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ORIGINAL ARTICLE

Improved lentiviral vectors for Wiskott–Aldrich syndrome gene therapy mimic endogenous expression profiles throughout haematopoiesis

C Frecha[1,2], MG Toscano[1], C Costa[2], MJ Saez-Lara[3], FL Cosset[2], E Verhoeyen[2,4] and F Martin[1,4]

[1]*Immunology and Cell Biology Department, Institute of Parasitology and Biomedicine López Neyra-CSIC, Parque Tecnológico Ciencias de la Salud, Granada, Spain;* [2]*Université de Lyon, F69000; Inserm, U758, Human Virology Department, F-69007; Ecole Normale Supérieure de Lyon, F-69007; Université Lyon 1, F-69007, Lyon, France and* [3]*Department of Biochemistry and Molecular Biology, School of Pharmacy, University of Granada, Granada, Spain*

Wiskott–Aldrich syndrome (WAS) gene therapy requires highly efficient and well-controlled vectors. Here we studied the performance of a lentiviral vector (LV) harbouring a 500-bp fragment of the WAS *proximal promoter (WW), which we previously characterized as haematopoietic-specific and capable of restoring WAS phenotype in patients' T cells. We used an LV (WE) expressing* eGFP *to evaluate whether this promoter was following the expression pattern of endogenous WASp. Transgene expression was analysed in WE-transduced hCD34⁺ population and its progeny after* in vitro *and* in vivo *differentiation in the* $Rag_2^{-/-}$, $\gamma c^{-/-}$ *humanized mouse. We revealed very poor expression from the WE internal promoter in macrophages and erythroid cells. Therefore, we designed a novel LV including a fragment of the alternative* WAS *promoter in WE vector (AWE). This new vector sustained high transgene levels along the whole lymphoid lineage* in vivo*. Most importantly, the performance of AWE vector was highly superior to WE vector since AWE clearly improved transgene levels in* in vitro *and* in vivo *hCD34⁺-derived macrophages, erythroid cells, megakaryocytes and B cells while supporting a high expression in human T cells. This emphasizes that it is a suitable LV backbone for gene therapy of haematopoietic diseases such as WAS.*

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Introduction

The Wiskott–Aldrich syndrome (WAS) is an X-linked condition clinically characterized by profound and progressive immunodeficiency, eczema and thrombocytopaenia.[1,2] It is caused by heterogeneous mutations within the *WAS* gene,[3–6] leading to absence of protein function in most of the cases.[2,7] WASp expression is restricted to haematopoietic cells,[8–10] so its absence affects all lineages of the haematopoietic system. WASp deficiency affects mainly T cells, dendritic cells, macrophages and platelets. WAS T cells are unable to respond to T-cell receptor-mediated activation and proliferation,[11–14] macrophages and dendritic cells cannot form podosomes in response to activating stimuli,[3,15] platelets also have impaired function.[7,16] This broad range of manifestations is explained by WASp function, which is tightly associated with the actin cytoskeleton formation, especially in actin polymerization.[3,11,17,18] A successful therapy for WAS can be achieved by allogenic bone marrow (BM) stem cell transplantation[19,20] or unrelated umbilical cord blood transplantation when a compatible donor is not available,[21] but the risk of graft vs host disease and rejection cannot be excluded in any case.[19–21] To overcome these limitations, gene therapy using autologous haematopoietic stem cells (HSCs) is a real alternative for treating WAS patients.

Gene therapy vectors for WAS must be well regulated, as transgene expression will be needed in the whole haematopoietic lineage at a certain concentration depending on the cell type. Recently, we have shown that overexpression of WASp in non-haematopoietic cells reduces cell viability by disturbing the cytoskeleton.[22] Moreover, overexpression of WASp homologues may contribute to cancer cell invasion and metastasis.[23] This points out the importance of gene therapy vectors with the capacity to express the transgene in the right cells, at the right levels. We and others demonstrated that an human immunodeficiency virus-based, SIN lentiviral vector (LV) carrying a 500-bp fragment of the endogenous *WAS* proximal promoter, upstream of the *WAS* cDNA (WW), achieved transgene levels equivalent to normal cells that completely restored the abnormal

Correspondence: Dr F Martin, Institute of Parasitology and Biomedicine López Neyra-CSIC, Parque Tecnológico Ciencias de la Salud. Avenida del conocimiento s/n. Armilla, Granada 18100, Spain.
E-mail: fmartin@ipb.csic.es or
E Verhoeyen, EVIR, INSERM, U758, Ecole Normale Supérieure de Lyon, 46 Allée d'Italie, Lyon 69007, France.
E-mail: everhoye@ens-lyon.fr
[4]These authors contributed equally to this work.
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phenotype of T-cell lines derived from WAS patients.[24,25] However, WAS gene therapy vectors should be able to give a physiologic-like profile of expression in all the haematopoietic lineages as T cells are not the only affected cell type. The expression levels of WASp are well documented in a wide variety of mature blood cells.[8,10] However, little is known about *WAS* promoter regulation and WASp expression profile during haematopoiesis. We and others have demonstrated that transduction of either human[24,26] or murine[27,28] progenitor cells is possible using LV that harbour *WAS* different promoters and that the differentiation potential is not perturbed by the presence of the inserted vector.[26]

However, up to date, no report exists describing in detail the expression profile of LVs bearing different *WAS* promoter fragments during the differentiation from human stem cells/early progenitors to all human haematopoietic lineages *in vitro* and *in vivo*. Thus, we focused on the performance of WE vector, derived from WW, in which *WAS* cDNA was replaced by enhanced green fluorescent protein (eGFP) and expressed from the 500-bp fragment of the *WAS* proximal promoter. We studied the transgene regulation during *in vitro* myeloid differentiation by comparing vector-driven eGFP with the endogenous WASp expression profile before and after haematopoietic differentiation, revealing very low expression levels in macrophages and erythroid cells. We therefore designed a new vector (AWE), by inserting into WE vector a 386-bp fragment of the alternative *WAS* promoter, which is proposed to be important during myeloid differentiation.[29,30] Here we present for the first time an extensive comparison, *in vitro* and *in vivo*, of the expression profiles of LV bearing proximal and alternative *WAS* promoter fragments throughout human haematopoiesis.

Results

Reduced expression from the 500-bp fragment of the WAS proximal promoter in hCD34⁺-derived macrophages and erythroid cells

A successful gene therapy for WAS could be achieved by *ex vivo* modification and re-infusion of patient's corrected HSCs. It was previously demonstrated that *WAS* mRNA and protein are expressed at similar levels among adult blood-circulating cells.[10] Therefore, we wanted to evaluate if our previously characterized WAS gene therapy lentivector could maintain a physiologic profile of expression throughout haematopoiesis. Due to the very low availability of HSCs from WAS patients, we applied healthy donor HSCs to study vector behaviour. The LVs WE, in which the *WAS* gene was replaced by eGFP under the control of a 500-bp fragment of the *WAS* proximal promoter, and SE (eGFP under spleen focus-forming virus (SFFV) ubiquitous promoter) have been described before[24,31] and are shown in Figure 1a.

