fold ($P < 0.001$) with retroviral vectors containing a MFG 5′LTR (pMX, pOS) or MFG/MESV hybrid 5′LTR (pMOS, pMOWS). Insertion of WPRE into pOS further enhanced the vector titer up to 2.9×10^6 tU/ml (pOWS). A comparison of pMOWS and pMOWSdSV revealed that the presence or absence of the SV40 origin of replication in our hybrid retroviral vectors had only a minor effect on the titer generated. Thus, the MFG-derived U3 region, in particular in conjunction with WPRE, is highly efficient in supporting the production of high titers upon transient transfection in Phoenix-Eco cells.

Identification of retroviral vectors promoting efficient transgene expression in hematopoietic cell lines

Factor-dependent hematopoietic cell lines are frequently used to study mechanisms underlying human diseases. To test the potential of our hybrid retroviral vectors for rapid generation of cell lines stably expressing high transgene levels, supernatants harvested from Phoenix-Eco cells were used to transduce the IL-3-dependent pro-B cell line BaF3 by spin infection. As an indirect indicator for the transduction frequency, the percentage of GFP-expressing BaF3 cells was determined by flow cytometry (Figure 2, lower panel). This analysis revealed that except for pMOWS the transduction rates obtained with our hybrid retroviral vectors were similar to pMX and ranged from 14.2% to 21.0% GFP-positive BaF3 cells. Strikingly, with pMOWS as much as 30.1%, GFP-expressing BaF3 cells were achieved corresponding to an almost two-fold improvement compared with pMX and a more than three-fold increase in comparison to pSF. As a consequence of retroviral replication and chromosomal integration, the expression of retroviral transgenes in target cells is controlled by the enhancer/promoter formerly present in the 3′LTR of a retroviral vector. Accordingly, the presence of the SFFV 3′LTR in combination with the complete MESV-leader sequence in pMOS improved transgene expression in BaF3 cells 5.9-fold compared with pMX as measured by the increase in mean fluorescence intensity (Figure 2, upper panel). Interestingly, pOS, a retroviral vector that harbors the MoMuLV-SD and -PBS in combination with a shortened MESV-leader sequence supported higher GFP expression levels than pMOS. Further addition of WPRE generated the hybrid retroviral vectors (pOWS and pMOWS) that showed as much as a 7.9-fold (pOWS) or a 8.7-fold (pMOWS) increase in transgene expression compared with pMX. Similar results regarding the efficiency of the novel hybrid retroviral vectors were obtained in 32D cells, another factor-dependent hematopoietic model cell line (data not shown). In summary, the presence of the SFFV 3′LTR and WPRE in combination with either the shortened (pOWS) or most notably the complete (pMOWS) MESV-leader sequence significantly enhances transgene expression levels in hematopoietic model cell lines.

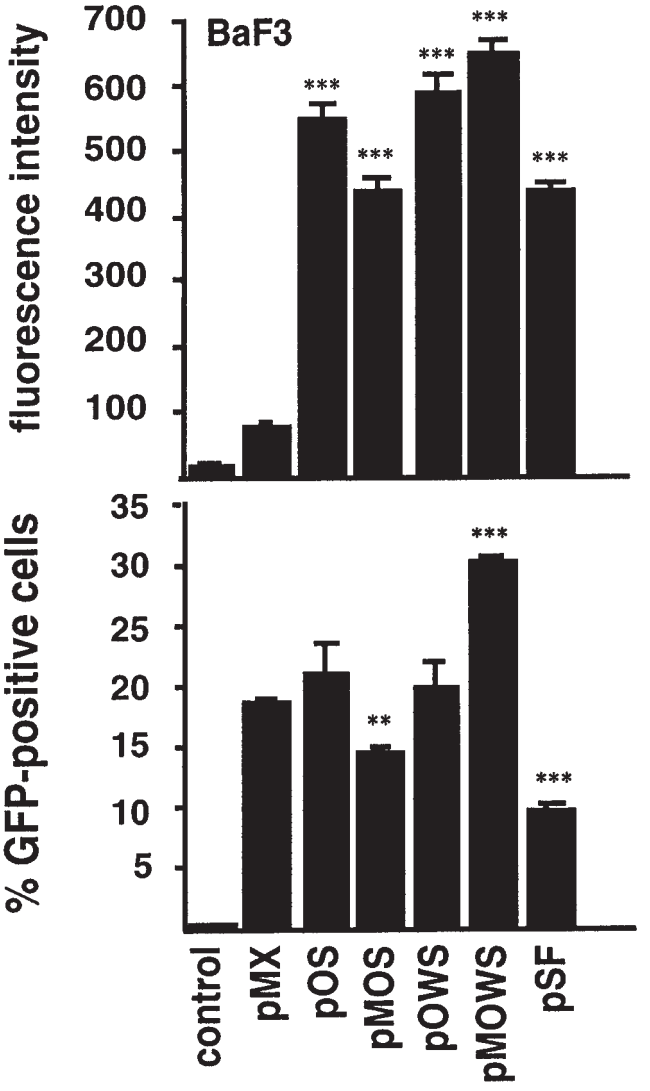

Figure 2 *Gene transfer and transgene expression in a model hematopoietic cell line. BaF3 cells were transduced by spin infection with supernatants from Phoenix-Eco cells transiently transfected with the indicated retroviral vectors. Forty-eight hours after transduction GFP expression in BaF3 cells was quantified by flow cytometry. The mean fluorescence intensity (upper panel) and percentage of GFP-positive cells (lower panel) determined in three independent transductions is shown. The experiment was performed five times with comparable results. (Significance compared to pMX are marked by asterisks: ***$P \leqslant 0.001$).*

Table 1 Titer of retroviral expression vector constructs[a]

Vector	*5′LTR*	*3′LTR*	*tU/ml*
pMX	MFG	MoMuLV	2.0 ± 0.1
pOS	MFG	SFFV	2.3 ± 0.5
pMOS	MFG/MESV	SFFV	2.3 ± 0.5
pOWS	MFG	SFFV	2.9 ± 1.0
pMOWS	MFG/MESV	SFFV	2.2 ± 0.6
pMOWSdSV	MFG/MESV	SFFV	1.8 ± 0.5
pSF	MESV	SFFV	0.6 ± 0.2

[a]Titration was performed on NIH3T3 cells with various vector supernatant dilutions. The number of GFP-positive cells was plotted against dilution and the titer has been determined in the linear range of the plot. The titer is given as transducing units/ml (tU/ml $\times 10^6$) calculated from five different transductions performed with two different plasmid preparations.

