

# Murine Retroviral but not Human Cellular Promoters Induce *In Vivo* Erythroid-specific Deregulation that can be Partially Prevented by Insulators

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We are developing lentiviral vectors for gene therapy of red blood cell disorders that co-express a transgene in an erythroid-specific manner and the *O*<sup>6</sup>-methylguanine-DNA-methyltransferase (MGMT) selective gene in a constitutive way. We report that transduction of murine hematopoietic stem cells (HSCs) with a human phosphoglycerate kinase promoter-based vector at low multiplicity of infection (MOI) does not result in a selective *in vivo* expansion in the presence of alkylating agents. In contrast, by replacing this cellular promoter with the powerful retroviral-derived myeloproliferative sarcoma virus enhancer, negative control region-deleted, *dl587rev* primer-binding site substituted promoter, the vector allowed efficient chemoprotection of transduced HSCs at low MOI. However, this promoter interacted with the erythroid HS40/ankyrin enhancer/promoter driving green fluorescent protein, leading to an unexpected loss of erythroid specificity. A partial restoration of tissue-specific expression was obtained by interposition of insulator sequences between the expression units. Alternatively, we found that the strong human cellular elongation factor1- $\alpha$  promoter allows similar chemoprotection but without any deregulation of the erythroid-specific promoter in the absence of insulators. These data demonstrate that the level of *in vivo* deregulation induced by a promoter is not correlated with its transcriptional activity.

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## INTRODUCTION

Hematopoietic stem cell (HSC) gene therapy is rapidly becoming a highly effective approach for inherited diseases in which transgene expression confers a strong selective advantage.<sup>1-3</sup>

However, for most blood disorders, there is no selective advantage of genetically corrected HSCs and therefore the full correction of the disease phenotype remains a challenge. The main constraint limiting successful gene therapy protocols is the relatively low number of corrected cells compared to deficient resident bone marrow cells (BM cells) in the absence of myeloablation. One potential way to overcome this major constraint is the use of vectors that allow the transduced cells to undergo selection *in vivo*. The most promising selective strategy for *in vivo* selection of HSCs is based on the human *O*<sup>6</sup>-methylguanine-DNA-methyltransferase (MGMT) gene. This gene encodes the MGMT enzyme, which can protect cells by removing alkyl adducts from the *O*<sup>6</sup> position of guanine before reactions leading to irreversible cell death occur.<sup>4,5</sup> Several MGMT mutants have been developed in which specific amino-acid substitutions confer resistance to inactivation by *O*<sup>6</sup>-benzylguanine (BG).<sup>6-8</sup> Selection and expansion of transduced HSCs have now been achieved for the first time using xenografted<sup>9</sup> or large animal models.<sup>10</sup> The aim of this project is the development of lentiviral vectors that co-express a therapeutic gene in an erythroid-specific manner and the MGMT selective gene in a constitutive way for *in vivo* selection of HSCs. The erythroid-specific enhancer/promoter (ESp) based on the chimeric HS40-enhancer and ankyrin promoter has been developed by us and found to be highly erythroid-specific.<sup>11,12</sup> We have also demonstrated that long-term gene expression limited to erythroid progeny was possible *in vivo* with this ESp in the area of the constitutive human phosphoglycerate kinase promoter (PGKp) driving MGMT gene without loss of specificity.<sup>13</sup> However, the transductions were performed at high multiplicity of infection (MOI) resulting in several transgene integrations in HSCs, elevating the risk of proto-oncogene activation from random vector insertions.<sup>14</sup> The incorporation of insulator sequences in gene therapy vector is a promising strategy to prevent this risk of proto-oncogene activation.

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Indeed, one of the major functional properties of chromatin insulator sequence is its ability to interfere with enhancer-promoter communication when placed between them. The best characterized of these insulators is the chicken  $\beta$ -globin 5'HS4 insulator (cHS4).<sup>15</sup> The enhancer blocking activity of cHS4 is contained in a 250 bp fragment, the *core*, and is associated with binding of the zinc-finger CTCF protein (CCCTC-binding factor).<sup>16</sup> To confer an efficient *in vivo* chemoprotection at one or two copies per cell, the vector needs to contain strong ES elements. In this present study, we replaced the PGKp with the powerful murine leukemia virus-derived MND promoter (myeloproliferative sarcoma virus enhancer, negative control region deleted, *dI587rev* primer-binding site substituted).<sup>17</sup> This vector allowed an efficient chemoprotection of transduced HSCs at low MOI. However, this retroviral promoter interacts with the erythroid one, leading to a loss of erythroid-specificity, probably via a transactivation mechanism. To block this enhancer activity, the full-length cHS4 insulator sequence or its minimal *core* was inserted between the two transgenes. The restoration of erythroid-specific expression as well as chemoprotection against alkylating agents were investigated *in vivo*. The first vectors developed in this study combine a high efficiency of chemoprotection afforded by the MNDp even at one copy per cell with a decrease in promoter deregulation owing to insulator properties. Alternatively, we evaluated the efficiency of the powerful human cellular elongation factor (EF)1- $\alpha$  promoter as compared to the MNDp: we found it was capable of similar high chemoprotection level but interestingly, without any deregulation of the erythroid-specific promoter. These data demonstrate that the level of *in vivo* deregulation induced by a promoter is not correlated with its transcriptional activity but possibly with the presence of its strong enhancer elements. Our study provides critical information for risk assessment in the development of viral gene therapies.