Highly purified CD34⁺ cells were transduced with WE and SE vectors at multiplicity of infection (MOI) of 10 and part was kept in serum-free medium under proliferating conditions while the rest of the cells were differentiated into myeloid lineages using a semisolid medium that allows the development of myeloid cells (see Materials and methods). To allow a true comparison between vector expression levels, we routinely confirmed by quantitative PCR that a transduction efficiency of 20–40% correlated with 1–2 copies of vector per transduced cell (WE mean values for integrated copies per transduced cell were 1.5 in proliferating CD34⁺ cells, 1.3 in granulocytic colonies (CFU-G), 1.5 in monocytic/macrophagic colonies (CFU-M) and 1.9 in erythroid colonies (CFU-E); for SE vector, values were 1.0 in proliferating CD34⁺ cells, 0.8 in CFU-G, 1.0 in CFU-M and 1.4 in CFU-E; see Materials and methods). Both vectors achieved efficient and stable eGFP expression as well as mean fluorescence intensity (MFI) over time in culture under proliferating conditions (data not shown). However, at day 15 of myeloid differentiation, it was clear that the WE vector behaved differently depending on the colony type when analysed by fluorescence microscopy. Green fluorescence intensity from WE-transduced CFU-G colonies was much higher than that from WE-transduced erythroid (BFU-E) and CFU-M colonies, indicating reduced levels of eGFP expression in erythroid and macrophagic cells (data not shown).

To quantify transgene expression variation, an average of 100 colonies of each cell type was pooled and analysed by flow cytometry (FACS). In Figure 1b, a representative FACS experiment shows the different expression profiles of WE and SE vectors in undifferentiated CD34⁺ cells and in isolated BFU-E, CFU-M and CFU-G colonies. In each plot, we calculated the MFI increase (MFI-Inc) as the ratio between MFI of the eGFP-positive population and the MFI of the eGFP-negative population. MFI-Inc of CFU-G was as high as that for proliferating CD34⁺ cells for both vector types (MFI-Inc CFU-G = 16.7 vs MFI-Inc CD34⁺ = 21.7, for WE vector and MFI-Inc CFU-G = 362.2 vs MFI-Inc CD34⁺ = 247.5, for SE vector; Figure 1b). However, the WE vector exhibited a sevenfold drop in WE transgene expression for BFU-E and a 3.5-fold drop for CFU-M as compared to CD34⁺ cells (MFI-Inc BFU-E = 3.3 and MFI-Inc CFU-M = 6.5 vs MFI-Inc CD34⁺ cells = 21.7). In contrast, SE vectors showed no significant variation in expression levels in all colony types and they were similar to the ones obtained in CD34⁺ cells. As vector copy number was 1–2 per transduced cell for WE- and SE-transduced cells, we can relate the observed differences in eGFP expression levels to a distinct promoter activity. This may suggest a reduced WAS promoter activity of WE vector in monocytic and erythroid cells.

eGFP expression from WE vector mimics WASp endogenous expression profile in granulocytes but not in macrophages or erythroid cells

It will be important for gene therapy of WAS patients to correct the WASp deficiency in the different haematopoietic lineages to normal endogenous levels. Thus, to determine if the difference in expression within the WE-transduced population correlated with a physiologic behaviour of the promoter, we compared eGFP expression with endogenous WASp expression levels in the different myeloid colony types. We studied endogenous WASp expression by quantitative western blot, using the protein extracellular signal-regulated protein kinase as a loading control. A representative experiment is shown in Figure 1c, where it can be observed that there is barely any variation in the levels of endogenous WASp expression between proliferating CD34⁺ cells and mye-

loid colonies. In fact, the comparative analysis indicates that endogenous WASp levels barely changed upon differentiation, with a ratio close to 1 for CFU-G vs $CD34^+$ cells, ratio = 0.8 for BFU-E vs $CD34^+$ and ratio = 0.8 for CFU-M vs $CD34^+$ (Figure 1d, $N = 8$; P = not significant, dashed bars).

In parallel, we compared these ratios with the ratios in MFI-Inc of each WE- and SE-transduced colony type relative to the MFI-Inc of the $CD34^+$ population. In Figure 1d (white bars), it can be observed that the ratio between the MFI-Inc of WE-transduced CFU-G colonies and the proliferating $CD34^+$ cells (WE—CFU-G/CD34) is close to one, resembling endogenous WASp expression profile. This suggests that the 500-bp promoter fragment present in WE assures the same expression profile to the endogenous *WAS* promoter in granulocyte colonies. In contrast, WE-transduced BFU-E and CFU-M colonies do not achieve similar expression levels between eGFP and endogenous WASp (eGFP-MFI-Inc ratios for BFU-E/CD34 and CFU-M/CD34 are 0.27 and 0.32 respectively, as compared to 0.76 and 0.83 for endogenous WASp) (Figure 1d). Therefore, it cannot be excluded that the 500-bp promoter fragment might be lacking some regulatory sequences especially relevant in the erythroid and the monocyte/macrophage differentiation pathway.

Improved expression in erythrocytes, macrophages and megakaryocytes by insertion of a 386-bp fragment of the WAS alternative promoter into WE vector

To improve transgene expression in CD34-derived macrophages and erythroid cells, we inserted part of the *WAS* alternative promoter, which has been proposed to be especially relevant in myeloid differentiation.[29] We designed an LV (AWE), carrying a 386-bp fragment of the *WAS* alternative promoter immediately upstream of the 500-bp *WAS* promoter in the WE vector (Figure 2a). First, we transduced a panel of haematopoietic cells from lymphoid (Allo-N, Allo-W, Jurkat and Raji) and myeloid (THP-1, HL-60 and K562) lineage and we found that AWE vector achieved an improved expression in myeloid cells, and a fivefold improved expression in the erythroleukaemic K-562 cell line when compared with the WE vector (see Supplementary Material, Supplementary Figure S1).

Second, umbilical cord blood (UCB)-$CD34^+$ cells were transduced with SE, WE and AWE vectors and then differentiated into myeloid colonies *in vitro*. We confirmed that transduced $CD34^+$ cells and their derived colonies contained between 1 and 2 vector copies per cell for the three vector types (data not shown). The incorporation of the alternative *WAS* promoter into the