Efficient transduction of murine erythroid progenitor cells and elevated GFP-expression

Retroviral reconstitution of genes in cells of murine disease models facilitates functional studies in primary cells. To evaluate the efficiency of our retroviral vectors in establishing transgene expression in erythroid progenitor cells, lineage depleted (lin^-) fetal liver cells were transduced with supernatants harvested from Phoenix-Eco cells as described above. Flow cytometry analysis (Figure 3) revealed that the hybrid retroviral vectors facilitated efficient transduction of lin^- fetal liver cells grown under conditions primarily supporting the expansion of erythroid progenitor cells (presence of erythropoietin (Epo)). By applying a spin infection protocol as much as 86.6 to 91.5% GFP-positive cells were achieved at a moderate MOI (MOI 5) with the novel hybrid retroviral vectors and pMX, whereas pSF resulted in 71.9% GFP expressing cells. To compare the transgene expression efficiency accomplished with the different retroviral vectors in erythroid progenitor cells, the mean fluorescence intensity (MFI) of GFP expressing lin^- cells was determined by flow cytometry. Upon transduction with pMX a MFI of 777.3 was detected in GFP-expressing erythroid progenitor cells. The presence of the SFFV 3′LTR and the MESV-leader sequence in pSF, pOS and pMOS markedly improved transgene expression and MFI ranging from 1778.6 to 1883.7 was reached. Insertion of WPRE in our hybrid retroviral vectors augmented the effect resulting in an MFI of 2318.5 (pMOWS) and 2394.1 (pOWS). Similar transgene expression was achieved by deleting the SV40-puromycin resistancy cassette (pMOWSdSV: mean fluorescence intensity 2411.4 and pOWSdSV: mean fluorescence intensity 2443.1).

Thus, the novel retroviral vectors pOWS and pMOWS promote efficient reconstitution of transgenes in murine erythroid progenitor cells.

Enhanced transgene expression in primitive hematopoietic progenitor cells

To assess the potential of the hybrid retroviral vectors for gene therapy, transduction of murine and human primitive HPC was performed. Preparations of lineage-depleted murine fetal liver cells reproducibly contained 2.4–5.8% (mean 3.74%; $n = 6$) sca-1^+ c-kit^+ double-positive cells that represent primitive HPC in the mouse (Figure 4a). Lin^- fetal liver cells were transduced with the retroviral vectors indicated in Figure 4b and cultivated in IL-6, SCF and Flt3L, conditions that favor growth and survival of hematopoietic stem cells. In four independent experiments at a moderate MOI (MOI 5) using a spin infection protocol as much as 50 to 61% transduced sca-1^+c-kit^+ cells were obtained with the retroviral vectors tested, except for pSF and MSCV that yielded 40% or 36% GFP-expressing sca-1^+c-kit^+ cells (Figure 4b). When transduced at a low MOI (MOI 1) favoring less than one retroviral copy per cell (Figure 4c), the parental retroviral vector pMX yielded a MFI of 285.4 in GFP expressing sca-1^+ c-kit^+ cells. The presence of the SFFV 3′LTR and the incomplete MESV-leader sequence in pOS or the complete MESV-leader sequence in pMOS improved transgene expression in sca-1^+ c-kit^+ cells and resulted in MFI of 886.4 or 1072.9, respectively. The insertion of WPRE further elevated this effect resulting in a MFI of 1279.7 for cells transduced with pOWS, whereas surprisingly pMOWS (MFI 981.8) did not further improve transgene expression compared with pMOS. The analysis of pMOWSdSV in this assay revealed that the presence of the SV40 puromycin resistance cassette had no major effect on transduction rates or transgene expression (data not shown). Therefore, pOWS represents a hybrid retroviral vector that is significantly improved compared with the parental vector and mediates efficient transgene expression in murine primitive HPC.

