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RESEARCH ARTICLE

Lentiviral vectors transcriptionally targeted to hematopoietic cells by WASP gene proximal promoter sequences

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The development of vectors that express a therapeutic transgene efficiently and specifically in hematopoietic cells (HCs) is an important goal for gene therapy of hematological disorders. In order to achieve this, we used a 500 bp fragment from the proximal WASP gene promoter to drive the expression of the WASP cDNA in the context of a self-inactivating lentiviral vector. Single-round transduction of WASp-deficient herpesvirus saimiri (HVS)-immortalized cells as well as primary allospecific T cells from Wiskott–Aldrich syndrome (WAS) patients with this vector (WW) resulted in expression levels similar to those of control cells. Non-HCs were transduced with similar efficiency, but the levels of WASp were 135–350 times lower than those achieved in HCs. Additionally, transduction of WASp-deficient cells with WW conferred a selective growth advantage in vitro. Therefore, lentiviral vectors incorporating proximal promoter sequences from the WASP gene confer hematopoietic-specific, and physiological protein expression.
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Introduction

Gene therapy has demonstrated its clinical potential in hematopoietic disorders with significant success in the treatment of patients with severe combined immunodeficiency[1,2] and adenosine-deaminase deficiency.[3] These successful therapies were based on the reinfusion of gene-modified hematopoietic stem cells (HSCs). However, it has been demonstrated by several groups that purified HSCs may differentiate into or fuse with several non-hematopoietic cell (HC) types.[4–10] The ectopic expression of hematopoietic genes in non-hematopoietic tissues raises safety concerns. Further improvements in vector design to achieve specific and efficient expression of transgenes in target tissues would increase the safety of the procedure and are therefore desirable. This may also facilitate the application of gene therapy to other hematopoietic diseases.

Lentiviral vectors are among the most versatile of all integrating vector systems.[11] They are stable, easy to concentrate and have a broad tropism. Virtually all cell types analyzed, including those nondividing, can be efficiently transduced. The latest generation of lentiviral vectors are self-inactivating (SIN) and drive the expression of the transgene only through an internal promoter.[11] This has facilitated the development of transcriptionally regulated vectors.[12–14]

The Wiskott–Aldrich syndrome (WAS) is a X-linked condition characterized clinically by profound and progressive immunodeficiency, eczema and severely decreased number of platelets (reviewed in Snapper and Rosen[15] and Thrasher[16]). Patients with classical WAS have heterogeneous mutations in the *WASP* gene,[17–19] which usually result in the absence or very diminished expression of its encoded gene product (WASp), or otherwise give rise to non-functional truncated proteins.[20,21] Expression of the *WASP* gene is restricted to cells of the hematopoietic lineage.[17,22,23] Petrella and co-workers[24] have characterized a 170 bp fragment containing four potential binding sites for hematopoietic transcription factors (two for Ets-1, one for c-Rel and one for PU.1) sufficient to confer hematopoietic-specific expression in transient assays.[24] Therefore, the *WASP* gene promoter is a good candidate to achieve hematopoietic-specific expression in the context of a SIN lentiviral vector.

Experimental models to study primary defects and to evaluate therapeutic alternatives when patient cells are not readily available include allospecific T-cell lines[25] as well as herpesvirus saimiri (HVS)-immortalized T-cell lines.[26] Very recently, we have demonstrated that HVS-immortalized T cells from WAS patients can efficiently be transduced by lentiviral vectors and are therefore a

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useful model for WAS gene therapy.[27] In this study, we show that a 500 bp fragment from the proximal *WASP* gene promoter, in the context of a SIN lentiviral vector, achieves hematopoietic-specific and physiologically relevant levels of WASp.

Results

Efficient transduction of hematopoietic and non-HCs by WW lentiviral vectors

The WASp-expressing lentiviral vectors used in this study are shown in Figure 1a. WPRE and enhanced green fluorescent protein (eGFP) sequences from the HRSIN-CSEW plasmid (SE)[28] were excised to obtain the SIN lentiviral backbone used to construct SW and WW vectors. cDNA sequences encoding *WASP* were cloned after the spleen focus-forming virus (SFFV) promoter to generate SW as described in Materials and methods. To construct WW, a 500 bp fragment from the proximal *WASP* gene promoter (extending from bases −1 to −499 from the transcriptional start site) was amplified by PCR and inserted in place of the SFFV promoter contained in the SW vector.