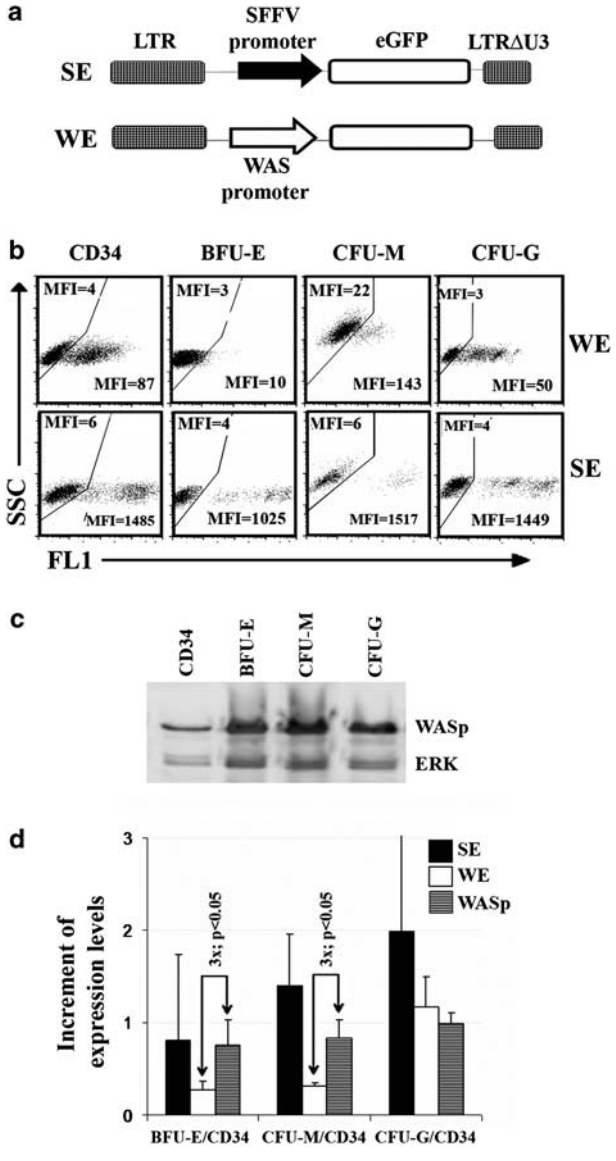

Figure 1 eGFP expression from the *WAS* proximal promoter is defective in $CD34^+$ *in vitro*-derived macrophages and erythroid cells. (**a**) Schematic representation of the SIN lentiviral vectors used. The WE vector contains a 500-bp fragment of the human *WAS* proximal promoter driving the expression of the reporter gene eGFP. In the SE vector, eGFP is under the control of the strong spleen focus-forming virus (SFFV) LTR promoter. (**b**) FACS analyses of WE- and SE-transduced $CD34^+$ cells and their derived myeloid colonies. Transduction was performed at MOI of 10, which resulted in 1–2 vector copies per transduced cell. An average of 100 myeloid colonies of each myeloid cell type (BFU-E, CFU-M or CFU-G) was picked at day 15 of myeloid differentiation, pooled and analysed by FACS for GFP expression. The mean fluorescence intensities (MFI) of the GFP^+ and GFP^- population are indicated in the dot plots (a representative experiment is shown; $N = 8$). (**c**) Detection of endogenous WASp expression by western blot in $CD34^+$ cells and their progeny after myeloid differentiation. As described in (**b**), an average of 100 colonies of each colony type was analysed for endogenous WASp expression by quantitative western blot using the ERK protein as loading control. (**d**) Comparison of expression patterns of SE and WE lentiviral vectors with endogenous WASp expression in BFU-E, CFU-M and CFU-G, relative to their expression in proliferating $CD34^+$ cells. The increment of eGFP expression after differentiation was calculated as the ratio of the eGFP MFI increase of the myeloid colonies and the MFI increase of the $CD34^+$ cells (MFI increase = MFI of the $eGFP^+$ population/MFI of the $eGFP^-$ population for each FACS plot). Increment of endogenous WASp expression was determined as the ratio of endogenous WASp expression levels between mature myeloid colonies vs undifferentiated $CD34^+$ cells, from the data obtained in (**c**). The fold difference between the increments in eGFP expression for WE vector as compared to the increment of endogenous WASp expression is indicated in the graph ($N = 8$). CFU-G, granulocytic colonies; CFU-M, monocytic/macrophagic colonies; eGFP, enhanced green fluorescent protein; FACS, flow cytometry; MOI, multiplicity of infection; WAS, Wiskott–Aldrich syndrome.

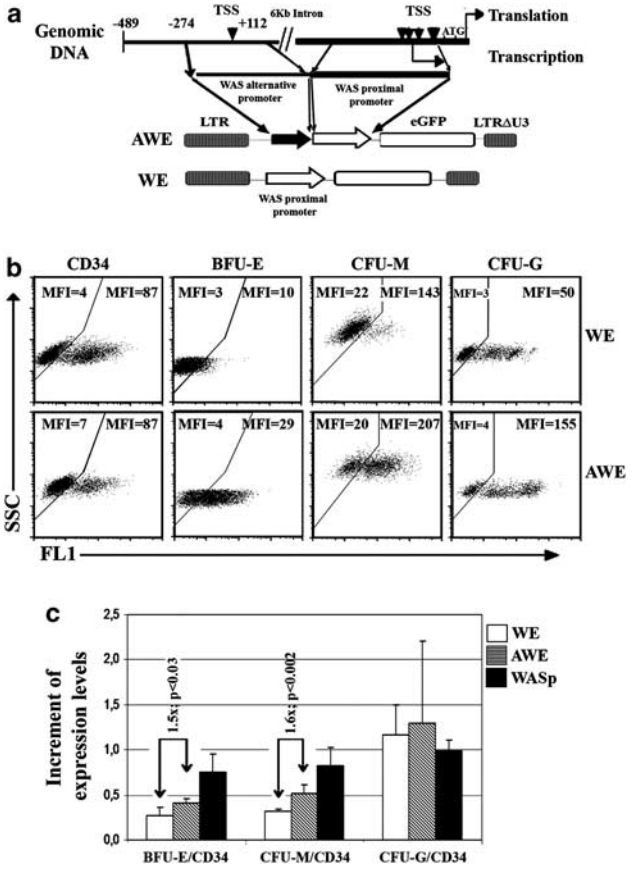

Figure 2 The AWE lentivector harbouring a fragment of the alternative and the proximal *WAS* promoter improves expression in CD34⁺-derived macrophages and erythroid cells. (**a**) Schematic representation of AWE vector. AWE vector was engineered by inserting a 387-bp fragment of the *WAS* alternative promoter immediately upstream of the 500-bp *WAS* proximal promoter in the WE vector. (**b**) FACS analyses of WE- and AWE-transduced CD34⁺ cells and their derived myeloid colonies. Transduction was performed at MOI of 10, which resulted in 1–2 vector copies per transduced cell. An average of 100 myeloid colonies of each myeloid cell type (BFU-E, CFU-M or CFU-G) was picked at day 15 of myeloid differentiation, pooled and analysed by FACS for GFP expression. The mean fluorescence intensity (MFI) of the GFP⁺ and GFP⁻ population are indicated in the dot plots (a representative experiment out of four is shown). (**c**) Comparison of eGFP expression pattern of the AWE and WE lentiviral vectors with endogenous WASp expression in BFU-E, CFU-M and CFU-G, relative to their expression in proliferating CD34⁺ cells. The increment of eGFP expression after differentiation was calculated as the ratio of the eGFP MFI increase of the myeloid colonies and the MFI increase of the CD34⁺ cells (MFI increase = MFI of the eGFP⁺ population/MFI of the eGFP⁻ population for each FACS plot). Increment of endogenous WASp expression was calculated from data obtained by quantitative western blot analysis of the different colonies and the one of proliferating CD34⁺ cells (see Figure 1c). The fold difference between eGFP expression achieved by WE as compared to AWE vectors is indicated in the graph ($N = 4$). CFU-G, granulocytic colonies; CFU-M, monocytic/macrophagic colonies; eGFP, enhanced green fluorescent protein; FACS, flow cytometry; MOI, multiplicity of infection; WAS, Wiskott–Aldrich syndrome.