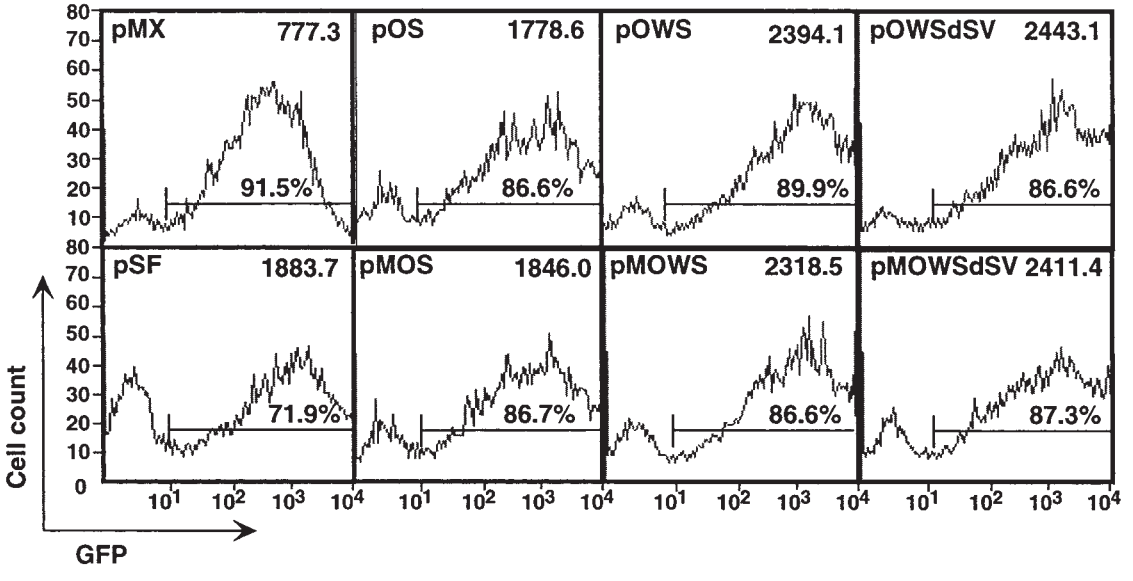

Figure 3 *Effective transduction and high-level transgene expression in primary murine erythroid progenitor cells. Flow cytometry of lin^- fetal liver cells (FLC) transduced with the indicated retroviral vectors at a moderate MOI (MOI 5). After 20 h cultivation in the presence of erythropoietin (EPO), GFP expression was quantified in viable cells identified according to forward and side scatter discrimination. Indicated is the percentage of GFP expressing (>10 fluorescence units) and the mean fluorescence intensity (MFI). The experiment was repeated three times with comparable results.*

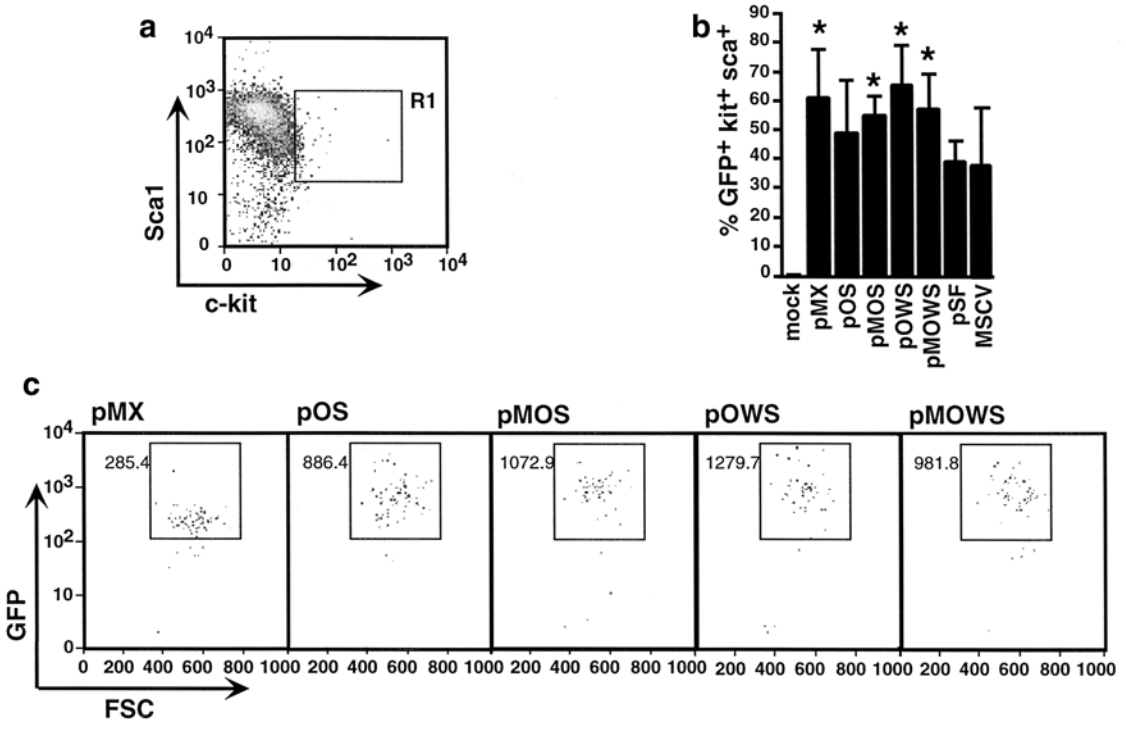

Figure 4 *Elevated transduction and improved transgene expression in murine primitive HPC harboring the hybrid retroviral vector pOWS. (a) Preparation and identification of murine primitive HPC. Fetal liver cells were depleted for lineage marker expressing cells (Gr1, Mac1, CD4, CD8, CD14, B220, Ter119) by rat antibodies. In the lin$^-$ fraction primitive HPC expressing sca-1 and c-kit were detected by incubation with anti-sca-1 coupled to PE and anti-c-kit coupled to APC. In six independent experiments, 2.4–5.8% (mean 3.74%) of lin$^-$ fetal liver cells were sca-1$^+$ and c-kit$^+$ double positive as identified by gate R1. (b) Elevated gene transfer in primitve sca-1$^+$ c-kit$^+$ HPC. Lin$^-$ fetal liver cells were transduced with the retroviral vectors indicated at a moderate MOI (MOI 5) and at day 7 in the culture period the percentage of GFP-expressing sca-1$^+$ c-kit$^+$ was determined by flow cytometry. Depicted is the mean percentage of GFP expressing sca-1$^+$ c-kit$^+$ cells from four independent experiments. (Significance compared with pSF is marked by asterisks: *$P \leqslant 0.05$). (c) High transgene expression levels in primitive sca-1$^+$ c-kit$^+$ HPC. GFP expression in primitive sca-1$^+$ c-kit$^+$ HPC transduced with the indicated retroviral vectors at a low MOI (MOI 1) was quantified at a day 7 in the culture period by FACS analysis. The numbers to the left of the gate represent the detected mean fluorescence intensity in GFP-expressing sca-1$^+$ c-kit$^+$ HPC. The figure shows the result of an experiment representative for three independent transductions performed.*