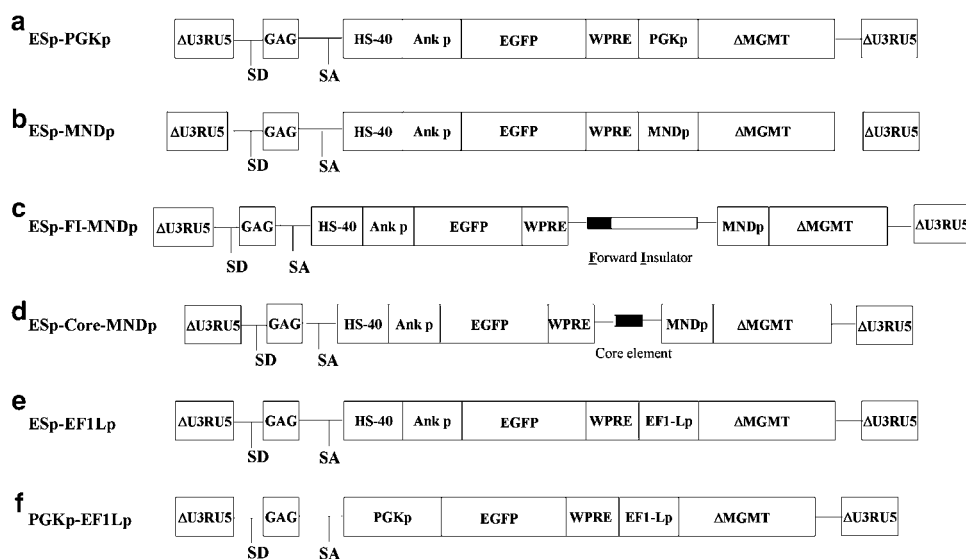
## RESULTS

### Development of lentiviral vectors with insulators for transduction of murine HSCs

The Erythroid Specific ES<sub>p</sub>-PGKp control vector (**Figure 1a**) contains two units of expression: an ES<sub>p</sub> driving the expression of the enhanced green fluorescent protein (EGFP) and an ubiquitous human PGKp driving the expression of the  $\Delta$ MGMT gene. To improve the efficiency of *in vivo* selection, we designed the ES<sub>p</sub>-MNDp vector (**Figure 1b**) in which the PGKp was replaced with the strong long terminal repeat (LTR)-derived MNDp. To block the enhancer activity observed in preliminary experiments with this promoter, we interposed between the two expression units the 1.2 kb fragment of cHS4 in forward orientation (ES<sub>p</sub>-FI-MNDp, **Figure 1c**) or its minimal 250 bp *core* (ES<sub>p</sub>-Core-MNDp, **Figure 1d**). VSV-G-pseudotyped lentiviral vectors were produced by transient transfection of 293T cells and titers were determined on K562 cells. Titers from supernatant before ultracentrifugation were equal to  $1.4 \pm 0.8 \times 10^6$  and  $2.2 \pm 0.2 \times 10^6$  transducing units (TU)/ml ( $n = 3$ ; mean  $\pm$  SD) for the ES<sub>p</sub>-PGKp and ES<sub>p</sub>-MNDp vectors, respectively. The insertion of insulators resulted in a moderate, but significant drop of titers, ( $0.8 \pm 0.1 \times 10^6$  and  $1.1 \pm 0.3 \times 10^6$  TU/ml for cHS4 and *core*, respectively, vs  $2.2 \pm 0.2 \times 10^6$  for ES<sub>p</sub>-MNDp,  $P < 0.05$ ). Reduction in vector titers roughly correlated with the size of the inserts.<sup>18</sup>

### *In vivo* chemoprotection of transduced cells

To analyze the ability of the vectors to selectively enrich for transduced cells, *in vivo* experiments were performed on purified 5FU/Sca1<sup>+</sup> cells from BM cells of BALB/c donor mice. Transduced 5FU/Sca1<sup>+</sup> cells were injected into myeloablated recipient mice. Mice were randomly divided into BG/BCNU-treated and untreated groups. Red blood cells (RBCs) were monitored for EGFP expression over time after bone marrow



**Figure 1** Schematic representation of the different bipromoter self-inactivating lentiviral vectors shown in the proviral form. EGFP gene is expressed under the control of the Erythroid-Specific HS40/ankyrin ES<sub>p</sub> and the  $\Delta$ MGMT cDNA under the control of the (a) ubiquitous human PGKp or (b-d) under the ubiquitous LTR-modified MND promoter. The 1.2 kb 5'-HS4 chromatin Insulator from the chicken  $\beta$ -globin locus is inserted in c Forward orientation between the two transgenes. Construct D contains the 0.2 kb Core element including the insulator activity. (e and f) Schematic representation of the EF1 $\alpha$ -based vectors, ES<sub>p</sub>-EF1Lp driving  $\Delta$ MGMT cDNA with EGFP under the control of the e ES<sub>p</sub> or the f constitutive PGKp.

transplantation (BMT). Mice were killed 16 weeks after BMT for fluorescence-activated cell sorting (FACS) analyses. Southern blots showed stable integration of unrearranged proviral genomes with expected band size for all vectors (not shown). Transduction of HSCs was performed at low MOI to obtain a low proviral copy number. FACS analyses of EGFP expression in RBCs demonstrated that the amplification of transduced cells with the ES<sub>p</sub>-PGKp vector was not efficient (Figure 2). Only one of 12 treated mice responded to the treatment (86.7% EGFP<sup>+</sup> RBCs), explaining the low mean percentage of EGFP<sup>+</sup> RBCs observed after 16 weeks ( $21.9 \pm 23.6\%$ ,  $n = 12$ ). In contrast, a strong selective growth advantage of transduced EGFP<sup>+</sup> cells was observed with the vector ES<sub>p</sub>-MNDp after each drug injection. The selection efficiency led to  $75.1 \pm 8.6\%$  vs  $34.8 \pm 11\%$  EGFP<sup>+</sup> RBCs for treated ( $n = 3$ ) vs untreated group ( $n = 3$ ), respectively. A similar high enrichment was observed with the two MNDp-based vectors with insulators ( $67.1 \pm 13.1\%$  and  $94.2 \pm 1.6\%$  EGFP<sup>+</sup> RBCs from the treated group vs  $9.0 \pm 1.6\%$  and  $1.8 \pm 0.7\%$  EGFP<sup>+</sup> RBCs from the untreated group for ES<sub>p</sub>-FI-MNDp and ES<sub>p</sub>-Core-MNDp vectors, respectively). These data demonstrate that none of the insulators disturb the MNDp activity. As expected by the low MOI used, BM proviral copy number/cell determined by Southern blot analyses was low ( $1.6 \pm 0.4$ ;  $n = 10$  for mice treated with BG/BCNU). The only exception is the BG/BCNU-responding mouse grafted with the ES<sub>p</sub>-PGKp-transduced BM (3.7 copies/cell). This result indicates that the PGKp requires at least three copies/cell for chemoprotection to be efficient. In contrast, a highly efficient selective amplification of transduced cells at low copy number (1 or 2) was obtained with all MNDp-based vectors.

### Insulators partially prevent deregulation of the erythroid-specific promoter in murine cells

To determine whether insulator elements can prevent promoter deregulation, analyses of EGFP expression were performed in different BM lineages from efficiently selected mice 16 weeks after BMT. Analyses were performed on white blood cells from the BG/BCNU-treated groups of mice containing at least 80% of EGFP<sup>+</sup> RBCs. BM cells were analyzed for EGFP expression in erythroid cells (Ter-119<sup>+</sup>), granulocytes (Gr-1<sup>+</sup>), myeloid (CD11b<sup>+</sup>), and B cells (B220<sup>+</sup>) (Figure 3a). Thymic cells were analyzed for EGFP expression in T cells (CD3<sup>+</sup>). Two parameters were chosen to characterize this deregulation: the percentage of EGFP<sup>+</sup> cells (Figure 3a) and the mean fluorescence intensity (MFI) of EGFP expression in non-erythroid cell lineages (Figure 3b).

For the ES<sub>p</sub>-PGKp vector, EGFP expression was restricted to erythroid BM cells for the only selected mouse (86.7% EGFP<sup>+</sup> Ter119<sup>+</sup> cells vs 1.1%, 1.7%, 1.2%, and 0.1% EGFP<sup>+</sup> Gr-1<sup>+</sup>, CD11b<sup>+</sup>, CD3<sup>+</sup>, and B220<sup>+</sup> cells, respectively,  $P < 0.005$ ), but also for the non-responding mice ( $6.4 \pm 2.9\%$  EGFP<sup>+</sup> Ter119<sup>+</sup> cells vs  $1.2 \pm 0.5\%$ ,  $1.4 \pm 0.3\%$ ,  $1.9 \pm 0.6\%$ ,  $1.5 \pm 0.4\%$  EGFP<sup>+</sup> Gr-1<sup>+</sup>, CD11b<sup>+</sup>, CD3<sup>+</sup>, and B220<sup>+</sup> cells, respectively,  $P < 0.05$ ). The ES<sub>p</sub>-MNDp vector allowed a complete *in vivo* selection ( $90 \pm 3.6\%$  EGFP<sup>+</sup> Ter119<sup>+</sup> cells). However, the percentage of EGFP<sup>+</sup> cells was similar in erythroid and non-erythroid lineages, demonstrating a loss of tissue specificity ( $88.6 \pm 8.1\%$ ,  $86.6 \pm 8.1\%$ ,  $66.4 \pm 33.8\%$  EGFP<sup>+</sup> GR1<sup>+</sup>, CD11b<sup>+</sup>, and CD3<sup>+</sup> cells, respectively). Interestingly, in B cells (B220<sup>+</sup>), no *in vivo* ES<sub>p</sub> deregulation was observed (Figure 3a).