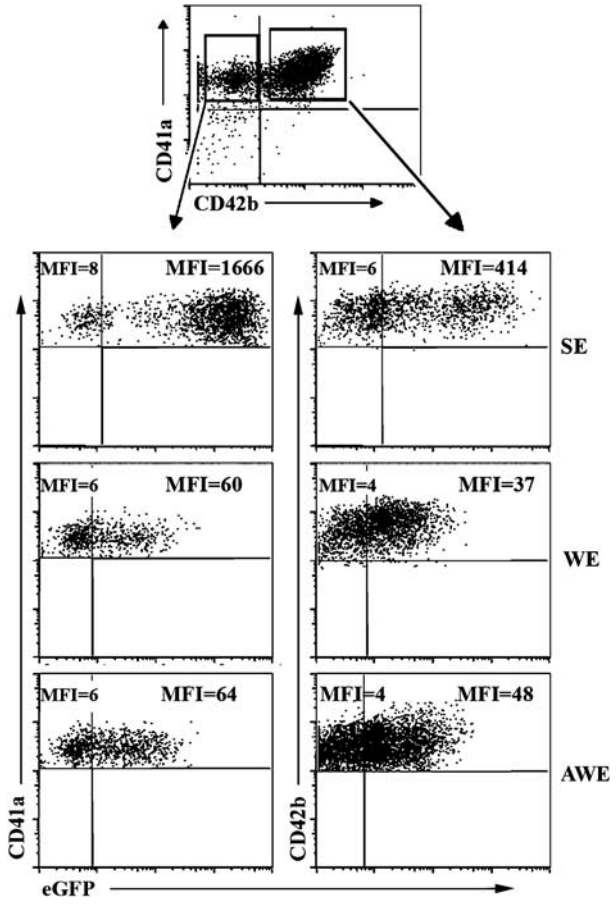

Figure 3 Expression profile of SE, WE and AWE lentivectors in *in vitro*-differentiated megakaryocytes. UCB-CD34⁺ cells were transduced with SE, WE or AWE vectors at an MOI = 10–20 and seeded in megakaryocyte-differentiation medium (see Materials and methods). A double staining with CD41a-APC and CD42b-PE markers was performed and allowed to distinguish between the immature (CD41a⁺/CD42b⁻) or mature (CD41a⁺/CD42b⁺) megakaryocytes. For each gated sub-population, the MFI is indicated in the dot plots. MFI, mean fluorescence intensity; MOI, multiplicity of infection.

AWE LV resulted in a significant increase of eGFP expression in erythroid cells as compared to WE vector (Figure 2b). Indeed, MFI-Inc ratio of BFU-E vs CD34⁺ rose from 0.27 for WE vector to 0.40 for AWE (Figure 2c, left bars), getting closer to the WASp protein ratio of BFU-E vs CD34⁺ (0.7) (Figure 2c, left black bar). AWE also achieved a better expression upon macrophage differentiation compared to WE vectors, as the MFI-Inc ratio of CFU-M vs CD34⁺ raised from 0.32 to 0.50, after incorporating the additional promoter fragment (Figures 2c, middle bars).

An additional important feature of WAS disease is the reduced platelet numbers released from megakaryocytes. Therefore, we found it was essential to compare WE and AWE expression profiles in *in vitro*-generated megakaryocytes derived from transduced CD34⁺ cells. Both vectors achieved stable expression in immature (CD41a⁺/CD42b⁻) and mature (CD41a⁺/CD42b⁺) megakaryocytes, with AWE vector showing a slightly increased MFI (Figure 3). In conclusion, the overall transgene expression profile of the AWE vector in myeloid lineages, derived *in vitro* from transduced

$CD34^+$ cells, was improved as compared to the original WE vector.

Both WE and AWE vectors assure high stable expression in lymphoid lineages in vivo

Recently, a successful new mouse model ($Rag2^{-/-}$, $\gamma c^{-/-}$ Balb/c mice) that allows the full and functional reconstitution with all human haematopoietic lineages was reported.[32,33] This mouse model allows efficient *in vivo* differentiation into T cells, which is difficult and cumbersome to achieve *in vitro*. Repopulation of these mice with $hCD34^+$ cells, transduced with the different eGFP-encoding LVs, permitted us to evaluate expression of the transgene in BM $CD34^+$ cells and their derived lymphoid and myeloid lineages. UCB-$CD34^+$ cells were transduced with the LVs AWE, WE and SE at MOI of 25 (using at least three different vector batches per vector) and injected intra-hepatically into sublethally irradiated 2- to 3-day-old $Rag2^{-/-}$, $\gamma c^{-/-}$ mice. The efficiency of engraftment and gene transfer was determined 8–10 weeks after transplantation. As indicated in Table 1, we obtained for all vectors, high engraftment with human $CD34^+$ cells in the BM (on average 31%) and the spleen (on average, 27%) and, where a thymus was present, it was almost completely humanized (>90%). In all haematopoietic organs, we obtained multilineage engraftment and transduction of all haematopoietic lineages (Figures 4 and 5).

Detailed FACS revealed that for all vectors (WE, AWE and SE), we obtained a normal distribution of the human thymic sub-populations with 81–94% immature $CD4^+$/$CD8^+$ cells (DP) and a low percentage of single-positive $CD4^+/CD8^-$ cells (SP4; 1.1–5.5%) and $CD4^-CD8^+$ cells (SP8; 2–6.1%) (Figure 4a, upper panels). For WE, as for AWE vectors, the more mature SP4 and SP8 sub-populations expressed eGFP at similar high MFI (Figure 4a), which were 1.5- to 2-fold higher than the expression obtained by these vectors in the BM $CD34^+$ population (Figures 5b and c). It is worth noting that for WE as well as for AWE vector, eGFP expression levels in mature $CD3^+$ T cells from the peripheral blood or spleen were maintained or even higher than in BM-$CD34^+$ cells (Figures 5b and c). In contrast, SE vector expression for thymic sub-populations dropped dramatically as compared to the $CD34^+$ cells in the BM and other lymphoid lineages such as B cells (for example, MFI-Inc SP4 = 115 ± 59 vs MFI-Inc $CD34^+$ = 561 ± 500; and MFI-Inc spleen $CD19^+$ = 428 ± 262; Figure 5a). Moreover, $CD3^+$ T cells in the spleen and peripheral blood showed a similar low-level expression (Figure 5a).

Next, we compared AWE and WE expression profiles in B cells and natural killer cells. AWE eGFP expression levels were slightly higher than those obtained by WE in peripheral and splenic B lymphocytes (Figures 4b, 5b and c). However, the MFI-Inc of both AWE and WE vectors in BM $CD19^+$ were similar and did not decrease when compared to the MFI-Inc of early progenitor $CD34^+$ cells or $CD13^+$ cells (Figures 5b and c). In addition, both WE and AWE vectors behave similarly in BM and splenic $CD56^+$ cells maintaining similar expression profiles as compared to progenitor cells. In summary, both AWE and WE vectors support high-level expression in lymphoid lineages after long-term *in vivo* human haematopoietic reconstitution.

AWE vector strongly improves expression in in vivo*-differentiated myeloid lineages*

We observed a different behaviour of WE vs AWE in myeloid lineages as compared to lymphoid lineages. It can be first outlined that SE-driven eGFP levels barely changed between $CD34^+$ cells and the derived myeloid progeny (Figures 5a). On the contrary, we observed a downregulation in eGFP expression of WE in certain cell types from the myeloid lineage (Figures 4c and 5b, black bars). In fact, we observed a striking downregulation in MFI-Inc for the WE vector in $CD14^+$ cells from the spleen (Figures 5b, black bar) relative to the eGFP MFI-Inc levels found in WE-transduced $CD34^+$ cells from the BM (Figures 5b, dashed bar; MFI-Inc S-$CD14^+$ = 9.34 vs MFI-Inc BM-$CD34^+$ = 37.21), which is in agreement with our *in vitro* differentiation data (Figure 1). However, we found that $CD14^+$ cells from BM (BM-$CD14^+$) behaved differently to S-$CD14^+$, as the former downregulated expression only 1.3-fold (Figure 5b). Finally, in megakaryocytes ($CD41^+$ cells), there is also a 1.8-fold decrease in WE expression levels as compared to BM-$CD34^+$ cells (Figure 5b).