To test the relevance of these results for the human system, transduction of human hematopoietic progenitor cells (CD34$^+$) was performed. CD34$^+$ peripheral blood stem cell progenitor cells (PBSC) were prepared from leukapheresis samples by positive selection for CD34 expression. The purity of the enrichment was 96.7 ± 2.1% (n = 6) of CD34$^+$ PBSC as determined by flow cytometry (not shown). Transient transfection of the retroviral vectors pOS and pOWS in Phoenix-Ampho cells in the absence of serum reproducibly resulted in the production of high titers of 3×10^5 tU/ml (data not shown). Transduction of CD34$^+$ PBSC was conducted in three consecutive transduction cycles under serum-free conditions. The analysis of transduced CD34$^+$ PBSC by flow cytometry revealed that gene transfer rates obtained with pOS and pOWS were similar and ranged from 32.1% to 58.2% (mean 44.6 ± 10.4%) for pOS and from 29.5 to 62.7% (mean 46.0 ± 13.4%) for pOWS in six different samples (data not shown). To assess transgene expression in cells that remained CD34$^+$ during the *ex vivo* cultivation period, double staining with a PerCP conjugated anti-CD34 antibody was performed. The GFP expression levels detected in CD34$^+$ PBSC and cells lacking CD34 expression were comparable (Figure 5). The number of GFP-expressing cells is given in the upper left and upper right quadrants, and the percentage of GFP expressing cells in the CD34$^+$ fraction (upper right and lower right quadrant) is shown in the upper right quadrant. Upon using the retroviral vector pOS, 38.6% of the analyzed cells expressed GFP and 42.4% in the CD34$^+$ fraction expressed GFP (Figure 5), whereas gene transfer mediated by pOWS resulted in 62.7% GFP expressing cells and 66.3% were GFP$^+$ CD34$^+$. While the transduction

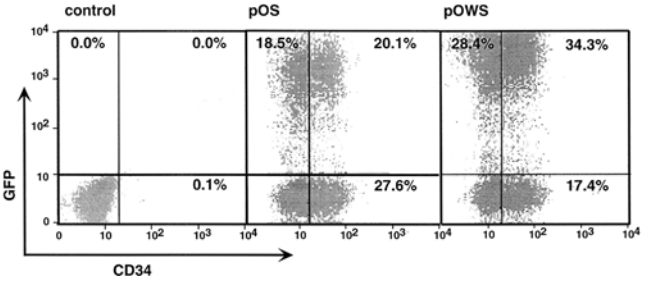

Figure 5 *Efficient transduction and transgene expression in human CD34$^+$ HPC. Primitive hematopoietic cells were obtained from leukapheresis samples and enriched for CD34 expression. Transduction was performed in serum-free conditions with a control vector harboring no GFP and with the vectors pOS and pOWS. The cells were stained with anti-CD34 conjugated with PerCP and fluorescence intensities were compensated in the FL1-FL3 channels. The results presented are representative for six independent experiments. Reproducibly, a significant increase in fluorescence intensity of cells transduced with pOWS compared with pOS was observed. The negative control shows untransduced cells.*

rates achieved by both retroviral vectors in CD34+ PBSC was within a similar range, the fluorescence intensities obtained with pOWS were significantly enhanced (Figure 5).

Therefore, in both systems our hybrid retroviral vectors, in particular pOWS, facilitate efficient transgene delivery and high levels of transgene expression in HPC.

High levels of transgene expression in retroviral transduced murine embryonic stem cells

In ES cells, inefficient transcription of proviruses has been primarily attributed to the NRE-binding site overlapping with the MoMuLV-PBS. Accordingly, transduction of ES cells with retroviral vectors containing the MoMuLV-PBS, such as pMX and pOS yielded only very few GFP-expressing ES cells (1.1% and 6.3%; Figure 6). Insertion of WPRE in pOWS partially compensated the effect and resulted in an increase to up to 27.1% GFP-expressing ES cells ($P < 0.01$). However, applying our hybrid retroviral vectors pMOS and pMOWS that harbor the MESV-PBS dramatically enhanced the yield of GFP-expressing ES cells (50.9% and 64.6%, $P < 0.002$). Surprisingly, the retroviral vector pSF that contains the MESV-PBS was less efficient (16.2% GFP-expressing cells) than pMOS and pMOWS and was even reduced compared with pOWS. This suggests that the absence of the NRE-binding site is not the sole determinant for efficient transgene expression in ES cell and can be partially compensated by WPRE insertion. Thus, whereas in primitive HPC, hybrid retroviral vectors harboring the MoMuLV-PBS and -SD are comparable or superior, in ES cells transgene expression is greatly facilitated by the presence of the MESV-PBS and -SD. The effect of the MESV leader sequence is further enhanced upon insertion of the WPRE and therefore our novel hybrid retroviral vectors, in particular pMOWS, provide valuable tools facilitating efficient transgene expression in ES cells.

Discussion

In this study, we report a novel set of hybrid retroviral vectors optimized for highly efficient gene transfer and transgene expression in hematopoietic cell lines, primitive HPC and ES cells. Major improvements compared with MoMuLV-based vectors are achieved by combining the high promoter activity of the MFG 5́LTR in 293T-derived packaging cell lines with the myeloid-erythroid potency of the SFFV-LTR, the MESV-leader and the WPRE. We observe that in primitive HPC a hybrid retroviral vector retaining the MoMuLV-PBS is highly efficient, whereas in ES cells inactivation of the NRE is crucial for efficient transgene expression.