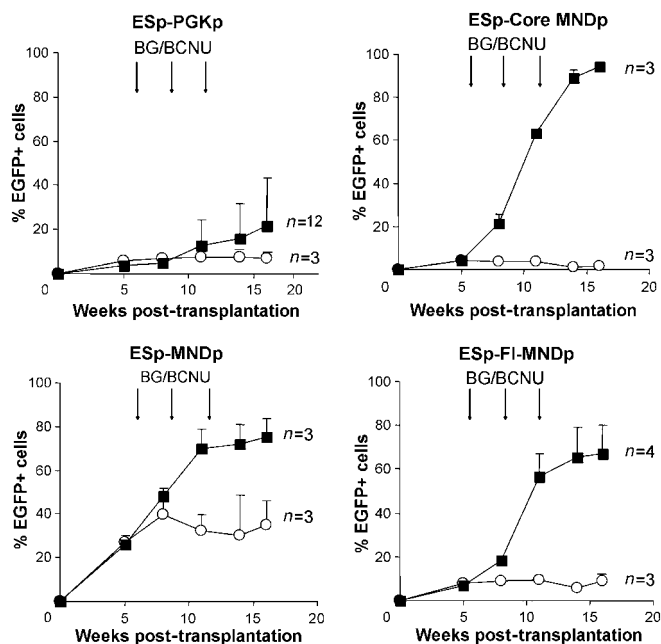


Figure 2 *In vivo* expansion of EGFP<sup>+</sup> peripheral RBCs after drug exposure. Percentages of EGFP<sup>+</sup> RBCs were monitored over time by FACS. The mice were randomly assigned to two different groups: drug-exposed (■) and non-exposed groups (○). Mice were injected intraperitoneally with 30 mg/kg BG followed by 5 mg/kg BCNU three times 1 month apart as indicated by black arrowheads. Values represent mean  $\pm$  SD.  $n$  = Number of mice per group.

The addition of cHS4 to the vector in forward orientation resulted in a 2-fold reduction of EGFP expression in granulocytes and myeloid BM cells ( $83.2 \pm 5.6\%$  EGFP<sup>+</sup> Ter-119<sup>+</sup> cells vs  $33.8 \pm 15.4\%$ ,  $30.1 \pm 14.8\%$  and  $53.2 \pm 11.7\%$  EGFP<sup>+</sup> Gr1<sup>+</sup>, CD11b<sup>+</sup>, and CD3<sup>+</sup> cells, respectively). In contrast, the insertion of cHS4 insulator in reverse orientation did not allow a significant reduction of EGFP<sup>+</sup> cells in granulocytes and myeloid lineages demonstrating that “insulator” sequences do not simply act as spacers (data not shown). When the core replaced cHS4 in this vector, the percentage of EGFP<sup>+</sup> cells detected in Gr-1<sup>+</sup> and CD11b<sup>+</sup> populations was reduced 2-fold and 4-fold, respectively ( $89.1\% \pm 3.3\%$  EGFP<sup>+</sup> Ter-119<sup>+</sup> cells vs  $27.4 \pm 14.8\%$  and  $17.5 \pm 6.5\%$  EGFP<sup>+</sup> Gr1<sup>+</sup>, and CD11b<sup>+</sup>, respectively).

To accurately quantify the ES<sub>p</sub> deregulation, the second parameter studied was the MFI of EGFP expression in erythroid and non-erythroid cells (Figure 3b). As expected, ES<sub>p</sub>-PGKp-transduced BM cells showed a high EGFP expression level in erythroid Ter-119<sup>+</sup> cells (MFI = 100.9) compared with the level in non-erythroid cells (MFI = 3.4, 3.8 and 5.1 for GR1<sup>+</sup>, CD11b<sup>+</sup>, and CD3<sup>+</sup> cells, respectively). Erythroid transduced cells express EGFP at a similar level for all ES<sub>p</sub>-MNDp vectors (MFI =  $82.6 \pm 23.6$ ;  $126.3 \pm 28.5$ ;  $113.4 \pm 12.4$  for ES<sub>p</sub>-MNDp, ES<sub>p</sub>-FI-MNDp and ES<sub>p</sub>-Core-MNDp, respectively). However, EGFP expression was also observed in non-erythroid cells for ES<sub>p</sub>-MNDp vector (MFI =  $46.2 \pm 7.3$ ,  $50.8 \pm 8.0$  and  $33.8 \pm 4.1$  for Gr-1<sup>+</sup>, CD11b<sup>+</sup> and CD3<sup>+</sup> cells, respectively) confirming the loss of tissue specificity with the MND promoter. The comparison of MFI of EGFP between erythroid and non-erythroid cells showed that this deregulation is important as the ES<sub>p</sub>-MNDp vector exhibits about half of RBC EGFP expression level in non-erythroid cells. Inclusion of cHS4 insulator resulted in a 2-fold drop in the level of EGFP expression in non-erythroid lineages (MFI =  $21.4 \pm 7.3$ ,  $20.1 \pm 7.9$  and  $14.2 \pm 0.8$  for GR1<sup>+</sup>, CD11b<sup>+</sup> and CD3<sup>+</sup> cells, respectively,  $n = 4$ ,  $P = 0.04$ ,  $0.05$ , and  $0.01$  vs ES<sub>p</sub>-MNDp corresponding MFI). The ES<sub>p</sub>-Core-MNDp vector led to the highest decrease of EGFP expression level as the level of EGFP expression was 5-fold lower in GR1<sup>+</sup> cells (MFI =  $9.3 \pm 4.6$ ,  $n = 3$ ,  $P = 0.01$ ), 6-fold lower in CD11b<sup>+</sup> cells (MFI =  $8.5 \pm 4.2$ ,  $n = 3$ ,  $P = 0.01$ ), and 2-fold lower in CD3<sup>+</sup> cells (MFI =  $18.5 \pm 3.9$ ,  $n = 3$ ,  $P = 0.05$ ). However, the difference observed between cHS4 and core element-based vectors was not statistically significant.