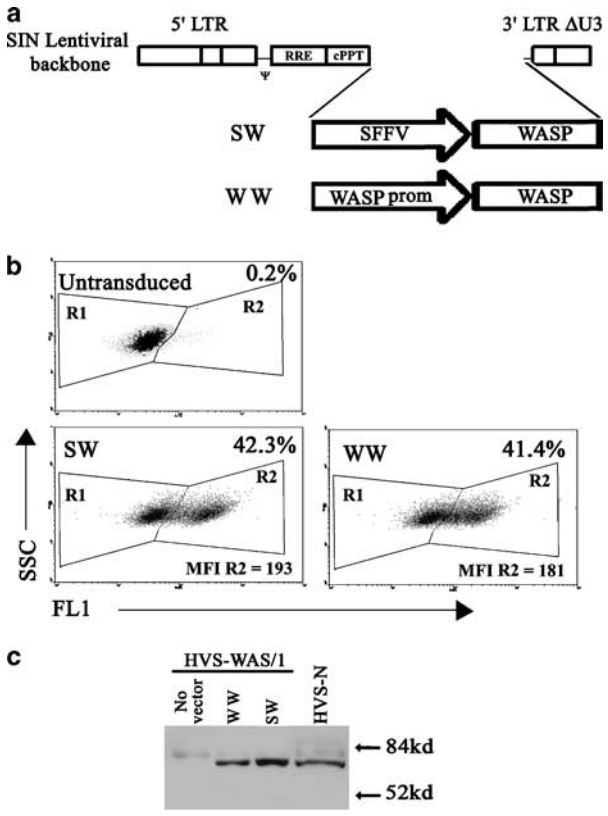

Figure 1 *Efficient transduction of target cells by lentiviral vectors expressing WASp. (a) Schematic maps of lentiviral vectors SW and WW. The SW vector drives the expression of the WASP cDNA by the SFFV LTR in a self-inactivating lentiviral vector backbone. The WW vector uses the proximal WASP gene promoter (extending from bases −1 to −499 from the transcriptional start site) to drive the expression of the WASP gene. (b) Flow cytometry analysis showing HVS-WAS/1 cells transduced with WW and SW vectors at an MOI = 1. WW- and SW-transduced (bottom panels) and untransduced (top panel) cells were incubated with the α-WASp B9 mAb and an FITC-labeled goat anti-mouse second antibody. The percentage of WASp⁺ cells and MFI within the gated-positive region (R2) are shown. (c) Western blot of cell lysates from HVS-N cells as well as untransduced HVS-WAS/1 (no vector) and HVS-WAS/1 transduced with WW and SW vectors from (b). Membranes were probed with the α-WASp D1 mAb.*

SW and WW lentiviral vectors expressed WASp protein in HVS-WAS/1 cells (derived from a WAS patient completely deficient in WASp) (Figure 1b, top panel) at the expected molecular weight (Figure 1c). Both vectors exhibited similar transduction efficiencies on hematopoietic HVS-T cells (Figure 1b), with titers over 10^6 TU/ml (Table 1). As expected, these titers were lower than those obtained with the WPRE-containing vector SE (Table 1). Nevertheless, the intensity of WASp expression was slightly higher in SW- than in WW-transduced cells as suggested by the mean fluorescence intensity (MFI) within the R2-positive gate (Figure 1b). In marked contrast, we did not detect any WASp expression in the non-hematopoietic RKO cells transduced with WW vectors (Table 1), even though they were efficiently transduced (data not shown and Figure 3). As expected, SW and SE vectors containing the SFFV promoter transduced RKO cells with high efficiency (titers over 10^7 TU/ml) (Table 1).

Targeted WASp expression to HCs by WW lentiviral vectors

In order to determine whether the proximal *WASP* gene promoter confers hematopoietic-specific expression of WASp, two HC lines from different WAS patients (HVS-WAS/1 and ALLO-WAS/2) and three non-HCs (RKO, follicular dendritic cell (FDC) and HUVEC) were transduced with SW and WW lentiviral vectors at identical multiplicity of infection (MOI). HCs were transduced with an MOI = 1 (as determined on HVS-WAS/1 cells) and the more permissive non-HCs with an MOI = 0.2 in order to achieve similar transduction efficiencies. WASp expression levels were determined by flow cytometry based on the difference of MFI between cells in the positive R2 gate and negative R1 gate (Figure 2a). Hematopoietic T cells deficient in WASp, HVS-WAS/1 (Figure 2a and b) and ALLO-WAS/2 (Figure 2b) were efficiently transduced by SW and WW vectors and WASp expression levels were readily detected. Interestingly, non-HC RKO (Figure 2a and b), HUVEC and FDC (Figure 2b) transduced with the WW vector showed almost undetectable WASp expression. In contrast, all SW-transduced cells expressed WASp regardless of their origin (Figure 2a and b).

Table 1 Titration of lentiviral vectors

	Vector titers (TU/ml)	
	RKO	*HVS-WAS/1*
SE	1.70×10^8	7.00×10^6
SW	2.50×10^7	3.00×10^6
WW	ND	2.10×10^6

Exponentially growing target cells were transduced with serial dilutions of SE, SW and WW vectors. Viral titers were calculated by flow cytometry as indicated in Materials and methods. ND: not determined due to lack of WASp expression in nonhematopoietic RKO cells.

Hematopoietic specificity of the WW vector was further analyzed by comparing WASp protein expression levels and vector copy number in two hematopoietic (HVS-WAS/1 and ALLO-WAS/2) and two nonhematopoietic (FDC and RKO) cell lines. The different cell lines were transduced with SW and WW vectors at varying MOIs (ranging from 0.01 to 3 depending on the cell type) in two separate experiments (Experiments 1 and 2 of Figure 3) and allowed to grow for 1 month before analyzing the number of vector integrations per cell by quantitative PCR, and protein expression by semiquantitative Western blot. In Experiment 1, we analyzed SW- and WW-transduced HVS-WAS/1 T cells *versus* nonhematopoietic FDC cells (Figure 3, left panels). Densitometric analysis revealed that WASp signal in WW-transduced HVS-WAS/1 cells containing 0.09 vector per cell (v/c) was 30 times stronger than the signal observed in WW-transduced FDC cells containing 1.05 v/c (Figure 3, bottom left panels). Therefore, we calculated that WASp expression driven by WW vector was 350 times more efficient in HVS-WAS/1 cells than in FDC. In Experiment 2, we performed a similar study using allospecific T cells from a second WAS patient (ALLO-WAS/2) and non-hematopoietic RKO cells. The WASp signal in WW-transduced ALLO-WAS/2 cells containing 0.3 v/c was 2.5 times stronger than the signal observed in WW-transduced RKO cells containing 16.2 v/c (Figure 3, bottom right panels). Therefore, WASp expression driven by the WW vector was at least 135 times more efficient in ALLO-WAS/2 T cells than in RKO cells. In sharp contrast to the WW vector, WASp protein levels in SW-transduced cells were equivalent (ALLO-WAS/2 *versus* RKO) or slightly lower (3–10 times) in non-HCs (FDC *versus* HVS-WAS/1) (Figure 3, top panels).