Two blocks for provirus expression by MoMuLV-based vectors in HPC[19] and ES cells[7,20] have been reported. The primary block that is mediated by *trans*-acting factors can be overcome by sequence alterations in the LTR, increasing the affinity for positive factors and disrupting the recruitment of negative factors. The SFFV-LTR, present in our retroviral hybrid vectors, is particularly efficient in cells of myeloid-erythroid origin and possesses in comparison to the MoMuLV-LTR a high-affinity binding site for the Sp1 transcription factor. This alteration concomitantly results in the inactivation of the binding site for a transcriptional repressor, the embryonic long terminal repeat binding protein (ELP) that is contained in the MoMuLV-LTR.[11] Similar sequence alterations have been identified in the MPSV-,[19,21] as well as the MSCV-LTR.[7,22] A MSCV-based retroviral vector has been reported to

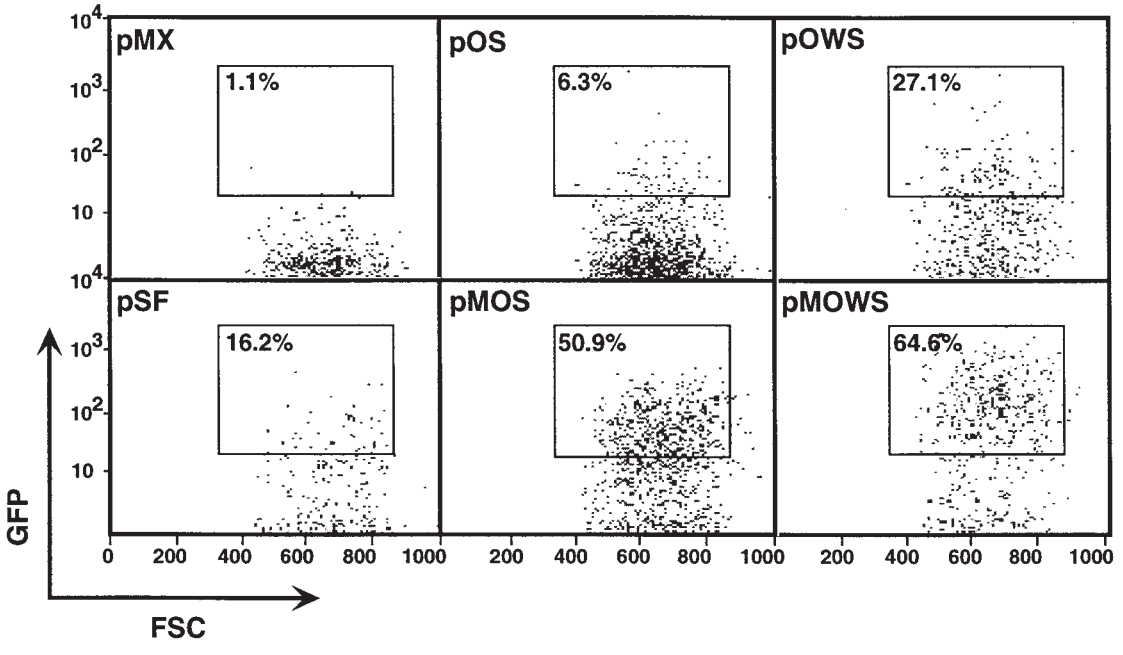

Figure 6 *High gene transfer rates and transgene expression in ES cells by the hybrid retroviral vector pMOWS. ES cells were seeded on gelatin-coated plates 24 h before transduction and transduced at a moderate MOI (MOI 5) by spin infection as described in Materials and methods. After 5 days in culture with LIF, the cells were analyzed by flow cytometry. The experiment shows forward scatter discrimination to GFP fluorescence. The number indicates the of GFP positive ES cells (>10 fluorescence units). The results shown are representative of three independent transductions.*

maintain stable retroviral vector expression in HSC upon serial adoptive transfer.[7] In comparison to MSCV our hybrid retroviral vectors harboring the SFFV-LTR showed improved transduction rates and transgene expression in primitive HPC. The highest level of transgene expression was achieved with pOWS that comprises the MoMuLV-PBS and -SD. However it was observed that in ES cells the activating mutations in the LTR are not sufficient to over-ride the repressor activity of the NRE coinciding with the MoMuLV-PBS.[22] Thus vectors such as MESV harboring both mutations in the LTR and the NRE are transcriptionally active in ES cells.[8] In agreement with this our hybrid retroviral vectors pMOWS harboring the MESV-PBS mediated the highest transgene expression levels in ES cells. Surprisingly, despite harboring a MoMuLV-PBS, pOWS is transcriptionally active in ES cells suggesting that insertion of the WPRE in a retroviral vector can at least partially compensate for the negative effect of the NRE. Despite the reported similarities in mechanisms regulating retroviral transgene expression in primitive HPC and ES cells,[5] successful utilization of a MoMuLV-based retroviral vector in gene therapy trials suggests that compared with ES cells the presence of the NRE is less detrimental for transgene expression in primitive HPC. Accordingly, we observe that, whereas inactivation of the NRE is important for maximal transgene expression in ES cells, the presence of the MoMuLV-PBS and -SD in our hybrid retroviral vector pOWS is beneficial in primitive HPC.

Long-term expression of integrated proviruses is repressed by *cis*-acting mechanisms including *de novo* methylation. It was demonstrated that ES cells possess high *de novo* methylation activity resulting in transcriptional repression of MoMuLV-proviruses.[23] Expression of silenced proviruses in the tissue of transgenic animals derived from MSCV-transduced ES cells could be reactivated upon treatment with demethylating agent 5-azadeoxycytidine.[7] Furthermore, inactivation of the *Dnmt1* methyltransferase prevented long-term transcriptional silencing of MSCV-based retroviral vectors in ES cells.[7] Others reported that methylation is responsible for partial repression of MESV-controlled transgene expression in undifferentiated ES cells, whereas at early stages of differentiation MESV-provirus silencing is mediated by an unknown methylation independent mechanism.[20] In primitive HPC long-term retroviral transcription is regulated by comparable mechanisms and silencing of MoMuLV-derived retroviral vectors in HSC has been associated with *de novo* methylation.[19] The role of methylation for retroviral silencing is not entirely understood, but it has been observed that insertion of a hypomethylation signal in retroviral vectors can significantly improve transgene expression.[19] Presence of a Sp1 binding site was reported to enhance demethylation of surrounding sequences and to partially protect from *de novo* methylation in F9 cells.[24] Our hybrid retroviral vectors possess a Sp1 binding site in the SFFV-LTR and support, compared with conventional vectors, significantly improved transgene expression in ES cells. Thus it will be of interest in the future to assess whether these improvements are sufficient to promote sustained retroviral transgene expression upon *in vitro* differentiation and in transgenic animals.

