

Comparison of Insulators and Promoters for Expression of the Wiskott–Aldrich Syndrome Protein Using Lentiviral Vectors

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Abstract

Gene therapy for the treatment of Wiskott–Aldrich syndrome (WAS) presents an alternative to the current use of allogeneic bone marrow transplantation. We describe the development of a self-inactivating lentiviral vector containing chromatin insulators for treatment of WAS and compare a gammaretroviral (MND), human cellular (EF1 α), and the human WASp gene promoter for expression patterns *in vivo* during murine hematopoiesis using the green fluorescent protein (GFP) marker. Compared with the EF1 α and the WASp promoters, expression from the MND promoter in mouse transplant recipients was much higher in all lineages examined. Importantly, there was sustained expression in the platelets of secondary recipient animals, necessary to correct the thrombocytopenia defect in WAS patients. Analysis of WAS protein expression in transduced human EBV-immortalized B-cells and transduced patient peripheral blood mononuclear cells also demonstrated stronger expression per copy from the MND promoter compared with the other promoters. In addition, when analyzed in an *LMO2* activation assay, the addition of an insulator to MND-promoter-containing constructs reduced transactivation of the *LMO2* gene. We propose a clinical trial design in which cytokine-mobilized, autologous, transduced CD34⁺ cells are administered after myelosuppression.

Introduction

WISKOTT–ALDRICH SYNDROME (WAS) is an X-linked recessive immunodeficiency that occurs at a frequency of five in every million live births. Patients present with thrombocytopenia, eczema, recurrent infections, and a high frequency of autoimmune disease and malignancy and have a shortened life expectancy (Imai *et al.*, 2004; Bosticardo *et al.*, 2009). Patients with mild WAS may be treated with supportive care. The only cure for WAS is bone marrow (BM) transplantation. Patients with matched sibling donors have an 88% survival rate 7 years post-treatment (Ozsahin *et al.*, 2008). Previously, patients who received a matched unrelated donor or mismatched related donor graft have had a poorer prognosis (71% and 55%, respectively) (Filipovich *et al.*, 2001), but recent analyses suggest that the outcome has improved (Moratto *et al.*, 2011; Mahlaoui *et al.*, 2012; Shin *et al.*, 2012).

Recent clinical trials indicate that various immunodeficiencies, including WAS, can be successfully treated by retroviral-mediated gene transfer into repopulating hematopoietic cells (Cavazzana-Calvo *et al.*, 2000; Boztug *et al.*,

2010; Hacein-Bey-Abina *et al.*, 2010; Gaspar *et al.*, 2011). The WAS gene therapy trial used a gammaretroviral vector to facilitate delivery of the human WAS protein (hWASp) cDNA driven by the MMP promoter (a derivative of the myeloproliferative sarcoma virus promoter) (Klein *et al.*, 2000) into the patient's own CD34⁺ cells, which were subsequently transplanted back to the patient after chemomyelosuppression (Boztug *et al.*, 2010). This resulted in an increase in platelet counts, emergence of corrected lymphoid cells, and remission of other disease manifestations, demonstrating the effectiveness of this treatment strategy. However, 4 of 10 patients treated to date have developed leukemia (Braun *et al.*, 2011; Paruzynski *et al.*, 2011). All four patients were shown to have a vector integration close to the LIM domain only two (*LMO2*) locus causing transactivation of this proto-oncogene, a phenomenon that has also been seen in the treatment of severe combined X-linked immunodeficiency using similar vectors (Hacein-Bey-Abina *et al.*, 2003a,b; Hacein-Bey-Abina *et al.*, 2008; Howe *et al.*, 2008).