### EF1 $\alpha$ Lp allows efficient *in vivo* chemoprotection of transduced cells at low-copy number without any promoter deregulation

As the promoter deregulation could not be completely prevented by insulators, particularly, in T-cell lineage, we decided to search for a powerful cellular promoter that would allow an efficient *in vivo* selection of transduced cells at low copy number, but with a lower deregulation activity of ES<sub>p</sub> than that observed with the MNDp. Four EGFP-expressing lentiviral vectors were constructed to compare the transcriptional activities from PGKp, MNDp and a Short and Long version of EF1 $\alpha$  promoter (EF1Sp without intron I and EF1Lp with intron I, respectively) (Figure 4a). Transduction was performed at low-copy number in the

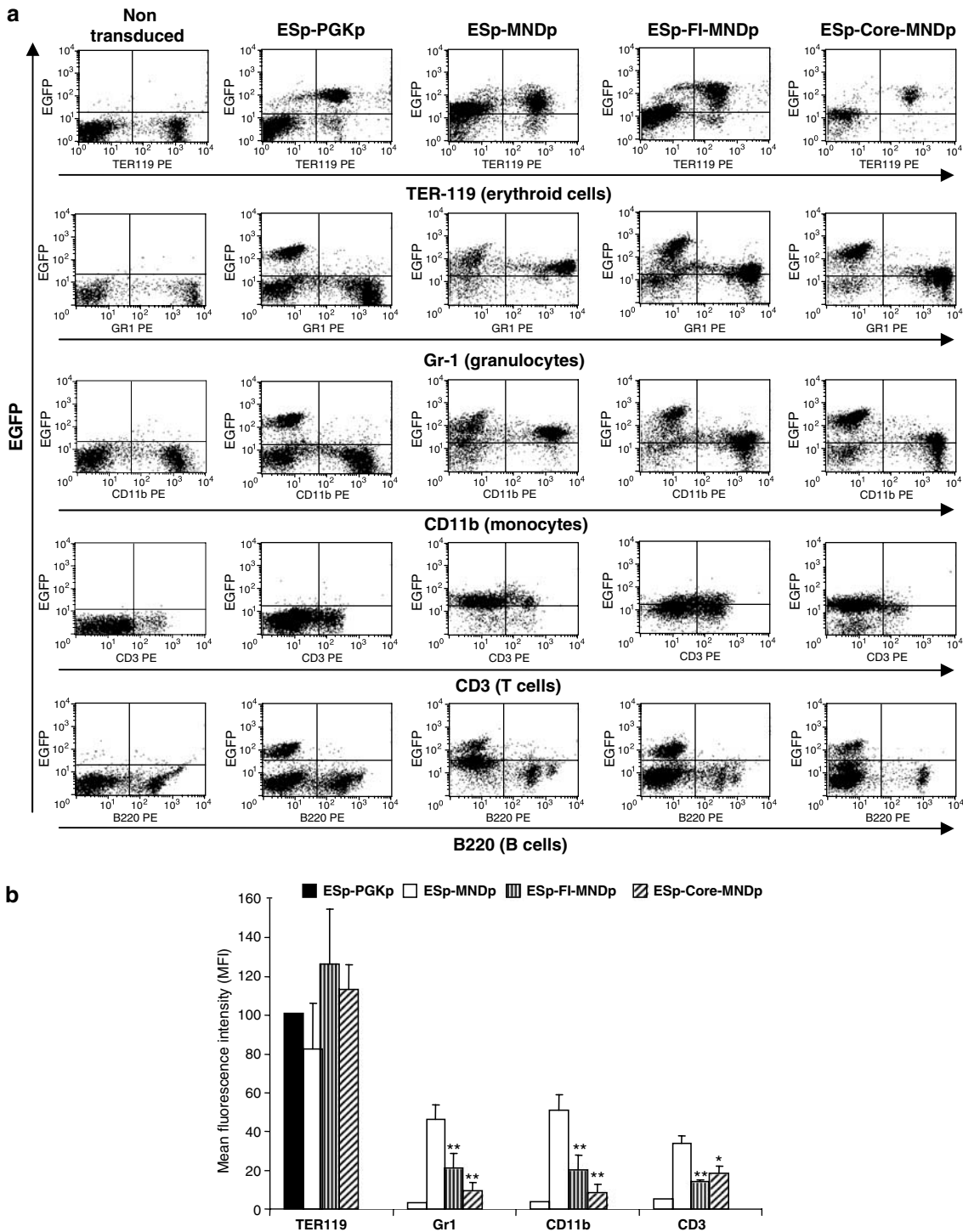
human K562 cells, primary human CD34<sup>+</sup> and murine Sca-1<sup>+</sup> cells, and the MFI of EGFP cells was analyzed 7 days after transduction. The PGKp and EF1Sp had a lower activity in all cell types than MNDp and EF1Lp (Figure 4b). Interestingly, the highest activity was obtained with the intron-containing EF1Lp: the MFI of EGFP was 1.5- to 3-fold higher compared with EF1Sp in primary human and murine hematopoietic cells, respectively). These data demonstrate the importance of the intron in EF1Lp to improve gene expression in HSCs. (Figure 4b). Therefore, we chose the EF1 $\alpha$ Lp to replace the MNDp in our bipromoter vectors in order to get the ES<sub>p</sub>-EF1Lp vector (Figure 1e).

Then, we evaluated the efficiency of this cellular promoter to obtain a potent amplification of the transduced cells (Figure 5a). As suggested by the *in vitro* experiments, this promoter allowed a strong selective growth advantage of transduced EGFP<sup>+</sup> cells after each drug injection. The selection efficiency led to  $86.0 \pm 5.0\%$  EGFP<sup>+</sup> RBCs for treated vs  $34.1 \pm 2.5\%$  EGFP<sup>+</sup> RBCs for untreated groups of mice. To evaluate if the ES<sub>p</sub> deregulation occurred with this EF1 $\alpha$ Lp, analyses of EGFP expression were performed in different BM lineages from selected mice (Figure 5b). BM cells were analyzed for EGFP expression in erythroid cells, granulocytes, monocytes, and lymphoid cells. As expected, the ES<sub>p</sub>-EF1Lp vector allowed a complete *in vivo* selection in erythroid BM cells ( $89.1\% \pm 2.0\%$  EGFP<sup>+</sup> Ter119<sup>+</sup> cells,  $n = 3$ ). Interestingly, no loss of erythroid specificity was observed in non-erythroid lineages ( $1.0 \pm 0.2$  EGFP<sup>+</sup> GR1<sup>+</sup> cells,  $1.0 \pm 0.3$  EGFP<sup>+</sup> CD11b<sup>+</sup> cells, and  $1.1 \pm 0.3$  EGFP<sup>+</sup> B220<sup>+</sup> cells).

### The erythroid-specific promoter deregulation in human hematopoietic cells

Finally, we investigated the tissue specificity of these vectors in human cell lines and primary human hematopoietic cells. Erythroblastic K562 and non-erythroid cell lines (T-lymphoblastic Jurkat and myelo-monoblastic HL-60) as well as primary cord blood cells were transduced with constitutive control vector (PGKp-EF1Lp) (Figure 1f), the ES<sub>p</sub>-MNDp, or the ES<sub>p</sub>-EF1Lp vectors. The transductions were performed for each cell line at an equal MOI for all vectors corresponding to a transduction rate of the constitutive control vector ranging from 20 to 30%. This low and similar MOI allowed comparison of the EGFP level of transduced cells at approximately one copy per cell. In all three cell lines, the PGKp-EF1Lp control vector (Figure 6a) allowed a constitutive expression. The ES<sub>p</sub>-MNDp vector led to a loss of tissue specificity in all the cell lines ( $22.8 \pm 2.7\%$  EGFP<sup>+</sup> K562 vs  $14.6 \pm 6.7\%$  and  $13.3 \pm 2.4\%$ , EGFP<sup>+</sup> Jurkat, and HL60 cells, respectively). In contrast, once again, the EF1 $\alpha$ Lp does not deregulate the erythroid-specific expression of EGFP ( $18.7 \pm 0.9$  vs  $0.8 \pm 0.3\%$  and  $3.6 \pm 0.6\%$  EGFP<sup>+</sup> K562 vs Jurkat and HL60 cells, respectively).