Besides transcriptional control mechanisms, transgene expression is influenced by the leader sequence present in retroviral vectors.[13] Although it was reported that the MFG-leader sequence supported augmented expression in hematopoietic cells,[16] we observe that the MESV-leader sequence is superior in promoting high levels of transgene expression in hematopoietic progenitor cells (data not shown). It is conceivable that additional sequence alterations affecting the three-dimensional structure of the leader sequence and/or splicing efficiency render the MESV-leader sequence particularly efficient.

Furthermore, incorporation of RNA transport elements such as the WPRE in retroviral vectors can significantly improve transgene expression and the production of high titers. The insertion of the WPRE in the 3′ untranslated sequence of retroviral vectors resulted, independent of the retroviral vector used, in a five- to eight-fold increase in transgene expression.[14] In our hybrid retroviral vectors, the presence of the WPRE significantly enhanced transgene expression in hematopoietic cell lines, primitive HPC and ES cells. The effect of the WPRE was comparable upon localization between the reporter gene GFP and the puromycin resistance cassette (pOWS and pMOWS) or immediately upstream of the 3′LTR (pOWSdSV and pMOWSdSV) and therefore in proximity to the polyadenylation signal. However, the relative efficiency of the WPRE was dependent on the cell- and vector-context. With the exception of BaF3 cells, the presence of the WPRE enhanced transgene expression more efficiently in pOWS than in pMOWS. Although the WPRE is presumably not directly involved in splicing, a synergistic effect with splice signals was noted.[15] Therefore it is possible that the WPRE cooperates more efficiently with the MoMuLV-SD present in pOWS than with the MESV-SD contained in pMOWS. The reason for the higher efficiency of pMOWS in the proB cell line BaF3 is unknown, but it could be related to the observation that MFG is more rapidly inactivated in lymphoid,[6] whereas MESV-based retroviral vectors exhibit high activity in T- and B-lymphocytes.[12] We show for the first time that in ES cells insertion of the WPRE in a retroviral vector can, at least partially, compensate the negative regulatory effect of the NRE. In summary, the efficiency of retroviral vectors can be significantly improved by combining several elements which are particularly effective in primitive HPC and/or ES cells and act synergistically.

To generate high titer supernatants, stable producer cell lines are frequently used, but due to shortened time and greater flexibility transient transfection approaches are becoming increasingly important. The use of human 293T cell-derived retroviral packaging cell lines and retroviral pseudo-typing could potentially reduce the risk of generating replication competent viruses. Upon transient transfection in these packaging cell lines MFG-derived retroviral vectors, such as pMX are particularly efficient.[3] Our hybrid retroviral vectors pMOWS and pOWS that retain the MFG U3 region in the 5′LTR and harbor the WPRE are improved compared with pMX and reliably support the production of extremely high titers, eliminating the necessity for retroviral pseudotyping. For gene therapy trials the production of transducing supernatants under serum-free conditions is important to ensure standardization and biological safety.[25] Up to now stable retroviral producer cell lines have been used, however it was noted that serum withdrawal can compromise the viability of retroviral producer cell lines and poten-

tially reduce the efficiency of gene transfer.[26] Here we report that high-titer serum-free supernatants can be generated by a transient transfection approach that limits the time producer cells are grown in the absence of serum.

Recently, protocols for *ex vivo* expansion and transduction of primitive HPC have been greatly improved by using low or serum-free conditions and fibronectin as a cell/virus colocalizer.[26] In general, transduction rates obtained for human primitive HPC are difficult to compare since different sources and methods are being used for the preparation of the cells and their subsequent analysis. With MoMuLV-based vectors gene transfer rates of up to 50% were achieved for $CD34^+$.[25] For FMEV-type retroviral vectors, transduction efficiencies ranging from 8% to 21%[26–30] were reported for $CD34^+$ cells. However, rather low gene transfer rates in $CD34^+$ cells were obtained with supernatants generated by transient transfection of FMEV-type vectors in Phoenix-Ampho cells.[28,29] We reproducibly achieve in a transient transfection approach under serum-free conditions with our improved retroviral vector pOWS up to 66% transduced $CD34^+$ cells expressing high transgene levels, thus eliminating the need to enrich for transduced cells by potentially immunogenic selection markers.

For gene therapy approaches, efficient gene transfer and effective transgene expression at low multiplicity of infection (MOI) are important to lower the risk of insertional mutagenesis.[31,32] In a controlled model system applying a VSV-pseudotyped retroviral vector at defined MOI, Wahlers *et al*[31] showed that gene transfer rates reach saturation at an MOI >5 and transgene expression remains comparable at MOI <1. In analogy, our studies revealed that at low multiplicity of infection (MOI 1) the hybrid retroviral vectors pOS and in particular pOWS supported significantly elevated levels of transgene expression in murine HPC when compared with the MFG-based retroviral vector pMX. At a moderate multiplicity of infection (MOI 5), we achieved with the hybrid retroviral vector pOWS as much as 61% GFP-expressing primitive HPC and up to 90% GFP-positive erythroid progenitors. When we compared our improved vectors with MSCV, a retroviral vector that supported long-term expression in HSC,[7] we observed with our hybrid retroviral vectors significantly higher gene transfer rates and increased transgene expression in every cell type tested in this study (data not shown).