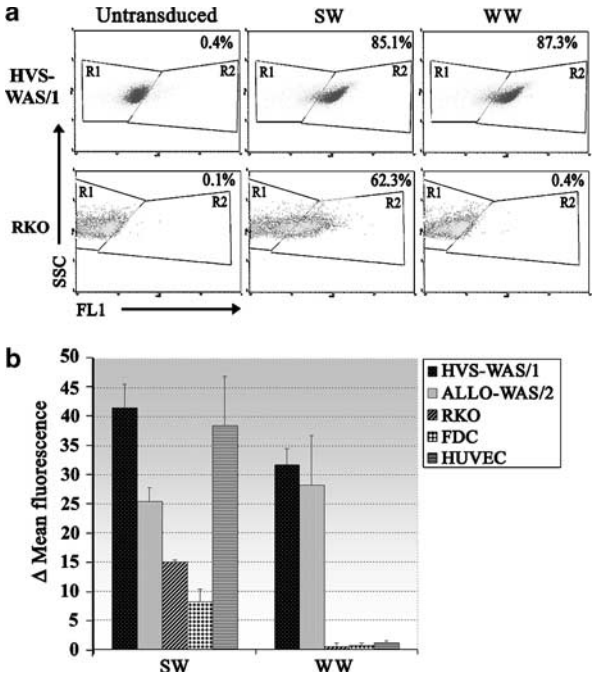

Figure 2 *Hematopoietic-specific expression of the WASP gene by WW lentiviral vectors. (a) Representative experiment (out of three) showing expression levels of WASp in untransduced (left panels), SW- (middle panels) and WW- (right panels) transduced hematopoietic HVS-WAS/1 (top row) (MOI = 1) and nonhematopoietic RKO cells (bottom row) (MOI = 0.2). Cells were stained 3 weeks after transduction with the B9 α-WASp monoclonal antibody. (b) Graph showing differences in MFI between cells in the positive R2 gate and negative R1 gate of transduced cells as shown in (a). WASp expression levels achieved by SW and WW lentiviral vectors were analyzed in two HC lines (HVS-WAS/1 and ALLO-WAS/2) (MOI = 1) and three non-HC lines (HUVEC, FDC and RKO) (MOI = 0.2). Data represent the average of increment (R2 R1) in MFI ± s.d. from three separate experiments.*

WW lentiviral vectors achieve physiological levels of WASp expression in WASp-deficient T cells

Full reconstitution of cellular functions may require WASp expression levels similar to those found in normal individuals. Thus, we compared WASp expression in WW-transduced HVS-T cells (MOI = 1) from a WAS patient (HVS-WAS/1) and their normal counterparts (HVS-N) (Figure 4, bottom panels). In addition, primary allospecific T cells from a second WAS patient (ALLO-WAS/2) were transduced with WW (MOI = 3) and WASp expression compared with allospecific cells from a healthy donor (ALLO-NORMAL) (Figure 4, top).

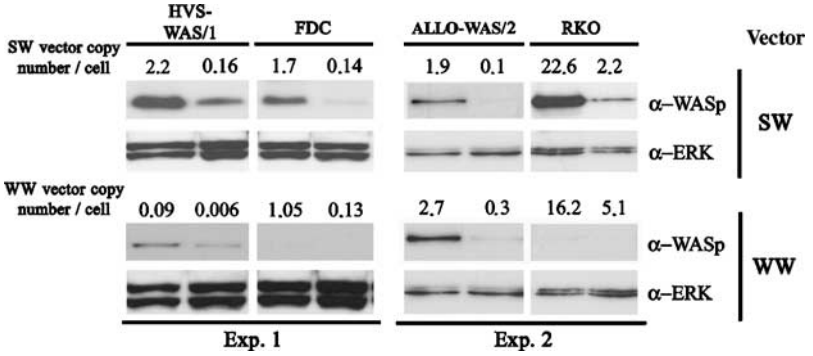

Figure 3 *WASp expression driven by WW vector is 135–350 times more efficient in HCs than in non-HCs. Western blot analysis showing WASp protein expression driven by SSFV and WASP promoters relative to vector copy number per cell. Two different experiments compared hematopoietic HVS-WAS/1 versus nonhematopoietic FDC cells (Experiment 1, left panels) and hematopoietic ALLO-WAS/2 versus nonhematopoietic RKO cells (Experiment 2, right panels). Cells lines were transduced with SW (top panels) and WW vectors (bottom panels) at two different MOIs (right lane of each sample is a 10-fold dilution of the MOIs used in left lanes). Samples were analyzed 1 month after transduction for WASp expression using the D1 α-WASp monoclonal antibody. Number of vector integrations per cell was determined by real-time PCR (indicated on top of each lane). Membranes were rehybridized with an α-ERK polyclonal antibody as internal loading control.*