Self-inactivating (SIN) lentiviral vectors have proven less oncogenic than gammaretroviral LTR-driven vectors in a

number of assays (Montini *et al.*, 2006, 2009; Ryu *et al.*, 2008; Modlich *et al.*, 2009). By switching to a SIN lentiviral vector for the treatment of WAS, the therapeutic benefit to the patient should be maintained while the oncogenic potential of the delivery system is reduced. The use of insulators to transcriptionally isolate the transgene may further reduce the oncogenic potential of the vector. Originally discovered in association with DNase I hypersensitivity sites within the β -globin locus control region (Chung *et al.*, 1993), insulators are able to prevent histone modification and DNA methylation, which may lead to heterochromatin formation and down-regulation or silencing of the transgene (Chung *et al.*, 1997). Insulators also prevent regulatory signals in the vector (either positive or negative) from acting on nearby genes, leading to their deregulation (Gaszner and Felsenfeld, 2006).

Lentiviral vectors are currently being developed by a number of groups to deliver the hWASp cDNA into autologous BM cells (reviewed by Galy and Thrasher, 2011). Initial studies used a strong retroviral LTR promoter to drive WASp gene expression and resulted in high levels of protein expression and functional correction (Dupre *et al.*, 2004). More recent studies have focused on the use of the endogenous WASp promoter (Martin *et al.*, 2005; Charrier *et al.*, 2007; Marangoni *et al.*, 2009). Functional correction has been achieved in the mouse model using a 1.6 kb WASp promoter fragment to drive the WASp cDNA (Dupre *et al.*, 2006; Marangoni *et al.*, 2009; Scaramuzza *et al.*, 2013). These data contrast with results obtained by others in which full correction of the WAS phenotype was achieved in the murine model with a vector having a retroviral promoter, whereas only partial correction was achieved with a vector having the 1.6 kb WAS promoter fragment (Astrakhan *et al.*, 2010). Methodologies have also been developed for the large-scale manufacture and characterization of the lentiviral vector having the 1.6 kb WAS promoter fragment for use in a gene therapy application (Merten *et al.*, 2011). Clinical trials have been opened at four sites over the period 2009–2011 and are currently recruiting and enrolling patients as summarized in Galy and Thrasher (2011).

We have compared expression levels from a retroviral promoter, the cellular EF1 α promoter, and the WASp gene promoter (P500 or P1600) in insulated lentiviral vectors in hematopoietic cells with the goal of identifying a vector design to be used in a future clinical trial that has been funded by the National Heart Lung and Blood Institute (P01HL53749).

Results and Discussion

Clinical trial

General design. All patients with WAS who lack a matched related or unrelated donor will be candidates for gene therapy. Only patients who are large enough to undergo apheresis (≥ 12 kg) will be eligible. Patients will be screened for evidence of cells containing WASp secondary to a spontaneous correcting reversion mutation, but such patients will be eligible for enrollment if they have persistent thrombocytopenia and immunodeficiency. Cellular WASp levels will be compared by fluorescence-activated cell sorting analysis before and after gene transfer to determine efficacy in patients with residual WASp production. A preliminary apheresis after G-CSF administration will be performed to

evaluate each individual patient's capacity to mobilize CD34⁺ cells. These cells will be used to evaluate gene transfer efficiency, and the majority will be cryopreserved as a backup for the subsequent apheresis and transduction procedures. Depending on the success of the initial mobilization, G-CSF only or G-CSF administered with AMD2100 will be used for the definitive cytopheresis mobilization. Purified CD34⁺ cells will be cultured under conditions previously described (Kim *et al.*, 2010). Patients who have residual manifestations of WAS after a stem cell transplant or who fail to engraft will not be eligible for protocol enrollment.