For the rational design of novel therapies, detailed knowledge of the molecular basis for human diseases is of advantage. Retroviral vectors are frequently used to stably introduce genes in model cell lines or more importantly in primary cells of murine disease models. Previously, gene transfer in murine hematopoietic progenitor cells required extensive infection times and selection of transduced cells before analysis.[33] However, with our improved vector, we achieved pOWS 89% and pMOWS 86% transduced murine erythroid progenitor cells within a 2-h incubation time, indicating that freshly prepared progenitor cells can be efficiently targeted before differentiation occurs. Therefore, gene transfer in primary cells of knockout mice and murine disease models should be greatly facilitated by the use of our hybrid retroviral vector pOWS and pMOWS.

In summary, the hybrid retroviral vectors we report here support rapid production of high titers in transient transfection approaches and mediate high transgene expression levels. In particular, the retroviral vector pOWS is very efficient for gene transfer in hematopoietic progenitor cells, whereas pMOWS is extremely efficient for gene expression in embryonic stem cells. Thus, in combination with improved transduction protocols, these vectors possess great potential for reconstitution of genes in the murine system and for gene therapy approaches.

Materials and methods

Construction of retroviral vectors

The design of the novel retroviral vectors was based on the retroviral vectors pMX[3] and pSFβ1.[5] The MoMuLV 3′LTR in pMX was first replaced with the MESV-LTR by introducing restriction sites for *Sal*I and *Eco*47III (5′-end) and for *Mlu*I and *Sap*I (3′-end) by PCR amplification using primers *MESV5LTR* (5′-*ATCGTCGACGAT AGCGCTGATAAAATAAAAGATTTTATTTAGTC*-3′) and *MESV3LTR* (5′-*AGGAAGCGGAAGAGCGCCACGCGTTT AATGCAGAATGAAAGACCCCCG*-3′) and pSFβ1 as a DNA template. The obtained PCR fragment was cloned into the *Sal*I and *Sap*I restriction sites in pMX. The MESV 3′LTR was replaced by the SFFV-LTR that was generated by PCR amplification from pSFβ1 using the primers *SFFV5* (5′-*GATAGCGCTCCTATCGATAGGCCTAAC*-3′) and *SFFV3* (5′-*GCCACGCGTTACTGAAAGACCCCC GAGG*-3′). The PCR fragment and vector were digested with the restriction enzymes *Eco*47III and *Mlu*I and ligated. The MESV-leader sequence was excised from pSFβ1with the restriction enzymes *Spe*I and *Sal*I and ligated to the *Spe*I and *Pml*I restriction sites and the resulting plasmid was named pOS. To construct a MFG/MESV hybrid LTR, the MFG-U3 region was fused to the MESV-R/U5 region and -leader sequence by overlap extension PCR. The first arm was generated by PCR amplification applying primers *MFG5* (5′-*AAGGGCGA-CACGGAAATGTTG*-3′) and *MFG3* (5′-*CGGAGGAC TGGCGCCCCGAGTGAGGGGT*-3′) and pMX as DNA template, while primers *MESV5* (5′-*ACCCCTCAC TCGGGGCGCCAGTCCTCCG*-3′) and *MESV3* (5′-GAC GGGCCCCCGCCAGATACAGAACTAGTTAGCCAAC TA-3′) and pSFβ1 as DNA template were used to amplify the second arm. The two PCR fragments were joined using the external primers *MFG5* and *MESV3*, and the resulting product was subcloned into pOS via the *Ssp*I and *Spe*I restriction sites to generate pMOS. The WPRE was amplified using the vector pCWT (kindly provided by Dr M Nassal, University Hospital, Freiburg, Germany) by PCR with primers *WPRE5* (5′-*GACGAATTCAAT-CAACCTCTGGA*-3′) and *WPRE3* (5′-*GACCTCGAG-GAATTCAATCAACCTCT*-3′) and subsequently cloned via the restriction sites *Eco*RI and *Xho*I into the retroviral vectors pOS and pMOS to generate pOWS and pMOWS. The SV40 origin/promoter-puromycin resistance gene (SV40-puro^R) cassette was excised from pOWS and pMOWS by digestion with the restriction enzymes *Sal*I and *Xho*I and the vector fragments was religated and the resulting plasmids were named pOWSdSV and pMOWSdSV. The enhanced green fluorescent protein (EGFP, Clontech, Palo Alto, CA, USA) has been modified by four amino acid exchanges (V163A, I167T, S175G, F223Y). The GFP cDNA was amplified using primer *GFP5bgl* (5′-*GACAGATCTAATGGTGAGCAAGGG*-3′) and *GFP3* (5′-*GCATCGAATTCTTAGGATCCCTTGTACAGC*

TCGT-3′) and the mutated GFP as a template. The resulting fragment was digested with *Bgl*II and *Eco*RI and ligated to pMX-derived retroviral vectors digested with *Bam*HI and *Eco*RI. GFP was inserted into pSFβ1 by ligation of the PCR amplified fragment using primers *GFP5not* (5′-*GACGCGGCCGCTGATCAACCATGGTGAGC AAGGG*-3′) and *GFP3* and the mutated GFP as DNA template. As a control vector in all transfection and transduction experiments, we have used the empty pMX vector without insertion of GFP.

Cell lines

BaF3 cells and 32D cells were cultured as described.[34] NIH3T3 and Phoenix cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS). All media were supplemented with penicillin (100 U/ml) and streptomycin (100 mg/ml). Transduced cell lines expressing the puromycin resistance gene were selected in the presence of 1.5 μg/ml puromycin (Sigma, St Louis, MO, USA). Embryonic stem cells B4[35] obtained from Dr Hong Wu (UCLA School of Medicine, Los Angeles, CA, USA) were cultured in DMEM knockout (GibcoBRL, Karlsruhe, Germany) with 15% FCS, sodium pyruvate, non-essential amino acids, L-glutamine, penicillin, streptomycin and 5 μM β-mercaptoethanol (all GibcoBRL) supplemented with 10^3 units/ml LIF (GibcoBRL).