Importantly, the opposite was true after the incorporation of the 386-bp fragment present in the AWE vector, which resulted in an overall increase in eGFP MFI in the myeloid cell types as compared to WE vector (Figures 4c, 5b and c). First of all, it strikingly improved eGFP MFI-Inc in $CD14^+$ cells from spleen. Indeed, the MFI-Inc for AWE was 65.04 vs 9.34 obtained with WE, resulting in a sevenfold improved expression level (Figures 5b and c). In addition, AWE BM-$CD14^+$ cells gave a threefold higher expression (Figures 5b and c; AWE MFI-Inc = 86.3 vs WE MFI-Inc = 29.1). AWE also ensured a remarkable improvement in transgene expression (>10-fold) in peripheral blood $CD14^+$ monocytes as compared to WE (Figures 5b and c). Finally, AWE improved by twofold the expression levels in megakaryocytes (AWE MFI-Inc = 39.92 vs WE MFI-Inc = 20.96; Figures 5b and c).

In summary, the insertion of part of the alternative promoter in AWE vector resulted in an overall improvement of expression in myeloid lineages while sustaining expression in the lymphoid lineage.

Discussion

Ex vivo gene therapy can be a real alternative for WAS patients. Among target cells, HSCs are the most suitable target to transduce as they give rise to the whole haematopoietic system.[34] An ideal gene therapy vector for WAS should transduce HSCs efficiently and maintain transgene levels throughout differentiation, giving the right levels of the transgene in each cell type. The *WAS* proximal promoter has a key role in haematopoietic-specific expression[29,30,35] and has been used before to drive WAS cDNA expression for WAS gene therapy.[24,25] However, there is no detailed study of the behaviour of these vectors through human haematopoiesis, in part due to the difficulties in obtaining WAS-deficient human HSCs. Therefore, we initially studied the behaviour throughout differentiation of an LV expressing *eGFP* from the *WAS* proximal promoter (WE vector).

In vitro, we detected a differential promoter activity according to cell lineage; indeed, in granulocytes, the inserted 500-bp promoter seemed to be regulated as the

Table 1 Engraftment of $Rag_2^{-/-}$, $\gamma c^{-/-}$ Balb-c mice with hCD34+ cells transduced with the different lentiviral vectors

Vector/animal no.	*UCB donor/ vector batch*	*Bone marrow*		*Spleen*		*THYMUS*	
		Total hCD45+ cells (%)	*Transduced hCD45+ cells (%)*	*Total hCD45+ cells (%)*	*Total hCD45+ cells (%)*	*Total hCD45+ cells (%)*	*Transduced hCD45+ cells (%)*
WE vector (MOI = 25)							
W1	U1/L1	42.9	53.2	21.1	27.5	92.04	—
W2	U1/L1	68.8	12.6	30.7	21.9	93.0	36.0
W3	U2/L2	11.8	26.0	10.7	19.1	90.0	—
W4	U2/L3	29.4	2.1	3.3	—	—	—
W5	U3/L2	46.3	31.5	68.4	22.5	86.3	12.4
SE vector (MOI = 25)							
S1	U4/L4	48.5	4.6	51.0	6.7	85.3	6.4
S2	U1/L5	33.4	6.8	26.6	7.2	98.3	11.1
S3	U2/L5	44.0	7.3	31.0	6.5	99.2	22.9
S4	U1/L6	23.0	18.9	22.9	15.0	96.1	0.8
S5	U3/L5	44.8	3.1	18.6	9.5	98.7	5.5
S6	U4/L5	7.0	35.7	7.7	—	94.2	—
AWE vector (MOI = 25)							
A1	U5/L7	69.3	23.1	61.9	55.1	95.5	56.7
A2	U6/L8	13.6	45.0	29.4	53.4	95.4	69.0
A3	U6/L8	17.8	63.3	26.9	72.8	98.9	83.7
A4	U7/L9	4.91	5.91	21.1	61.1	70.0	7.8
A5	U7/L9	14.2	31.1	17.7	49.7	96.2	52.6
A6	U6/L9	6.0	76.0	10.0	79.2	97.2	81.0

Abbreviations: eGFP, enhanced green fluorescent protein; FACS, flow cytometry; MOI, multiplicity of infection; rhSCF, recombinant human stem cell factor; rhTPO, recombinant human thrombopoietin.
Protocol: 2×10^5 CD34+ cells from cord blood (UCB) were transduced overnight with the indicated vectors (SE, WE, AWE) at MOI = 25 in the presence of rhSCF and rhTPO, rendering around 30% of transduction. Cells were washed and injected intrahepatically to sublethally irradiated 4-day-old $Rag_2^{-/-}$, $\gamma c^{-/-}$ Balb-c mice. The animals were killed after 8–10 weeks and bone marrow, spleen and thymus were extracted and analysed through FACS for human engraftment (% total hCD45+ cells) and eGFP-expressing human cells (% transduced hCD45+ cells). Independent experiments were performed with UCB-CD34+ cells from different donors as well as vector preparations (U1–U7 indicate different UCB donors, L1–L9 account for different vector batches).