The rationale for considering gene therapy rather than transplantation from a mismatched related or unrelated donor as the first therapeutic alternative for WAS patients lacking a matched sibling or fully matched unrelated donor is based on the poorer outcome after transplantation with a partially matched donor (Filipovich *et al.*, 2001) and the high frequency of long-term complications (Ozsahin *et al.*, 2008). Also, the gene therapy intervention can be implemented and, if unsuccessful, followed subsequently by an allogeneic transplantation procedure. The patients will be stratified based on age into two groups on the basis of the observation in X-linked severe combined immunodeficiency patients that the gene therapy intervention, although successful among infants, is significantly less successful in older patients (Thrasher *et al.*, 2005; Chinen *et al.*, 2007). Only one of five older patients had improvement in immunological function after infusion of genetically modified, primitive hematopoietic cells. The poorer outcome in older patients is thought to reflect diminished thymic function with age, a fact that may also be important in WAS patients, and therefore we propose an age stratification. We have somewhat arbitrarily chosen age 9 and younger for stratum I and age 10 and older for stratum II. Our goal is to enroll a minimum of five participants in each stratum. All patients will receive a myelosuppressive regimen consisting of Busulfan at a total dose of 8 mg/kg given at a dose of 2 mg/kg at 12 hr intervals before infusion of transduced CD34⁺ cells, as this dose has been used successfully in a prior gene therapy trial for WAS (Boztug *et al.*, 2010). Trial participants will be monitored periodically after the gene transfer procedure to evaluate reconstitution and determine the proportion of genetically modified cells in peripheral blood.

Objectives. The primary objective for each of the two strata is to investigate safety and efficacy with respect to hematological and immunological reconstitution in WAS patients transplanted with autologous CD34⁺ cells that have been transduced with a SIN lentiviral vector expressing the WASp coding sequences. Objective endpoints by 1 year are an increase in platelet count ($\geq 50,000$) and IgM [\geq lower limit of normal range for age (Orkin *et al.* 2009)]. The secondary objectives are (a) to estimate the incidence of, and describe serious side effects caused by lentiviral gene therapy; (b) to determine the integration site distribution of the WASp lentiviral vector in reconstituted peripheral blood cells; and (c) to observe for evidence of clonal dominance and/or the development of leukemia.

Inclusion criteria. Only patients who have a documented mutation in the WASp gene will be candidates for a gene

therapy intervention. All patients who are ≥ 12 kg and who have significant thrombocytopenia (platelet count $\leq 50,000/\text{mm}^3$ on ≥ 3 occasions) and any other manifestation of WAS will be eligible. Other manifestations may include the following: (a) two or more infections requiring chronic therapy; (b) significant eczema requiring chronic therapy; (c) autoimmune disease; (d) malignancy or premalignant condition; and (e) positive family history defined by a family member with WAS who died before 10 years of age.

Exclusion criteria. Exclusion criteria include the following: (a) has a suitable, available 6/6 HLA-matched sibling donor; (b) has a suitable, available 10/10 HLA allele-matched unrelated donor identified through the National Marrow Donor Program (potential participants will become eligible for the gene therapy trial if no donor has been identified within 90 days); (c) HIV infection; (d) a medical condition that would preclude anesthesia or sedation for apheresis or a BM harvest, if needed; (e) a medical condition precluding the administration of myelosuppressive Busulfan therapy; and (f) a prior allogeneic stem cell transplant.

Objectives and study design

Our intention is to perform a comprehensive series of studies to provide preclinical data for the clinical trial described above involving the use of a SIN lentiviral vector for transfer of the WASp gene into repopulating hematopoietic stem cells from WAS patients. Murine models are being used to evaluate promoter function. In our experiments, we have utilized the GFP marker in wild-type mice, as the use of this marker provides a facile methodology for evaluating promoter function in the series of animals while testing a significant number of promoters. Our results indicate that the MND γ -retroviral promoter gives consistently high levels of expression in multiple hematopoietic lineages.

We are collaborating with the Rawlings' laboratory to evaluate promoter function in the murine WAS mouse strain. This work is supported by a subcontract from our program project grant (P01HL53749). The initial results from the Rawlings' lab have been published (Astrakhan *et al.*, 2012). These studies demonstrated that the MND promoter gave full correction of the WAS phenotype in the murine model, whereas the vector with the 1.6 WASp gene promoter gave only partial correction. Ongoing studies provide further evaluation of the level and duration of expression of the WASp transgene in the knockout mouse model. A formal toxicity study is in the preliminary stages of planning.