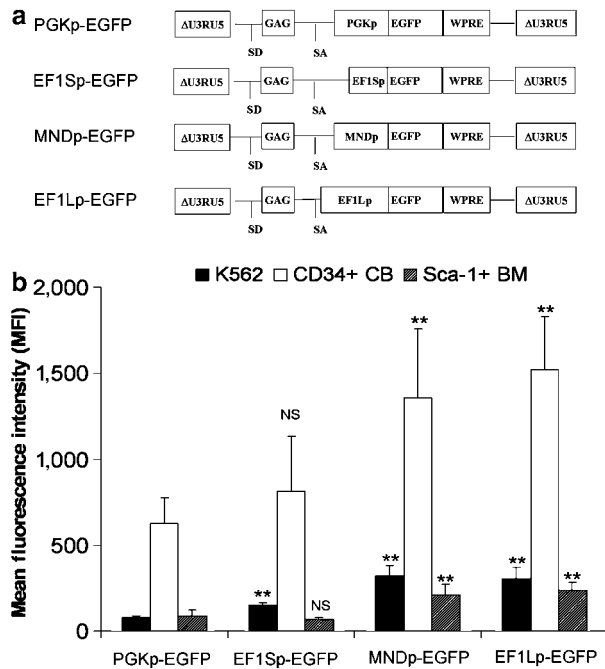
EGFP expression was analyzed in the differentiated progeny of transduced cord blood cells grown in liquid culture conditions that allow erythroid, myeloid differentiation, or T-cell expansion (Figure 6b). Consistent with cell line experiments, EGFP expression was restricted to the erythroid cells with the ES<sub>p</sub>-EF1Lp vector ( $19.5 \pm 2.5\%$  EGFP<sup>+</sup> GPA<sup>+</sup> cells vs  $1.8 \pm 0.8$  CD33<sup>+</sup> EGFP<sup>+</sup> cells and  $0.8 \pm 0.3$  CD3<sup>+</sup> EGFP<sup>+</sup> cells). In



**Figure 3** FACS analyses of EGFP expression in different BM hematopoietic cell lineages and T cells from the thymus. **(a)** FACS analyses were performed 16 weeks after BMT of purified HSC without transduction or transduced with the four vectors described in **Figure 1**. Results from one representative mouse are shown. Thymus and BM cells were harvested and stained with different antibodies: Ter-119 (erythrocytes), Gr-1 (granulocytes), CD11b (monocytes), B220 (B lymphocytes), and CD3 (T cells from thymus) to evaluate the specificity of EGFP expression. **(b)** MFI of EGFP expression in different lineages (mean  $\pm$  SD  $n = 3-5$  mice) for the ES<sub>p</sub>-PGKp vector, selection occurs only for one mouse. \* $P < 0.05$  and \*\* $P < 0.01$  vs ES<sub>p</sub>-MNDp by the Student's *t*-test.

contrast, the MNDp-based vector induces a strong ES<sub>p</sub> deregulation in human primary T-lymphoid cells ( $14.2 \pm 1.2$  CD3<sup>+</sup> EGFP<sup>+</sup> cells). However, only a mild deregulation in

human myeloid CD34<sup>+</sup>-derived cells was observed with this vector ( $2.9 \pm 1.9\%$  EGFP<sup>+</sup> CD33<sup>+</sup> cells vs  $21.7 \pm 6.2\%$  EGFP<sup>+</sup> GPA<sup>+</sup> cells).



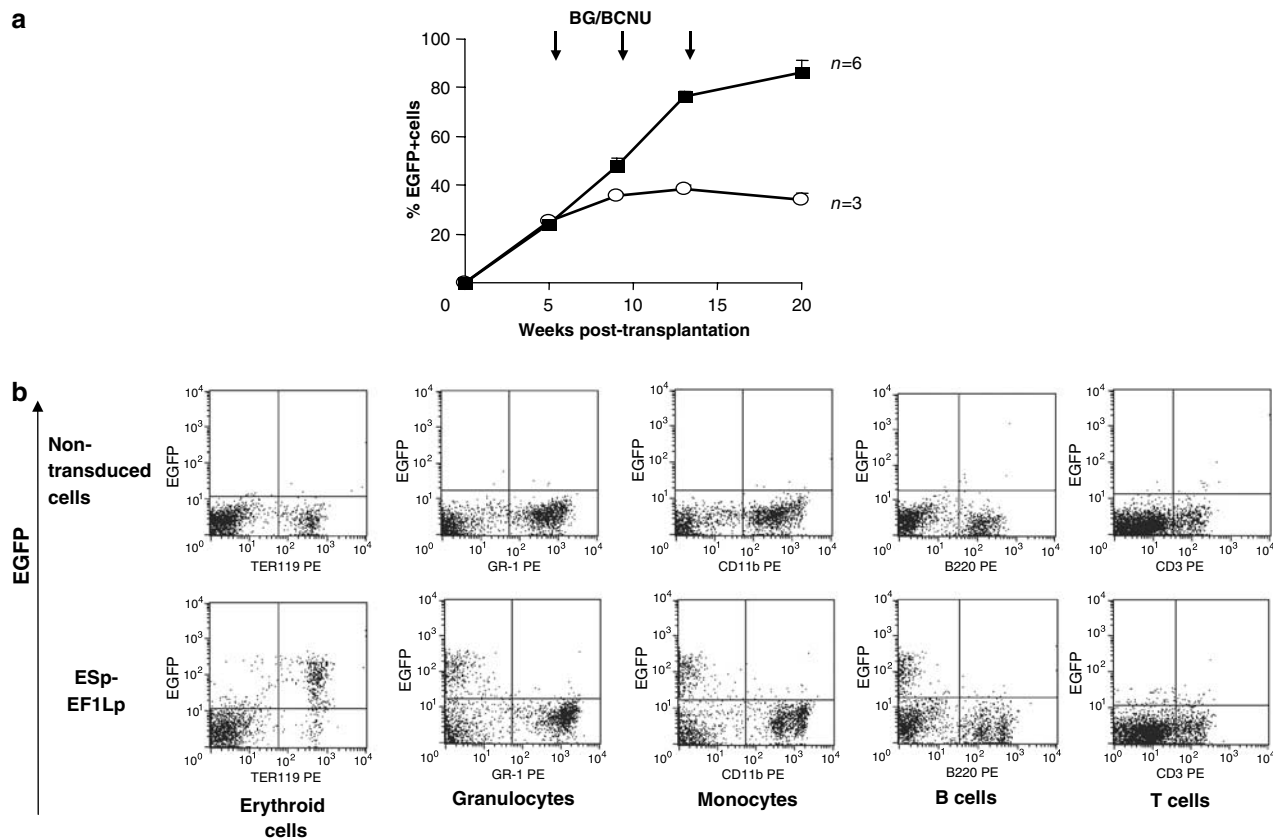
**Figure 4** Comparison of the transcriptional activities measured by EGFP expression from the different PGK, MND, EF1S, and EF1L promoters. **(a)** Schematic representation of self-inactivating lentivectors shown in the proviral form. **(b)** MFI of EGFP 7 days after transduction from the four vectors at low-copy number in the human K562 cell line, human primary CD34<sup>+</sup> cells, and murine Sca-1<sup>+</sup> BM cells (mean  $\pm$  SD of three independent experiments). \*\* $P < 0.01$  vs PGKp-EGFP for each type of cells, NS not significant by the Student's *t*-test.