Isolation of murine hematopoietic progenitor cells

For preparation of primary murine hematopoietic cells, fetal liver of 13.5-day-old embryos from Balb/c mice were prepared as described.[36] Fetal liver cells were depleted of cell surface marker expressing cells by a rat antibody cocktail (anti-Mac1, anti-Gr1, anti-Ter119, anti-B220, anti-CD4, anti-CD8, anti-CD14; all purchased from Pharmingen (San Diego, CA, USA) with exception of anti-Gr1 and anti-Ter119, which were kindly provided by Dr Albrecht Mueller (Julius-Maximilians-University, Dürzburg, Germany)) and subsequently sorted by AutoMACS with anti-rat MACS beads (Miltenyi Biotech, Bergisch Gladbach, Germany). Antibodies for characterization of lineage negative cells were anti-sca-1 coupled to Phycoerythrin (Pharmingen, San Diego, CA, USA) and anti-c-kit coupled to APC (Pharmingen). For analysis of expression in the erythroid lineage, fetal liver cells were either plated in 0.8% methylcellulose (Stem Cell, No. 03230; Vancouver, Canada) for colony assays or cultured in IMEM with 30% FCS and 50 μM β-mercaptoethanol for flow cytometry, both supplemented with 0.4 U/ml erythropoietin (Cilag-Jansen, Bad Homburg, Germany). The colonies were stained with benzidine (Sigma) to assess hemoglobinization. For expansion of murine hematopoietic progenitor cells, lineage-depleted cells were cultured in DMEM/10% FCS with 50 μM β-mercaptoethanol supplemented with 30 ng/ml Flt3L, 20 ng/ml SCF and 10 ng/ml IL-6 (all from R&D Systems, Minneapolis, MN, USA) for up to 14 days.

Purification and culture of human hematopoietic progenitor cells

Peripheral blood stem cell progenitor (PBSC) cells were enriched from leftover leukapheresis samples scheduled for discard according to procedures approved by the institutional review board of the Freiburg University Hospital. Mononuclear cells were obtained by Ficoll–Hypaque (Biochrom, Berlin, Germany) density centrifugation. $CD34^+$ cells were purified by positive selection using a MACS progenitor enrichment kit according to the manufactureŕs recommendations (Miltenyi Biotec). The purity of enriched cells and the phenotype of cultured cells were analyzed by a FACScalibur cytometer (Becton Dickinson, Heidelberg, Germany) using following monoclonal antibodies: CD34-PE, and CD34-PerCP (Becton Dickinson). Enriched $CD34^+$ cells were cultured in serum-free medium consisting of CellGro SCGM (CellGenix, Freiburg, Germany) supplemented with 20 ng/ml IL-3, 100 ng/ml SCF and 100 ng/ml Flt3L (CellGenix).

Transfection and determination of retrovirus titers

The retroviral vectors were transfected by calcium-phosphate precipitation into the Phoenix-Eco or Phoenix-Ampho packaging cell line as previously described.[34] Fourty-eight hours later, the supernatant was harvested and filtered through a 0.45-μm filter. To determine the titers generated, 1 ml of filtered transducing supernatant in various dilutions with 8 μg/ml polybrene (Sigma) was added to adherent NIH3T3 cells seeded at a density of 5×10^4 cells per well in six-well plates 24 h before the experiment. The plates were centrifuged for 3 h at 340 *g* at 20°C. The titer was calculated from the linear slope of a plot of the percentage of GFP-positive cells against vector dilution. Multiplicity of infection (MOI) was determined as the number of viral particles used divided by the number of cells in the transduction. When fixed MOI values are indicated, the vector supernatant used for transduction was re-titered on NIH3T3 cells to ensure accuracy of the MOI used. For all transductions, four different plasmid preparations were used and no significant difference among them was observed. Equal DNA concentrations of each plasmid preparation was determined in a spectral photometer and adjusted before each single transfection.

Transduction of cell lines and primary cells

Cells (5×10^5) (BaF3 or 32D) were incubated with filtered transducing supernatant and 12 μg/ml polybrene (Sigma) for 2 h in an Eppendorf centrifuge spun at 340 *g* at 20°C. Fetal liver cells (1×10^5 cells) were transduced in 96-well plates in a total volume of 150 μl supernatant by centrifugation at 32°C and 340 *g* for 2 h in the presence of 12 μg/ml polybrene. For transduction of human $CD34^+$ cells, 5×10^4 cells were pre-stimulated with 20 ng/ml IL-3, 100 ng/ml Flt3L and 100 ng/ml SCF for 2 days. Transiently transfected Phoenix-Ampho cells were washed and medium was changed to CellGro SCGM 12 h before transduction. Serum-free supernatant was diluted 1:2 with medium, added to $CD34^+$ cells and centrifuged for 3 h at 900 *g* at 25°C in the presence of 4 μg/ml Polybrene. The transduction cycle was repeated on 3 consecutive days. The cells were analyzed for GFP fluorescence using flow cytometry (FACScan, Becton-Dickinson). For double fluorescence, the cells were incubated with antibodies against CD34 conjugated with PerCP (Becton-Dickinson). For triple fluorescence of murine hematopoietic progenitor cells, the cells were incubated with antibodies against sca-1 coupled to phycoerythrin (PE) and against c-kit coupled to APC. Compensation for GFP, PE and APC was performed with control cells incubated with single antibodies. Embryonic stem cells were seeded on 0.1% gelatine (Sigma) precoated 24-well plates 24 h before transduction at a density

of 10^4 cells/well. Vector supernatant was added and the plates were centrifuged for 2 h at 32°C and 360 *g*. Vector supernatant was replaced by ES cell medium and cells were analyzed by flow cytometry after 5 days.

Statistical analysis

The mean and standard deviations were calculated from three independent transductions. Significance's were calculated with a two-sided paired Student's *t* test.

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