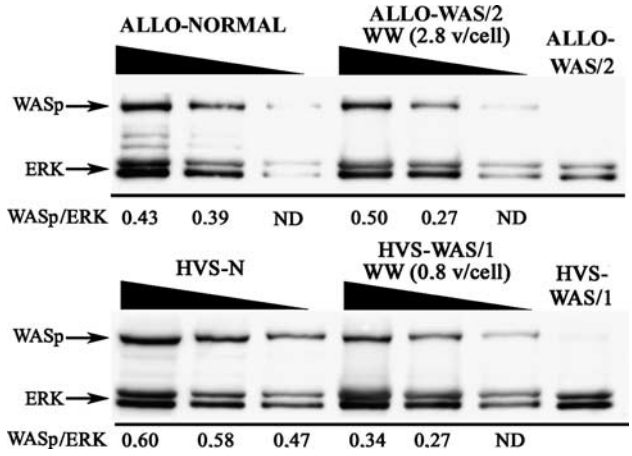

Figure 4 *Physiological expression of WASp protein in WW-transduced WAS T cells. Allospecific T cells (ALLO-NORMAL) (top left) and HVS-T cells (HVS-N) (bottom left) derived from normal individuals were compared for WASp protein expression with WW-transduced allospecific T cells (ALLO-WAS/2) (MOI = 3) (top right) and HVS-T cells (HVS-WAS/1) (MOI = 1) (bottom right) from two WAS patients. Two-fold serial dilutions were analyzed 1 week after transduction for WASp expression by quantitative Western blotting as described in Materials and methods. Membranes were simultaneously hybridized with an α-ERK polyclonal antibody as internal loading control. WASp/ERK ratio is indicated at the bottom of each lane. Number of WW vector integrations per cell (v/cell) was determined by real-time PCR.*

1 week thereafter, cells were analyzed for copy number by quantitative PCR, and for protein by quantitative Western blotting as described in Materials and methods. This indicated that WASp expression levels in WW-transduced HVS-WAS/1 cells were 0.55 times of those found in HVS-N T cells. Likewise, WW-transduced ALLO-WAS/2 cells expressed 0.93 times the levels found in normal allospecific cells (ALLO-NORMAL). Considering the vector copy number in the transduced population, we can conclude that a single transduction event in HVS-WAS/1 T cells by the WW lentiviral vector achieves 0.65 times the levels of WASp expressed in HVS-N cells. Similarly, a single insertion of a WW vector in ALLO-WAS/2 cells achieved 0.34 times the levels expressed by ALLO-NORMAL cells.

WASp expression confers a selective growth advantage to WW-transduced WASp-deficient T cells

We explored the possibility that expression of WASp could confer a selective advantage to transduced cells as it has been previously described in other systems.[29–33] Therefore, HVS-WAS/1 cells were transduced with the WW lentiviral vector at MOIs of 0.01, 0.1 and 1 and kept in culture for 39 days. The percentage of WASp-expressing cells was analyzed by flow cytometry at days 7, 27 and 39 after transduction. Figure 5a shows a robust increase in the percentage of $WASp^+$ cells over time in culture, indicating a selective growth advantage of transduced cells even in experiments where the starting $WASp^+$ population was lower than 1% (MOI of 0.1, solid circles). The cells were cultured in parallel with HVS-WAS/1 cells that had been transduced with the WE vector. Expression of eGFP driven by the *WASP* promoter did not affect cell growth characteristics (data not shown).

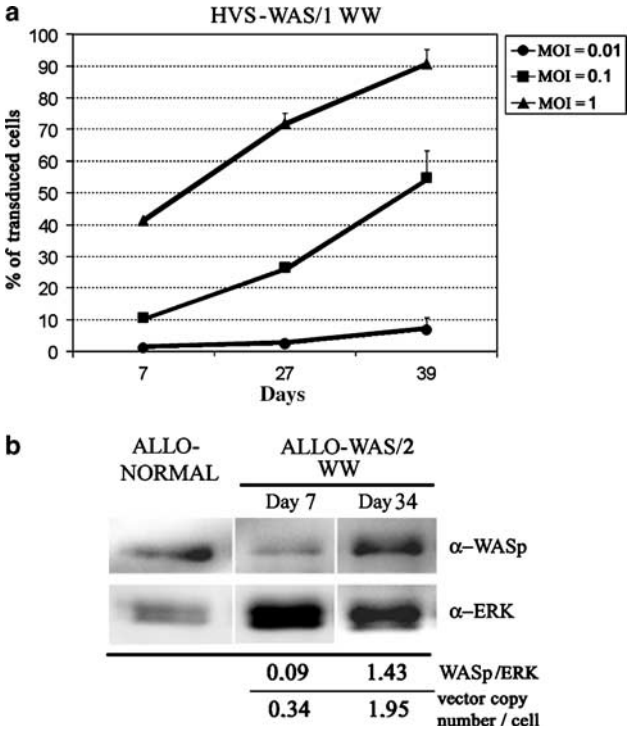

Figure 5 *WASp expression confers a selective growth advantage to T cells. (a) HVS-WAS/1 cells were transduced at day 0 with the WW lentiviral vector at MOIs of 0.01 (solid circles), 0.1 (solid squares) and 1 (solid triangles). Expression of WASp was determined by flow cytometry at indicated days after transduction. Data represent mean ± s.d. of three separate experiments. (b) Allospecific T cells from a WAS patient (ALLO-WAS/2) were transduced with WW vectors 5 days after allostimulation. At days 7 and 34 after transduction, cells were analyzed for vector copy number per cell by real-time PCR and WAS/ERK protein ratio by quantitative Western blotting. Cells were grown by weekly Raji allostimulations as described in Materials and methods.*

In order to corroborate these results, we performed a similar experiment with primary allospecific T cells from a second WAS patient (ALLO-WAS/2). ALLO-WAS/2 cells were transduced 5 days after stimulation and analyzed at days 7 and 34 after transduction. Vector copy number and WASp levels were analyzed by quantitative PCR and quantitative Western blotting as before. Figure 5b shows that WASp levels increased about 15 times from day 7 to day 34 (WASp/ERK ratio was 0.09 at day 7 and 1.43 at day 34). Vector copy number per cell was also increased about six times (Figure 6b, bottom), suggesting that expansion of cell populations with higher numbers of vector integrations had occurred.