## DISCUSSION

Effective gene therapy for RBC disorders is currently hampered by low rates of human HSC transduction and the absence of selective advantage for genetically corrected cells. To overcome these problems, we designed efficient bipromoter lentiviral vectors co-expressing *MGMT* constitutively and a therapeutic or marker gene in an erythroid-specific manner. Indeed, to date, the *MGMT* selection system is the most powerful tool for *in vivo* selection of transduced HSCs and is a promising approach for gene therapy of hematopoietic disorders.<sup>13,19,20</sup> However, relatively high levels of *MGMT* expression are required for cellular detoxification, because *MGMT* enzyme becomes irreversibly inactivated and degraded after transfer of the alkyl group from DNA to its active site (the so-called “suicide reaction”).<sup>21</sup> To overcome this limitation, a high *MGMT* expression level can be obtained by the use of high MOI leading in turn to a high vector copy number, directly correlated with transgene expression.<sup>22</sup> However, recent publications have shown that the accumulation of several transgene insertions increases the risk of transformation owing to combinatorial insertional mutagenesis of proto-oncogenes or other signaling genes.<sup>23</sup> Thus, for biosafety reason, the vector should be potent enough to mediate BM chemoprotection and stem cell selection, when introduced at only one or two copies per cell. In contrast with the weak human PGKp, we found that the powerful LTR-derived MNDp allowed efficient chemoprotection of transduced HSCs at low MOI. This LTR-derived promoter has been previously described as a very

strong constitutive promoter for transgene expression in murine and human hematopoietic cells.<sup>13,17</sup> Our data are consistent with a recent report showing that the internal PGKp, driving *MGMT* complementary DNA (cDNA) in a lentiviral vector, did not provide a significant growth advantage of transduced murine BM cells in an *in vitro* assay, in contrast with the use of a strong LTR-derived ES<sub>p</sub> derived from the spleen focus-forming virus.<sup>24</sup> In addition, we found that the MNDp, another powerful murine leukemia virus-derived ES<sub>p</sub>, interacted with the ES<sub>p</sub> driving EGFP, leading to a loss of erythroid specificity. We used the *G156A-MGMT* mutant in this study. Several other *MGMT* mutants have been developed that vary in stability, level of O<sup>6</sup>-benzylguanine resistance, and repair capacity.<sup>25</sup> In consequence, a mutant with a higher activity may allow the use of a weaker promoter.

Insulators are DNA sequence elements that can act either to block the extension of a condensed chromatin domain into a transcriptionally active region (barrier activity), or to prevent the interaction of a distal enhancer with a promoter when placed between them.<sup>26</sup> The efficiency of insulator elements at protecting transgene expression from the negative influence of chromosomal sequences flanking the site of integration has been studied previously both *in vitro* and *in vivo*.<sup>26,27</sup> In particular, several studies have demonstrated that incorporation of insulator elements into retroviral and lentiviral gene transfer vectors resulted in regulated, position-independent transgene expression.<sup>27,28</sup> In contrast, the latter property, called enhancer-blocking insulation, is experimentally more difficult to evaluate *in vivo* and has not been studied so extensively. A previous study proved the potential of the *cHS4* insulator to improve inducible or tissue-specific gene expression from adenovirus vectors.<sup>29</sup> Martin-Duque *et al.*<sup>30</sup> have also recently reported an interesting study using this insulator in an adenoviral vector containing two different expression cassettes. However, this *in vitro* study was performed with a bipromoter vector in which the strong constitutive promoter was located upstream of the deregulated erythroid-specific promoter, and in this model, a transcriptional fusion between the two promoters cannot be excluded.<sup>30</sup> In our study, a partial restoration of tissue-specific expression was obtained by the insertion of insulator sequences between the two expression units. To minimize the space devoted to insulation, we used the 250-bp *core* of the 1.2-kb HS4  $\beta$ -globin insulator.<sup>31</sup> This *core* contains the binding site of the CTCF, a protein that also interacts with the nuclear matrix.<sup>31,32</sup> Thus, the functional model proposed is that the insulator serves to generate loop domains that isolate enhancer and promoter in separate loops.<sup>31</sup> In our *in vivo* model, we have found that one copy of this *core* was enough to shield the erythroid transcription unit from the activating effects of the nearby LTR promoter and to give insulating activity at least similar to the entire 1.2-kb fragment. Previous studies have shown that the insulating activity of the *core* is multiplied when tandem copies are used.<sup>15</sup> Unfortunately, the insertion of a tandem repeat of this *core* in our lentiviral vectors always gave rise to strong proviral instabilities observed in Southern blot analyses. The use of duplicated sequences in retroviral vectors often lead to genetic instability,<sup>33</sup> and studies in *Drosophila* indicated that the use of two tandem copies of the



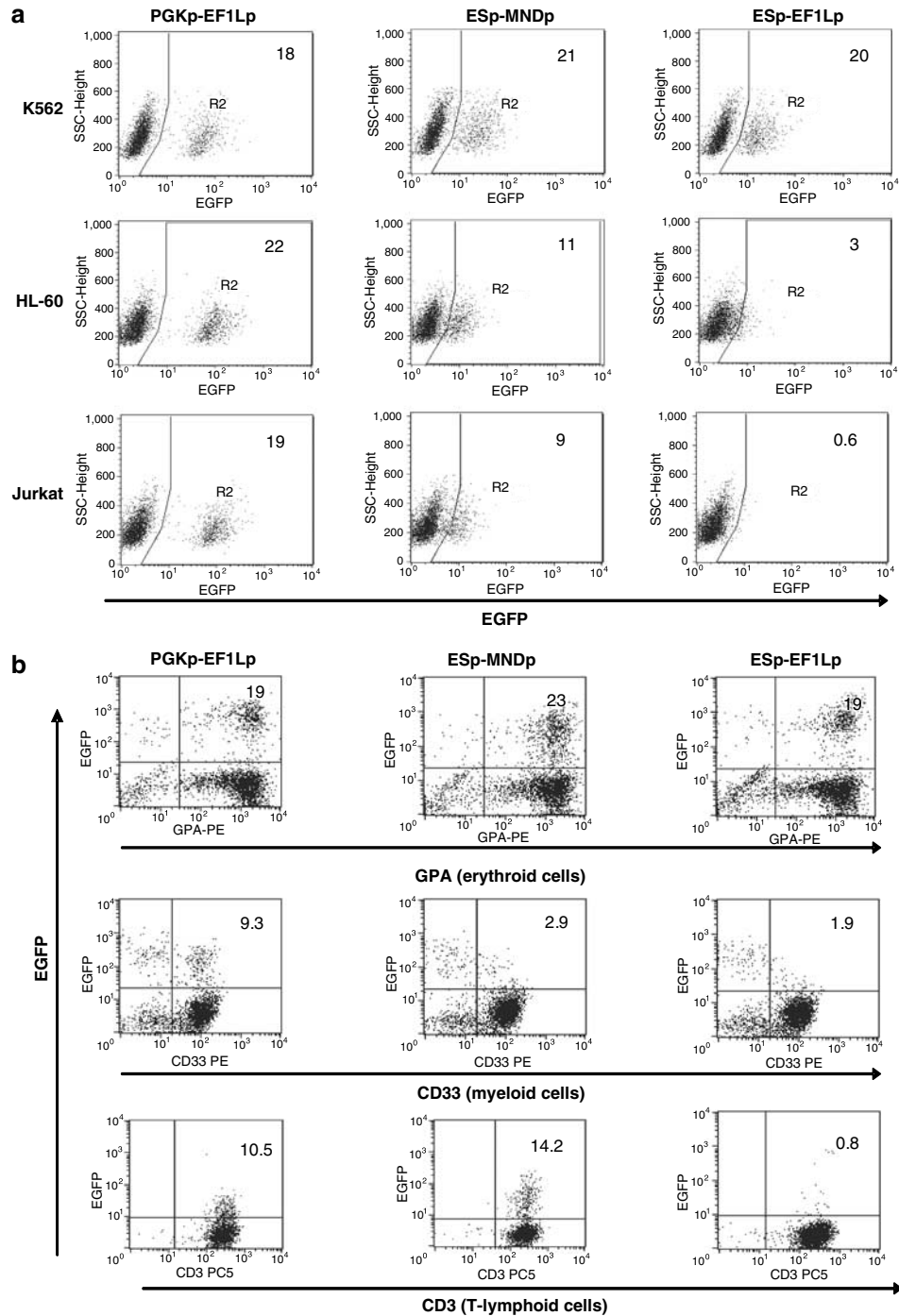
**Figure 5** *In vivo* amplification of Esp-EF1Lp-transduced cells by the MGMT system. **(a)** Percentages of EGFP + RBCs monitored over time by FACS in the drug-exposed (■) and non-exposed (○) groups of mice. **(b)** Representative FACS analyses of EGFP expression from BM cells stained with Ter-119 (erythroid), Gr-1 (granulocytes), and CD11b (monocytes) B220 (B-cells), and CD3 (T cells) 16 weeks after BMT. *n* = Number of mice per group.