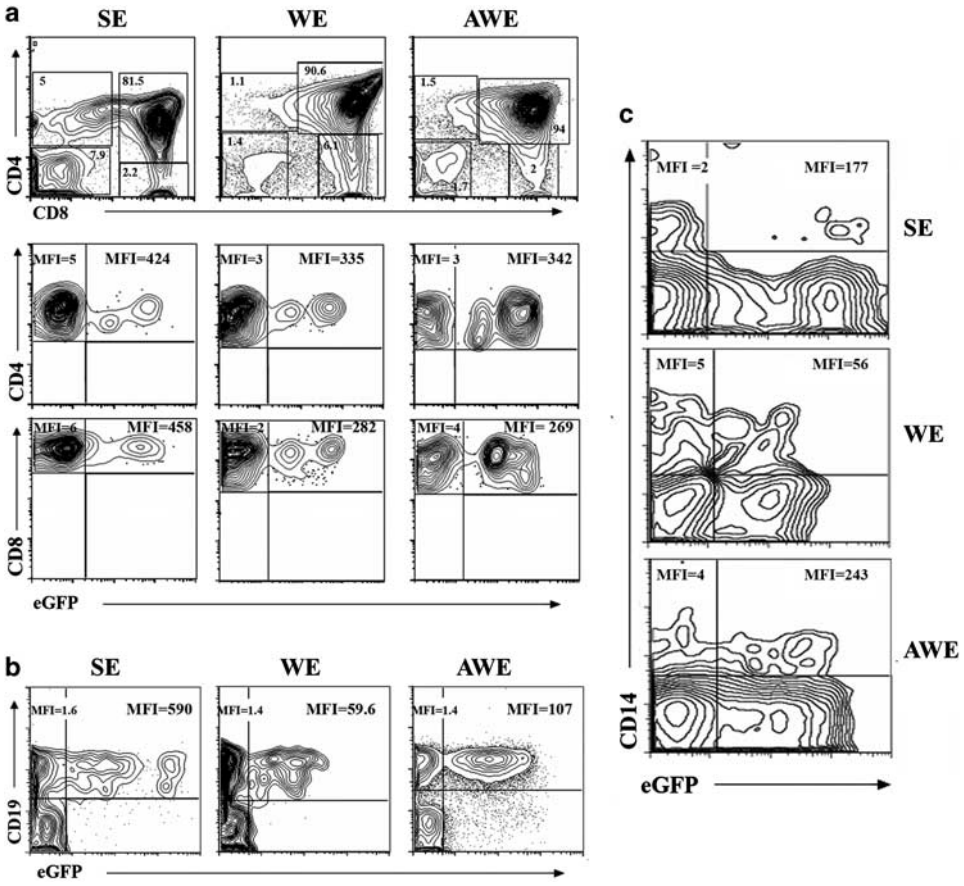

Figure 4 eGFP expression in human T-cell, B-cell and macrophage lineages of $Rag_2^{-/-}$, $\gamma c^{-/-}$ Balb-c mice reconstituted with WE-, AWE- and SE-transduced hCD34$^+$ cells. UCB-CD34$^+$ cells were transduced with SE, WE or AWE vectors at an MOI = 25 (resulting in a 20–30% eGFP transduction) and injected intrahepatically into sublethally irradiated 2- to 4-day-old $Rag_2^{-/-}$, $\gamma c^{-/-}$ Balb-c mice. (**a**) After 8–10 weeks of reconstitution, thymic tissue was extracted from the humanized mice and a two-colour marking (CD4/CD8) was done for discrimination of thymic sub-populations (top panels; SP4: CD4$^+$/CD8$^-$ cells; SP8: CD8$^+$/CD4$^-$ cells). The eGFP expression for the different vectors in the more mature SP4 and SP8 sub-populations is shown (middle and bottom panels). MFIs are indicated in the dot plots for the GFP$^+$ population (upper right quadrant) and the GFP$^-$ population (upper left quadrant). (**b**) FACS analyses of SE, WE and AWE eGFP expression in human peripheral B cells of humanized $Rag_2^{-/-}$, $\gamma c^{-/-}$ Balb-c mice. Peripheral blood was extracted after 8–10 weeks of reconstitution. Mononuclear cells obtained from a Ficoll gradient (see Materials and methods) were labelled with an anti-hCD19-APC antibody. A representative plot of hCD19-APC vs eGFP is shown for each vector type. MFIs are indicated in the dot plots for the eGFP$^+$ population (upper right quadrant) and the eGFP$^-$ population (upper left quadrant). (**c**) Spleens were extracted from the humanized $Rag_2^{-/-}$, $\gamma c^{-/-}$ Balb-c mice. Mononuclear cells were obtained from a Ficoll gradient (see Materials and methods). Cells were labelled with anti-CD14-APC antibody. A representative plot of hCD14-APC vs eGFP is shown for each vector type. MFIs are indicated in the plots for the GFP$^+$ population (upper right quadrant) and the GFP$^-$ population (upper left quadrant). eGFP, enhanced green fluorescent protein; FACS, flow cytometry; MFI, mean fluorescence intensity; MOI, multiplicity of infection.

endogenous *WAS* promoter. On the contrary, eGFP levels differed from endogenous WASp in monocytes/macrophages and erythroid cells, as we observed a transgene downregulation after differentiation. This could be due to a lack of regulatory sequences especially relevant for a correct expression in these cells. In the genome, endogenous human *WAS* gene is regulated by the *WAS* proximal and the *WAS* alternative promoter. The first one is 1.6-kb long and enriched in putative transcription factor-binding sequences such as Ets, PU.1, c-Rel, Rel-A and Sp-1, related to haematopoietic specificity and differentiation.[35–37] Recent studies have demonstrated that the full 1.6-kb version and the 500-bp fragment of the *WAS* proximal promoter achieved equivalent expression in the context of an LV in mature T, B and dendritic cells from WAS patients.[26] The alternative promoter is located 6-kb upstream the proximal promoter and less characterized, though it has been postulated to be especially relevant for myeloid differentiation.[29] In addition, we determined *in silico* using the TRANSFAC database, the presence of Sp-1, Ets-2, PU.1, AP-2 and c-Myb binding sequences. These sites were reported to play a role in early development stages[30] of myeloid differentiation.[38] Therefore, we included a 386-bp fragment of the alternative promoter immediately upstream the 500-bp *WAS* proximal promoter resulting in the novel AWE vector.

In vitro differentiation studies revealed that AWE vector achieved a significant increase in eGFP expression after differentiation of the CD34$^+$ cells into monocytic/macrophagic and erythroid cells, as compared to the WE vector. In addition, AWE also slightly increases eGFP expression in granulocytes and mature megakaryocytes. However, the inclusion of this extra regulatory fragment