Efficient transduction of hematopoietic progenitor cells by lentiviral vectors containing the WASP gene proximal promoter

We next explored whether WW vectors permit transduction of hematopoietic progenitor cells. Mouse $Sca1^+$ bone marrow cells transduced with WW in one single round (MOI = 1) resulted in up to 32% of colonies positive for vector integration as demonstrated by PCR of vector sequences (Figure 6). Transduction efficiencies achieved by SW and WW vectors were very similar, whereas with the SE vector it was almost double (up to 65% of colonies expressed eGFP).

A critical issue in HSC gene therapy approaches is to achieve adequate expression of the therapeutic gene in differentiated target cells. We therefore studied if the *WASP* promoter fragment used in the WW vector was able to drive transgene expression in human myeloid lineages derived from transduced hematopoietic progenitors. To determine this, we constructed a lentiviral vector (WE), which contains the backbone and promoter elements of WW vector but drives the expression of eGFP instead of WASp (see Materials and methods). CD34$^+$ hematopoietic progenitors transduced with WE vectors were differentiated in methylcellulose medium and the colonies analyzed under fluorescence microscopy. Expression of eGFP was readily detectable in colonies from myeloid and erythroid lineages (Figure 7a). These data demonstrate that the *WASP* endogenous promoter in a context of a SIN lentiviral vector is able to achieve expression of the transgene in different hematopoietic lineages after transduction of human progenitors. Transduction efficiency of WE vector ranged from 30–70 percent among colonies from different hematopoietic lineages after one single round of transduction at MOI = 2 (MOI estimated based on HVS-WAS/1 cells) (Figure 7b).

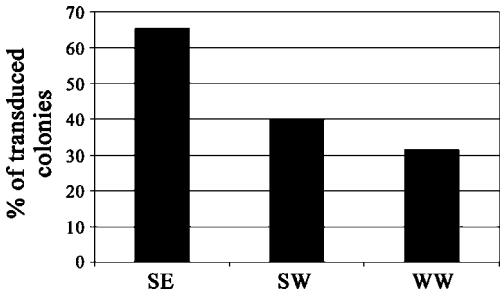

Figure 6 *WW vectors efficiently transduce mouse hematopoietic progenitors. Graph showing percentage of transduced colonies derived from murine Sca1$^+$ bone marrow cells transduced at MOI = 1 by SE (left bar), SW (center bar) and WW (right bar) vectors. Sca1$^+$ cells purified from Balb/c bone marrow were transduced and cultured in methylcellulose medium (see Materials and methods). Analysis of transduction efficiency was performed by PCR of single colonies (SW and WW vector) or eGFP expression (SE).*

Discussion

Gene therapy protocols for haematological and immunological disorders are often based in the re-infusion of gene-modified HSCs obtained using cell purification strategies. However, even purified HSCs populations may differentiate into or fuse with several non-HC types, such as hepatocytes, endothelial, epithelial, neuron or muscle cells.[5–8] The expression of hematopoietic-specific