chromatin insulator element *gypsy* could actually interfere with their activity.<sup>34</sup>

Our novel vectors are useful models in hematology, for carefully determining the effect of insulator sequences and for preventing the onset of deregulation in different white blood cell lineages. For example, we found that the erythroid-specific expression cassette remains “leaky”, in T cells, despite the presence of the insulator elements. Moreover, this is a novel system for evaluating the deregulation induced by an ES*p* *in vivo* in a murine model of BMT. Surprisingly, we found that the strong EF1*z*p allows similar chemoprotection but without any deregulation of the erythroid-specific promoter. The human 1.2 kb EF1*z*p used in this study has been previously described to be very powerful as an internal promoter in a self-inactivating-lentiviral backbone for transgene expression in HSCs.<sup>35</sup> Previously, we have successfully used an intron-deleted version of this human promoter in human CD34<sup>+</sup> cells.<sup>36,37</sup> However, this short version was too weak to allow an efficient *in vivo* chemoprotection of transduced HSCs (preliminary data not shown). This study confirms the interest of the intron to reach a level of gene expression obtained as high as with a strong viral ES*p* but without acting as long-distance enhancers. We observed a long-term transgene expression from EF1Lp in primary recipient mice. Serial BMTs, which are the most potent test for vector silencing, will

be necessary to study the very long-term stability of this promoter *in vivo*.

Our data have important implications for the design of gene therapy vectors. Indeed, our study underlines the interest and also the limits of insulators in the use of retroviral-derived promoters to prevent promoter deregulation. In addition, we did not observe any deregulation from EF1*z*p, neither in murine nor in human cells, showing an absence of correlation between the level of deregulation and the strength of the constitutive promoter. In contrast, a deregulation of the erythroid-specific promoter was observed in human T-lymphocytic, myelomonocytic cell lines, and human primary T cells transduced with the MNDp-based vector. Our *in vitro* and *in vivo* data demonstrate that the cellular, strong, EF1*z*p is more appropriate than retroviral promoters for designing a dual lentiviral vector for RBC disorders because of the absence of deregulation. It is noteworthy that a retroviral promoter induces a high level of *in vivo* deregulation compared with a cellular promoter with a similar level of transcription. One hypothesis is that murine leukemia virus has been selected over time through evolution by many passages through mice in which leukemias were induced by transactivation of proto-oncogenes. It is important to emphasize that the murine leukemia virus LTR has induced transactivation of the *LMO2* proto-oncogene promoter located upstream of the integration sites in T-cell lineage, and, which,



**Figure 6** EGFP expression in human cell lines and primary CD34<sup>+</sup>-derived cells. **(a)** Representative FACS analyses of EGFP expression in erythroblastic K562 and non-erythroid cell lines (myelo-monoblastic HL-60 and T-lymphoblastic Jurkat) transduced with the constitutive control (PGKp-EF1p), the ESp-MNDp and the ESp-EF1p vectors (three independent experiments). Numbers in the upper right panel are the % of EGFP<sup>+</sup> cells. **(b)** Representative FACS analyses of EGFP expression in human cord blood-derived cells transduced with the same vectors at MOI of 10 and grown in liquid culture, in conditions that allow erythroid, myeloid differentiation, or T-cell expansion. Numbers in the upper right panel are the % of EGFP<sup>+</sup> cells. 10 days after *in vitro* liquid culture expansion, cells were stained with either GPA (erythroid), CD33 (myeloid), or CD3 (T cells) antibodies (three independent experiments).

unfortunately has resulted in cases of leukemias in three children.<sup>38</sup> We do not yet know if there are specific genetic features of the murine leukemia virus-LTR that predispose to

transactivation of genes near the integration site, but if these features do exist, they would surely have been selected by the virus through evolution.

## MATERIALS AND METHODS

**Lentiviral vector constructs.** self-inactivating lentiviral vectors were derived from Ery-GFP<sup>12</sup> (Figure 1). To shorten vector names, EGFP, MGMT, and WPRE are not included in the abbreviations and HS40-ankyrine promoter labeled ES<sub>p</sub> (Erythroid Specific promoter). The MND<sub>p</sub> was removed from a lentiviral vector kindly provided by DB Kohn.<sup>17</sup> *G156A-MGMT* cDNA ( $\Delta$ MGMT) was obtained by polymerase chain reaction amplification as described.<sup>13</sup> The cHS4-containing vectors were constructed from ES<sub>p</sub>-MND<sub>p</sub> (HS40/ankyrine promoter–EGFP–WPRE–MND<sub>p</sub>– $\Delta$ MGMT) by inserting a 1.2 kb *SacI* fragment containing the chromatin Insulator cHS4 in Forward orientation (ES<sub>p</sub>-FI-MND<sub>p</sub>) between WPRE cassette and MND<sub>p</sub>. The cHS4 insulator was obtained from pJC13-1 plasmid (kindly provided by Dr G Felsenfeld).<sup>15</sup> The *core* element was restricted with *SacI*-HindIII and inserted with the same strategy (ES<sub>p</sub>-Core-MND<sub>p</sub>). The PGKp-EGFP (Figure 4a) is a shortened name of the pRRL-PGK-GFP-WPRE lentiviral vector backbone kindly provided by Dr D Trono. MND<sub>p</sub>-EGFP, EF1Sp-EGFP, and EF1Lp-EGFP were generated by replacing PGKp with the MND<sub>p</sub>, with the 0.25 kb containing EF1 $\alpha$ p from TEE kindly provided by P Charneau,<sup>39</sup> or with the 1.2 kb intron 1-containing EF1 $\alpha$ p from the pEF-BOS plasmid.<sup>40</sup> The constitutive control vector PGKp-EF1Lp is similar to the ES<sub>p</sub>-EF1Lp (Figure 1) but with the PGKp instead of the ES<sub>p</sub>. Cloning details are available upon request.