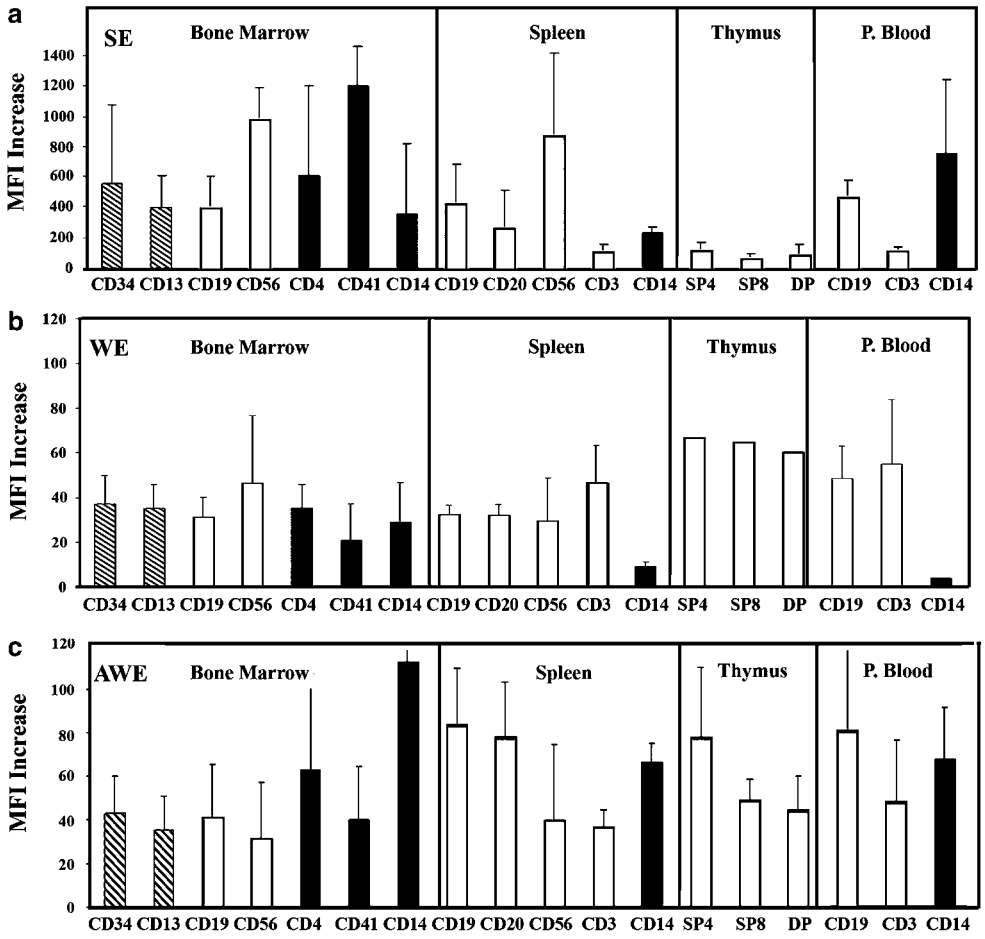

Figure 5 AWE vector ensures a high expression in all CD34⁺-derived haematopoietic lineages after *in vivo* differentiation in $Rag_2^{-/-}$, $\gamma c^{-/-}$ Balb-c mice. Bone marrow, spleen, thymus and peripheral blood (P. Blood) were extracted from $Rag_2^{-/-}$, $\gamma c^{-/-}$ Balb-c mice reconstituted with SE-, WE- or AWE-transduced human CD34⁺ cells. An extensive phenotype marking was done on each organ to study, by FACS, eGFP expression levels in a broad repertoire of progenitor, lymphoid and myeloid cells (represented on the *x* axis). MFI increase was calculated for each cell type as the ratio between the MFI of the eGFP⁺ population and MFI of the eGFP⁻ population of each dot plot (*y* axis). (**a**) SE vector. (**b**) WE vector. (**c**) AWE vector; Dashed bars: progenitor cells, white bars: lymphoid lineage, black bars: myeloid lineage; error bars = mean+s.d. These data include all the mice represented in Table 1. eGFP, enhanced green fluorescent protein; FACS, flow cytometry; MFI, mean fluorescence intensity.

did not achieve entirely the same profile of expression as compared to endogenous WASp.

To extend the study to the entire haematopoietic lineage, we used the $Rag_2^{-/-}$, $\gamma c^{-/-}$ immunodeficient humanized mouse, which is an excellent model for the engraftment and differentiation of hCD34⁺ cells into all lineages.[32,33] We first confirmed that both WE and AWE vectors achieved sustained transgene expression in all thymic sub-populations and mature T cells to the same level as detected in hCD34⁺ cells in the BM of the reconstituted mice. These results agree with functional restoration of human WASp expression in mouse T cells in a $WAS^{-/-}$ mouse model reconstituted with WW-transduced murine progenitor cells.[28] The *in vivo* performance of WE vector was markedly better in cells from lymphoid lineage than in myeloid cells. It is worth noting that AWE vector retained the high expression levels detected for WE vector in all lymphoid cell types. These results could mean that both promoters are well regulated in these cell types if we take into account that (1) previous mRNA studies carried out by Parolini *et al*.[10] showed no difference in *WAS* mRNA expression between CD34⁺ and peripheral lymphoid cells, and (2) we had previously shown that only 1.5–2 copies of the WW vector that carries the same backbone as WE, but encodes the full *WAS* cDNA instead of eGFP, were enough to reach normal transgene levels in $WAS^{-/-}$ patient T-cell lines.[24] It is worthnoting that we show that the SFFV promoter in the context of an LV is specifically downregulated in thymocytes and mature T cells. Nevertheless, it still induces higher expression levels than WE and AWE vectors, as shown also by Charrier *et al*.[26]

On the other hand, in accordance with our *in vitro* data, we found a striking decrease in *in vivo* transgene expression in WE-transduced CD14⁺ cells from the spleen and peripheral blood, but not for BM CD14⁺ population. These differences could be due to the fact that the spleen is highly enriched with CD14⁺ macrophages, while the BM contains mainly monocytes.[39,40] Importantly, AWE vector did improve eGFP expression levels by sevenfold in CD14⁺ cells from the spleen, by threefold in CD14⁺ cells from the BM and more than 10-fold in CD14⁺ cells from the peripheral blood. AWE

also slightly improved the expression in megakaryocytes *in vivo*, which is of importance as microthrombocytopaenia is a classic sign of WAS disease.[7,16] These data showed that, although the inclusion of AWE promoter does not entirely reconstitute the physiological WASp expression pattern, it improves significantly the expression profile in myeloid lineages. This effect on the myeloid lineages could be explained in part by the presence of additional 15 MZF-1 and 14 Sp1, 5 AML and PU.1 binding sites in the alternative promoter, which are certainly involved in myeloid development.[30,38,41–43] This might account for the improved expression of AWE in *de novo*-developed monocytes, erythroid cells and, to a lesser extent, in megakaryocytes.

Taken all together, this work gives new, valuable information about the performance of the 500-bp *WAS* proximal promoter fragment upon haematopoietic differentiation. Here we propose the addition of extra regulatory sequences contained in the 386-bp *WAS* alternative promoter for further vector optimization in the myeloid lineage, as both promoter fragments could be working together leading to a better transgene expression in myeloid cells, and specially in macrophages, without loosing efficiency of expression in the lymphoid lineage.

Materials and methods

Cell lines and culture media

Primary T cells from WAS patients (ALLO-W) and healthy controls (ALLO-N), both generated by allospecific stimulation, were kindly donated by Dr Molina. They were maintained by allospecific stimulation using mitomycin C treatment of Raji B-cell line weekly as described.[12] Haematopoietic cell lines, Jurkat (T-cell line), Raji (B-cell line) and THP-1 (monocytic cell line), were cultured in RPMI-1640, 10% fetal calf serum, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, sodium pyruvate and antibiotics. K562 (erythroleukaemic) and HL-60 (pro-myelocytic) cell lines were cultured in Iscove's medium, IMDM, with antibiotics, with 10% fetal calf serum and 20% fetal calf serum, respectively. UCB-derived $CD34^+$ human cells were seeded in StemSpan medium (Stem Cell Technologies, Vancouver, Canada) supplemented with 50 ng ml^{-1} recombinant human stem cell factor (rhSCF), 10 ng ml^{-1} recombinant human thrombopoietin (Peprotech EC, London, UK) and penicillin/streptomycin mixture (hereafter complete StemSpan). A commercial semisolid medium with recombinant cytokines (rhSCF, rhGM-CSF, rhIL-3, rhErithropoietin) for the differentiation of myeloid human cells (MethoCult, GF H4434; Stem Cell Technologies) was used for *in vitro* myeloid differentiation assays.

Plasmids, lentiviral constructs and vector production

The human immunodeficiency virus packaging (pCMVΔR8.91) and VSV-G (pMD.G) plasmids were kindly provided by D Trono. The LV plasmid SE (previously named pHRSIN-CSEW[31]) contains the SFFV LTR that drives the expression of the eGFP. The WE vector carries a 500-bp fragment of the WAS proximal promoter driving the expression of eGFP and it was already described.[24] For the construction of AWE vector, we performed a standard PCR using genomic DNA extracted from primary T cells using the following primer pair: F-AltPro-5: 5′-AAGTCAAAGGAGGA GAGGGCAACGC and R-AltPro-5: 5′-CTCAGTCTTTGT GAGCCCAGGAGTGC. The cycle of PCR was 1 × (5 min at 95 °C); 35 × (30 sec at 95 °C, 30 sec at 58 °C and 30 sec at 72 °C); 1 × (5 min at 72 °C). This primer pair amplifies a fragment of 386 bp from the *WAS* alternative promoter, which was cloned into pGEM-T easy vector (Promega, Madison, WI, USA). To obtain AWE vector, we cloned the 386-bp fragment into the *Eco*RI site of WE vector.