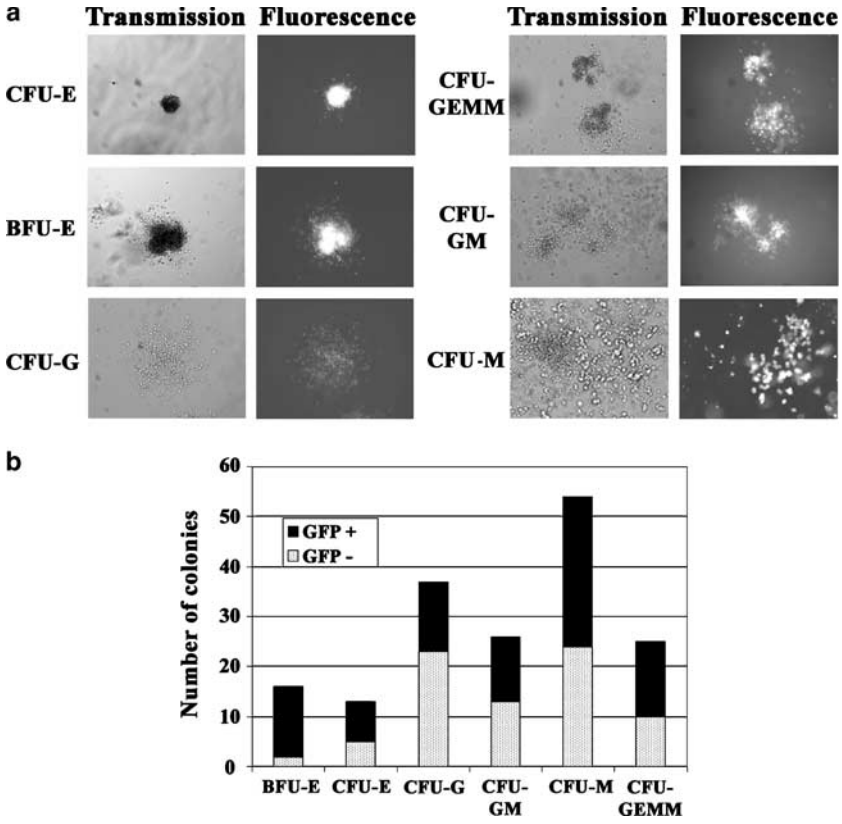

Figure 7 *WASP proximal promoter efficiently drives transgene expression in human hematopoietic precursors. Human CD34$^+$ cord blood cells were purified and transduced at MOI = 2 with the WE vector, a WW-derived vector where the WASP gene has been replaced by eGFP. Transduced progenitor cells were cultured in methylcellulose media for 10–16 days before analysis. (a) Light transmission and fluorescence microscopy of individual colonies (BFU-E, burst-forming unit-erythroid; CFU-E, CFU-erythroid; CFU-G, CFU-granulocyte; CFU-GM, CFU-granulocyte, macrophage; CFU-M, CFU-macrophage; and CFU-GEMM, CFU-granulocytic, erythroid, macrophage, megakaryocytic). (b) Graph showing the number of colonies of each hematopoietic lineage expressing the eGFP transgene (solid bars) over the number of colonies eGFP$^-$ (light bars).*

genes in non-hematopoietic tissues might result in undesired side effects (reviewed in Baum *et al*[34]). It would therefore be advantageous to develop vectors able to drive hematopoietic-specific expression of the therapeutic transgene.

Expression of WASp is normally restricted to HCs, including stem cells and progenitors.[22–24] WASp has well characterized roles in the functionality of immune cells and in the formation and survival of platelets. The biological activity of WASp in stem cell populations is witnessed by the apparent non-random distribution of X-inactivation in female carriers of a mutant *WASP* gene,[35] and the demonstration of homing and engraftment defects in mutant mice.[36] The widespread but hematopoietic-restricted expression profile of WASp is dictated by *WASP* gene regulatory elements which are therefore attractive for expression of many hematopoietic genes.

For this reason, we designed and tested lentiviral vectors incorporating proximal promoter sequences from the natural *WASP* gene. Previous functional characterization of the *WASP* gene promoter has been limited.[24,37,38] However, a 170 bp fragment 5′ upstream of the transcription starting site does contain four potential binding sites for hematopoietic transcription factors (two for Ets-1, one for c-Rel, and one for PU.1) and is sufficient to confer preferential hematopoietic expression in transient reporter gene assays.[24] An alternate *WASP* promoter has been found 6 Kb upstream of the proximal promoter containing c-Myb and PU.1 binding sites[37] and has been suggested to have a role in WASp expression during the earlier stages of hematopoiesis. This also has not been functionally evaluated in detail.

We have shown that WASp expression regulated by proximal sequences of the *WASP* gene promoter in the context of an integrating vector system is transcriptionally targeted to HCs. A detailed analysis of cells transduced by vectors incorporating the SFFV LTR or the *WASP* gene proximal promoter showed that the latter is 135–350 times more efficient driving the expression of WASp in hematopoietic than in non-HCs. Interestingly, the activity (in terms of protein expression) of the *WASP* gene promoter in T cells was equivalent to that of the SFFV LTR, a highly potent regulatory element containing the duplicated enhancer sequences characteristic of many gammaretroviral vector LTRs.[39] Likewise, we have shown that a single WW vector integration is able to express protein levels that are close (0.34–0.63 times) to those found in normal cells. Therefore, the proximal *WASP* gene promoter in the context of a SIN lentiviral vector can also achieve physiologically relevant levels of WASp in transduced cells. In addition, we have demonstrated efficient transduction of both mouse and human hematopoietic progenitors, with preservation of gene expression following myeloid and erythroid differentiation. The proximal *WASP* gene promoter in the context of a SIN lentiviral vector may have useful characteristics for clinical trials of gene therapy for WAS as it achieves hematopoietic-specific and physiologically relevant levels of WASp. Expression of the *WASP* transgene confers a selective growth advantage to transduced T cells as described in spontaneous reversions of WAS patients[29–31] as well as in other models of CD3-activated $WASp^+$ cells.[32,33] This observation indicates that the protein expressed in WW-transduced cells is functional. Furthermore, we have found that transduction of WAS-deficient T cells with the WW vector results in full reconstitution of cellular defects, including restoration of morphology and CD3-mediated responses (our unpublished data). We therefore propose that the *WASP* gene regulatory elements may provide a safer alternative to retroviral LTR sequences for the regulation of hematopoietic genes.

In summary, we have described the development of a SIN lentiviral vector that directs hematopoietic-specific, physiological transgene expression of a therapeutic gene (WASp) through the proximal *WASP* gene promoter. We believe that our data represent a significant step towards the goal of achieving controlled hematopoietic-specific transgene expression, an objective that is becoming of increasing interest in the light of vector-mediated cell transformation in animal models[34] and human clinical trials.[40]

Materials and methods

Cell lines and culture media

The generation and characterization of HVS-WAS/1 (derived from two WAS patients) as well as HVS-N (derived from a normal individual) have been described in detail.[26] Primary allospecific T cells from a second WAS patient (ALLO-WAS/2) (cared for by Dr N Matamoros, Hospital Son Dureta, Palma de Mallorca, Spain) and from a normal healthy individual (ALLO-NORMAL) were generated in our laboratory by mitomycin-C-treated Raji B-cell allospecific weekly stimulation as previously described in detail.[25] All HVS-T and allospecific cell lines were cultured in a mixture (1:1) of RPMI 1640 (Bio-Whittaker, Verviers, Belgium) and Panserin 401 (PAN Biotech, Aidenbach, Germany) media supplemented with 10% of fetal calf serum (FCS) (Gibco-BRL, Middlesex, UK), glutamine, penicillin–streptomycin and 50 UI/ml of recombinant human IL-2 (Hoffman-LaRoche, Nutley, NJ, USA; kindly supplied by the National Institutes of Health AIDS reagent program, Rockville, MD, USA); RKO (colon adenocarcinoma cells) were grown in Dulbecco's modified Eagle's medium (Gibco), supplemented with 10% FCS, glutamine and antibiotics as above; and HUVEC (primary umbilical cord cells) were grown in endothelial cell growth medium (PromoCell, Heidelberg, Germany). Primary FDCs were obtained from human tonsils and cultured in RPMI 1640, containing 20% FCS until the cell line was established (usually 2 weeks). Jurkat T cells were cultured in RPMI 1640 supplemented as above, without the addition of rIL-2.