**Production and titration of lentiviral vectors.** VSV-G pseudotyped lentiviral vectors were produced by triple-transient transfection of 293T cells, as described previously.<sup>12</sup> Infectious titers were determined by transducing the erythroblastic K562 cell line with serial dilution of viral supernatant. EGFP expression was quantified 5 days later by FACS. Lentiviral vector supernatants were tested for the presence of replication competent lentivirus as described,<sup>41</sup> and were found to be free of replication competent lentivirus.

**Murine HSC purification and transduction.** Donor BALB/c normal mice were injected intraperitoneally with 5-FU (150 mg/kg per mouse). Five days later, nucleated BM cells were harvested by flushing femurs and tibiae, purified by Ficoll-Paque Plus gradient separation (Amersham Pharmacia Biotech, Orsay, France) and sorted for Sca-1<sup>+</sup> cells using the MACS Sca-1 kit (Milteny Biotech, Auburn, CA) (5-FU/Sca-1<sup>+</sup> cells). These cells were prestimulated for 6 h in RM-B00 medium (MABIO-International Laboratories, Tourcoing, France), containing 10% fetal calf serum, 50  $\mu$ M deoxynucleoside triphosphates and supplemented with the following cytokines: rm stem cell factor (100 ng/ml), rh thrombopoietin (100 ng/ml), rhFlt3-L (100 ng/ml), rhIL-6 (10 ng/ml), and rmIL-3 (20 ng/ml) hereafter called RM/fetal calf serum medium. 5-FU/Sca-1<sup>+</sup> cells ( $4 \times 10^5$  cells/ml) were transduced with lentiviral vectors in the presence of 8  $\mu$ g/ml protamine sulfate (Sigma, St Louis, MO) at a MOI of 50. For *in vitro* studies (Figure 4), Sca-1<sup>+</sup> cells were transduced at MOI of 10 with PGKp-EGFP, MND<sub>p</sub>-EGFP, and EF1Lp-EGFP vectors in RM/fetal calf serum medium with 8  $\mu$ g/ml protamine sulfate.

**In vivo drug treatment.** Five weeks after transplantation, the mice were randomly assigned to the different groups and subjected to drug administration. Mice were injected intraperitoneally with 30 mg/kg BG followed by 5 mg/kg BCNU three times, 1 month apart, as described.<sup>13</sup>

**BMT.** Twenty-four hours after transduction, 5-FU/Sca-1<sup>+</sup> cells (7,000 to 15,000 cells) were injected into the tail vein of the 6–10-week-old female normal mice previously irradiated with two splits of 6 Gy, 24 h apart.

**Human hematopoietic cell line and primary human hematopoietic cell transduction.** The K562 (erythroblastic), HL60 (myelo-monoblastic),

and Jurkat (T-lymphocytic) cell lines were maintained in Rosewell Park Memorial Institute 1640 medium (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (BioWhittaker, Emerainville, France), 100 U/ml of penicillin and 0.1 mg/ml of streptomycin (Gibco) at 37°C in 5% CO<sub>2</sub> atmosphere. Cell lines were transduced in the presence of 8 mg/ml protamine sulfate (Sigma) to obtain a range of 20–30% transduction rate. On day 10 following transduction, cells were harvested and analyzed for EGFP expression by FACS.

Cord blood CD34<sup>+</sup> cells were isolated as described.<sup>42</sup> CD34<sup>+</sup> cells were suspended in RM-B00 medium containing the following cytokines: 100 ng/ml human stem cell factor, 100 ng/ml human Flt3-ligand, and 100 ng/ml human thrombopoietin. Lentiviral supernatants at an MOI of 10 were added in the presence of 8  $\mu$ g/ml protamine sulfate (Sigma). CD34<sup>+</sup>-derived cells were harvested 2 days after the infection for differentiation or analyzed for EGFP expression at day 10 by FACS.

**T-lymphoid expansion and transduction.** Mononuclear cord blood cells were placed in Rosewell Park Memorial Institute supplemented with 10% human plasma, 10 ng/ml rhIL-2 (R&D Systems, Minneapolis, MN), and 5 mg/ml phytohemagglutinin. After 4 days of culture, phytohemagglutinin was removed and  $3 \times 10^4$  cells were transduced once with lentiviral vectors at a MOI of 10. Controls were run in parallel, using non-transduced cells. T-lymphocytes were harvested 10 days after transduction, labeled with phycoerythrin-cyanine-5-conjugated anti-CD3 (Immunotech, Marseille, France) and analyzed by FACS for CD3 and EGFP expression.

**Myeloid and erythroid differentiations.** The differentiation culture conditions were started on day 2 after transduction.

The CD34<sup>+</sup>-derived cells were placed under erythroid culture conditions until day 4 in RM-B00 medium supplemented with the following cytokines: 25 ng/ml rh stem cell factor, 10 ng/ml rhIL6, 50 ng/ml rhIGF-1 (all R&D Systems), and 3 U/ml rhEPO (Eprex; Janssen-Cillag SA, Boulogne-Billancourt, France). Then, from days 4 to 10, cells were maintained in the same medium without rh stem cell factor. For myeloid differentiation, cells were maintained in RM-B00 supplemented with 25 ng/ml rh stem cell factor, 10 ng/ml rhIL6, and 10 ng/ml rhGM-CSF (R&D Systems). Staining with anti-GPA and anti-CD33 monoclonal antibodies conjugated to phycoerythrin (BD Biosciences Pharmingen, San Diego, CA), and FACS analysis for EGFP expression were performed on day 10.

**Flow cytometric analysis.** FACS analyses of EGFP expression were performed on a FACS Calibur (BD, San Jose, CA). Granulocytes and monocytes were gated by forward and side scatter and analyzed for EGFP expression. BM cells were labeled with phycoerythrin-conjugated anti-Ter-119, Gr-1, B220, and CD11b (BD Pharmingen). Thymus cells were labeled with phycoerythrin-conjugated anti-CD3 (BD Pharmingen). The percentage of EGFP-positive lineage cells was calculated as the % EGFP-expressing cells in the total phycoerythrin-positive gated region.

**Southern blot analysis.** Ten micrograms genomic DNA from BM cells was digested with *Bam*H1, gel fractionated, blotted, and hybridized with <sup>32</sup>P-labeled *EGFP* cDNA probe. Known amounts of digested plasmid DNA mixed with control mouse genomic DNA were run in the same gel. The vector copy number per genome was estimated by the amount of radioactivity compared with the standard curve (Phosphor Imager, Molecular Dynamics div. Amersham Biosciences, Sunnyvale, CA).

**Statistical method.** Student's paired *t*-test was used for comparison of differences between indicated groups. The null hypothesis was rejected when *P* < 0.05.

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