Lentiviral vectors were produced by co-transfection of 293T cells with three plasmids. Briefly, (1) Vector plasmid (SE, WE or AWE), (2) pCMVΔR8.9 and (3) Envelope plasmid pMD.G as described previously.[44] Viral titres (transduction units per ml) were calculated by FACS based on the initial amount of target cells (Jurkat cell line) and the percentage of $eGFP^+$ cells detected in the linear range of serial dilutions of the supernatant, ranging 5×10^6–1×10^7 transducing unit (TU) per ml in all the vector batches.

Isolation and transduction of human haematopoietic stem cells

Human umbilical cord blood was collected from healthy deliveries at Virgen de las Nieves Hospital (Granada, Spain), with consent of the mother. Samples were diluted 1:3 in Iscove's medium and low-density cells were separated over a Ficoll-hypaque gradient. $CD34^+$ cells were isolated by two rounds of magnetic cell sorting, using microbead-coated anti-CD34 antibodies (Miltenyi Biotech, Berqisch Gladbach, Germany), following the manufacturer's instructions. Cells were stained using a phycoerythrin-conjugated anti-CD34 antibody (Miltenyi Biotech). The purity of $CD34^+$ cells was higher than 95% in all the experiments. Previous to lentiviral transduction, 5×10^4 $CD34^+$ cells were seeded in 48-well plates and incubated overnight in complete StemSpam. Viral supernatants were added at an MOI from 10 to 30 for 8 h, then washed off with StemSpan.

In vitro *myeloid differentiation assay*

A total of 1–2 × 10^3 $CD34^+$ cells were seeded onto 35 mm petri dishes in 1.5 ml MethoCult containing rhSCF, rhGM-CSF, rhIL-3 and rhErithropoietin. The plates were incubated at 37 °C, 5% CO_2, for 14–16 days, then colonies were counted and analysed under a fluorescence microscope (Cell R IX 81; Olympus, Center Valley, PA, USA) using the same settings for non-transduced, SE- and WE-transduced colonies. Images were captured with a digital camera (Orca CCD; Hamamatsu, Japan).

To quantify eGFP expression achieved by the different vectors and to compare it with endogenous WASp content, the different colonies derived form SE-, WE- or AWE-transduced $CD34^+$ cells were picked. On average, 100 colonies from the same type (CFU-M, CFU-G and BFU-E) were pooled to obtain enough cells to analyse (1) eGFP expression by FACS, (2) WASp protein content by western blot and (3) number of vector integrations by quantitative PCR. An aliquot of $CD34^+$ cells was kept in StemSpan (Stem Cell Technologies, catalogue no. 09600) with rhSCF and recombinant human thrombopoietin through the whole experiment, and will be referred to as 'proliferating $CD34^+$ cells'. The MFI-Inc was calculated by dividing the MFI of the $eGFP^+$ population by the MFI

of eGFP$^-$ population and represents the strength of the promoter. To compare promoter behaviour after differentiation, we calculated the ratio between the MFI-Inc of each differentiated cell type and the MFI-Inc of the proliferating CD34$^+$ population.

In vitro *differentiation of megakaryocytes*

UCB-CD34$^+$ cells were transduced with WE, AWE or SE vectors at MOI = 25, as described above. After 4 days in culture, 3×10^5 CD34$^+$ cells were transferred to StemSpan medium containing 20 ng ml^{-1} of recombinant human thrombopoietin and 2.5 ng ml^{-1} of rhSCF. At day 10 of differentiation, cells were harvested and stained with antibodies against hCD41a-allophycocyanin (APC) and hCD42b-PE markers. eGFP percentage and MFI were analysed by FACS.

Sodium dodecyl sulphate/polyacrylamide gel electrophoresis and quantitative western blot

Cells were lysed in NP-40 lysis buffer containing protease inhibitor cocktail (Sigma, St Louis, MO, USA). Quantitative western blot analysis was performed using the ECL Advanced Western Blotting Detection Kit (Amersham Bioscience, Buckinghamshire, UK) and analysed at 440 nm with Quantity One version 4.5.0 software (Bio-Rad, Hercules, CA, USA). Contribution of each band was recorded and expressed as relative intensity per mm^2. We used mouse anti-WASp (clone D1; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rabbit anti-Erk antibodies (Anti-MAP Kinase 1/2; Upstate Biotechnology, Dundee, UK) followed by incubation with horseradish peroxidase-labelled anti-mouse and anti-rabbit antibodies (1:10 000 dilution; Caltag/Invitrogen, Carlsbad, CA, USA).

DNA preparation and quantitative PCR

Genomic DNA was extracted using a genomic DNA extraction kit (Promega). The real-time PCR were performed in the ABI Prism 7000 PCR Detection System (Applied Biosystems, Foster City, CA, USA) using hot-start DNA polymerase (Eppendorf AG, Hamburg, Germany). Vector copy number determination was done by amplification and detection of the eGFP-inserted gene. Primers: F 5′-GCCCGACAACCACTACCT-3′, R 5′-CGTCCATGCCGAGAGTGA-3′; TaqMan probe: 5′-FAM-CGGCGGCGGTCACGAACTCCA-TAMRA-3′. To standardize sample quantities, we amplified the genomic β-actin housekeeping gene, using F 5′-GCTATCCAGG CTGTGCTATC-3′, 5′-TGAGGTAGTCAGTCAGGTCC-3′, 5′-FAM-CCAGCCAGGTCCAGACGCAGG-TAMRA-3′. The parameters for the PCR were 1× (95 °C for 2 min), 45× (95 °C for 30 sec, 61.4 °C for 30 sec and 72 °C for 30 sec) and 1× (72 °C for 2 min). We then calculated the vector copy number per transduced cell taking into account the percentage of eGFP$^+$ cells.

Conditioning and reconstitution of Rag$_2^{-/-}$, γc$^{-/-}$ Balc mice

Freshly isolated UCB-CD34$^+$ were transduced overnight with WE, SE or AWE vectors, at MOI 25 as described above. At day 3 post-transduction, 2×10^5 cells were injected intrahepatically into sublethally irradiated 2- to 4-day-old newborn Rag$_2^{-/-}$, γc$^{-/-}$ mice (2×1.5 Gy). The animals were kept for 8–10 weeks under sterile conditions. The mice were killed in agreement with bioethical procedures and BM, spleen, thymus and peripheral blood were extracted. Cells were separated from the tissues using a mesh, and the mononuclear cell fraction was obtained from a Ficoll density gradient (Ficoll-Hypaque; Beckman-Coulter, Fullerton, CA, USA).

Flow cytometry analyses

PE-Cy5-conjugated anti-hCD45 antibody was used for the detection of total human cell engraftment in each haematopoietic tissue. APC-coupled antibodies were used for the detection of hCD3 (total T cells), hCD56 (natural killer), hCD19 (B cells), hCD34 (progenitor cells), hCD13 (more mature progenitors), hCD4 (monocytes/CD4 T cells). PE-coupled antibodies were used for the detection of hCD20 (mature B cells), hCD14 (myeloid cells), hCD41 (megakaryocytes), hCD8 (CD8 T cells). All antibodies were obtained from Pharmingen (San Jose, CA, USA).

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Conflict of interest

None.

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