Plasmids

The HIV packaging (pCMVΔR8.91) and vesicular stomatitis virus(VSV)-G (pMD.G) plasmids were kindly provided by D Trono. The packaging plasmid pCMVΔR8.91 encodes *gag*, *pol*, *tat* and *rev* genes. The pMD.G plasmid encodes the VSV-G protein. The lentiviral vector plasmid SE (previously named HRSIN-CSEW[28]) contains an SFFV LTR that drives an eGFP expression cassette that was used to construct WASp-expressing vectors.

Lentiviral vector constructs

The lentiviral vector backbone used for this study has been described previously.[28] To construct SW, the WPRE

sequence was eliminated from the SE vector and the *WASP* cDNA amplified by RT-PCR inserted into the SE vector in place of the eGFP. The SW vector drives the expression of the *WASP* gene by the strong promoter from SFFV LTR. The proximal *WASP* gene promoter (extending from bases −1 to −499 from the transcriptional start site) was derived by PCR from genomic DNA using the following primers: 5′CCGGAATTCGGGATT ACAGGTGTGAGC3′ and 5′CGCGGATCCGGTGCTTTC TGCCCTTGTCTTC3′. The WW vector was constructed by *Eco*RI and *Bam*HI restriction enzyme digestion and ligation of the SW vector and the PCR product. This excised the SFFV LTR promoter sequences and replaced them by the 500 bp fragment containing the 5′ proximal *WASP* promoter. WE vector was constructed by replacing the *WASP* gene cDNA from the WW vector with the *eGFP* cDNA from the pHRSin18-SFFV LTR plasmid[28] by double restriction enzyme digestion and ligation with *Bam*HI–*Xho*I.

Vector production

Lentiviral vectors were produced by cotransfection of 293T cells with three plasmids: (1) vector plasmid (SE, WE, SW or WW), (2) packaging plasmid pCMVΔR8.91 and (3) envelope plasmid pMD.G, as described previously.[41] Briefly, 293T cells (6×10^6) were plated on a 10-cm tissue culture-grade Petri dish (Sarstedt, Newton, NC, USA) the day before transfection to ensure exponential growth and 80% confluence. Vector plasmids, together with packaging and envelope plasmids (27 μg total DNA; plasmid proportions of 3:2:1, respectively), were resuspended in 1.5 ml of OPTI-MEM media (Gibco) and mixed at room temperature for 20 min with 60 μl of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) and then diluted in 1.5 ml of OPTI-MEM. The plasmid–lipofectamine mixture was added to prewashed cells and then incubated for 6–8 h. The producer cells were then washed and further cultured for 48 h in 10 ml of OPTI-MEM media. Viral supernatants were collected and filtered through a 0.45 μm filter (Nalgene, Rochester, NY, USA), aliquoted and immediately frozen at −80°C.

Cell transduction and vector titration

Exponentially growing target cells were washed in PBS, and 2×10^5 cells were seeded per well in 500 μl of their appropriate media in 24-well plates. Supernatants were added to the culture and incubated overnight. After 72-h incubation or when indicated, cells were collected, washed, fixed in 0.2% of paraformaldehyde and analyzed in a FACScan Flow Cytometer (Becton-Dickinson, San Jose, CA, USA). Viral titers (transduction units/ml) were calculated based on the initial amount of target cells and the percentage of GFP^+ or $WASp^+$ cells detected in the linear range of a serial dilution of the supernatant. The MOI is defined as the number of viral vectors per cell, and was estimated over the HVS-WAS/1 cell line. This was required since the WW vectors are not expressed in non-HCs such as 293T, and should therefore be assayed on a hematopoietic WASp-deficient cell line.

Mouse hematopoietic progenitors transduction and methylcellulose colony assay

Bone marrow cells pooled from four Balb/c mice were harvested and $Sca1^+$ cells selected using MACS immunomagnetic columns (Miltenyi Biotec, Germany). Briefly, 4×10^7 bone marrow cells were incubated with 50 μl of magnetic-labeled Sca-1 antibodies (Miltenyi) and passed through the columns. After two washes with PBS, $Sca1^+$ cells were eluted with RPMI containing 30% of FCS and supplemented with a cytokine cocktail (murine Scf 100 ng/ml; IL-6 20 ng/ml; mFlt-3L 10 ng/ml) (StemCell Technologies, Vancouver, Canada). Cells were plated at a concentration of 10^6 cells/ml and incubated O/N with lentiviral vectors' supernatant (MOI = 1 estimated over HVS-WAS/1 cells). Cells were then washed and incubated in Iscove's modified Dulbecco's medium (IMDM) media (Gibco) and 500 μl containing 10^4 cells were mixed with 2.5 ml of Metho Cult media (StemCell Technologies). Cells were cultured in 35 mm Petri dishes at 37°C and 5% of CO_2. Stem cell colonies were counted and analyzed after 20 days.

Human hematopoietic progenitors transduction and methylcellulose colony assay

Human cord blood was obtained from placental and umbilical tissues and diluted 1:3 in IMDM (Gibco). Mononuclear cells were collected by density centrifugation (Ficoll–Hypaque, Pharmacia Biotech, Uppsala, Sweden) and the $CD34^+$ subset isolated using α-CD34 MACS immunomagnetic columns (Miltenyi) as described above. Cells were analyzed by flow cytometry immediately after purification for $CD34^+$ expression using a PE-α-CD34 antibody (Miltenyi). The percentage of $CD34^+$ cells was typically higher than 85%. For transduction, 10^5 $CD34^+$ cells were seeded in 96-well plates in Stem-Spam media (StemCell Technologies) containing antibiotics and 10 ng/ml thrombopoietin (Peprotech EC, London, UK), incubated overnight with viral supernatants at indicated MOIs and then washed with IMDM. Human clonogenic progenitor assays were carried out by seeding 10^3–5×10^3 cells in two 10 mm Petri dishes in complete methylcellulose medium with recombinant cytokines (MethoCult GF-4434, StemCells Technologies). After 10–16 days in culture, colonies were analyzed in an inverted fluorescence microscope and images captured by a digital camera.

Immunostaining and flow cytometry

After 72 h or other times if indicated, cells were collected, washed and permeabilized with Citofix/cytoperm (Pharmingen, San Diego, CA, USA) following the manufacturer's recommendations. Cells were subsequently washed and preincubated in a PBS-blocking solution containing 2% normal goat serum and 0.05% saponin for 20 min at 4°C. After washes, intracellular staining of WASp was carried out by indirect immunofluorescence, using 2 μg of anti-WASp monoclonal antibody B9 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted in 200 μl of blocking solution and incubated at 4°C for 1 h with continuous agitation in an orbital shaker. Cells were washed in PBS–0.02% saponin and incubated again with 2 μg of an FITC-labeled goat anti-mouse IgG antisera (Caltag Laboratories, Burlingame, CA, USA) (diluted in PBS–0.05% saponin) for 45 min at 4°C in continuous agitation. Cells were washed 2× in PBS–0.02% saponin followed by a final wash in PBS alone. Cells were analyzed in the FACScan Flow Cytometer.

SDS-PAGE, semiquantitative and quantitative Western immunoblotting

Cells were lysed with 1% NP-40 lysis buffer containing protease inhibitor cocktail (Sigma, St Louis, MO, USA), resolved by SDS-PAGE (10% polyacrylamide gels, reducing conditions) and electrotransferred to Hybond-P PVDF membranes (Amersham, Buckinghamshire, UK). Membranes were blocked with 5% nonfat milk and probed for 1 h at room temperature with 1 μg/ml of the anti-WASp mAb D1 (Santa Cruz Biotechnology) followed by incubation with HRPO-labeled goat anti-mouse antibody (1:10 000 dilution) (Caltag). The blot was developed by chemiluminescence (ECL, Amersham) and exposed to autoradiographs (Amersham). Loading controls were carried out by rehybridization of stripped membranes with an α-Erk polyclonal antibody (anti-MAP kinase 1/2, Upstate Biotechnology, UK). Semiquantitative analysis was carried out by densitometric scanning of bands using the Quantity One version 4.5.0 software (Bio-Rad).

Quantitative Western blot analysis was performed using the ECL Advanced Western Blotting Detection Kit (Amersham Bioscience, UK). Membranes were blotted and probed as above, but were revealed by incubation for 1–5 min with a mixture of ECL Advanced reagents. Quantification of light emission was detected using a QuemiDoc XRS instrument (Bio-Rad Laboratories, Hercules, CA, USA) and analyzed at 440 nm with Quantity One version 4.5.0 software (Bio-Rad). Contribution of each band was recorded and expressed as relative intensity per mm^2.

DNA preparation, PCR and quantitative PCR

Genomic DNA of tissue culture cells or methylcellulose colonies was isolated using a genomic DNA extraction kit (Promega, Madison, WI, USA). DNA concentration was estimated by UV spectrophotometry. Transduction of methylcellulose colonies was determined by amplification of a 125 bp fragment from the *WASP* cDNA encompassing exons 9 and 10 from the *WASP* gene. The PCR were performed in a thermocycler (Mastercycler, Eppendorf AG, Hamburg, Germany) using the following primers: forward 5′-AGGCTGTGCGGCAGGAGAT-3′; and reverse 5′-CAGTGGACCAGAACGACCCTTG-3′ and parameters: $1\times$(95°C for 2 min), $30\times$(95°C for 30 s, 62°C for 30 s and 72°C for 30 s) and $1\times$(72°C for 10 min). PCR amplification products were observed in a 1% agarose gel with a Lambda 1 kb marker after staining with ethidium bromide.

The real-time PCR reactions were performed in the iCycler iQ Real-Time PCR Detection System (Bio-Rad). Samples were mixed with iQ™ Supermix (Bio-Rad) containing each dNTP, 50 U/ml iTaq DNA polymerase, 6 mM $MgCl_2$, SYBR green and 200 nM of the specific primers: forward 5′-GCTTAATACTGACGCTCTCGCA-3′; and reverse 5′-GGCTAACTAGGGAACCCACTG-3′, which amplify a 321 bp fragment containing the 5′ splice site and 89 bp from the 5′LTR of the vector. DNA from 1×10^5 HVS-WAS/1 cells were mixed with 10-fold increasing amounts of plasmid DNA (1 up to 1×10^5 copies) for the standard curve. The parameters for the PCR were: $1\times$(95°C for 2 min), $45\times$(95°C for 30 s, 61.4°C for 30 s and 72°C for 30 s) and $1\times$(72°C for 2 min). Melting temperature was optimized in preliminary experiments.

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