Promoter function in the studies described in this article was also evaluated in human B-cells (EBV immortalized B-cells) from normal individuals and WAS patients using a vector encoding WASp. Similar studies were done with peripheral blood mononuclear cells from both normal and individuals with WAS. Our studies are designed to provide a comprehensive evaluation of promoter function in an effort to identify the appropriate promoter for a clinical vector. We have also tested insulator elements, as the use of the MND promoter will almost certainly occur in the context of a vector with insulator elements.

Toxicity studies are being performed in a variety of assays. Described in this article are data using targeted integration to recreate vector insertions observed in patients with immuno-

deficiency treated with gene therapy, who then developed leukemia. Cre-mediated cassette exchange is used to place candidate vector genomes into these targeted integration sites. We have compared a vector with the MND promoter without insulator to comparable vectors having insulator elements.

Summary of data

Vector construction and promoter activity during hematopoiesis. Gene therapy for WAS requires that the promoter be active in all hematopoietic lineages except erythrocytes. This can be achieved relatively easily in B lymphocytes and T lymphocytes, where there is positive selective pressure on hWASp-expressing cells because of restoration of responses to proliferative signals (Klein *et al.*, 2003; Strom *et al.*, 2003; Westerberg *et al.*, 2008). However, hWASp-expressing cells of the myeloid lineage (megakaryocytes, monocytes, granulocytes, macrophages) do not have such an advantage, and therefore in these cells the promoter utilized must drive sufficient hWASp expression independent of selection pressure. In addition, it has been shown by two groups recently that if the T lymphocyte defect is corrected (which requires a low amount of hWASp) in WAS mice without correcting the B lymphocyte defect (which requires a higher amount of hWASp), a state of autoimmunity develops (Becker-Herman *et al.*, 2011; Recher *et al.*, 2012).

To compare the effect of promoters and insulators on hWASp (or a GFP marker) expression in lentiviral vectors, a series of vectors were constructed (Fig. 1) (Hanawa *et al.*, 2002, 2009). The insulators were derived from the canonical 1.2 kb chicken hypersensitive site four (cHS4) fragment from the β -globin locus (Emery *et al.*, 2000; Gaszner and Felsenfeld, 2006; Arumugam *et al.*, 2007). The 400 bp cHS4 insulator includes the 250 bp core element and adjacent 150 bp of sequences (Aker *et al.*, 2007), while the 650 includes the 250 bp core and 400 bp from the 3' end of the 1.2 kb insulator fragment (Arumugam *et al.*, 2009). It has previously been shown that the 400 bp cHS4 insulator placed in the reverse orientation within the LTR, combined with a 46 bp deletion that removes the residual nef sequence, reduces the frequency of polyadenylation read-through from a lentiviral LTR (Hanawa *et al.*, 2009), and therefore this configuration was used for our vectors. Similarly, the 650 bp cHS4 insulator was placed in the reverse orientation. For both the EF1 α and MND promoters, expression in the context of a 400 bp, 650 bp, or noninsulated lentivirus (400EF1 α , 650EF1 α , NIEF1 α , 400MND, 650MND, and NIMND) was tested, while for the endogenous hWASp promoter vectors (P500 and P1600), the 400 bp and noninsulated backbones were utilized (400P500, NIP500, 400P1600, and NIP1600) (Fig. 1). All vectors were based on the pCL20cw series of lentiviral vectors and contain a mutated WPRE (Hanawa *et al.*, 2009).

GFP vectors were used to examine promoter activity during murine hematopoiesis. Concentrated lentiviral vector stocks were produced for each vector and used to transduce $\text{lin}^- \text{CD45.1}^+$ BM cells at a multiplicity of infection (MOI) = 20 and transplanted into lethally irradiated CD45.2^+ mice. BM from primary recipients was harvested at 22 weeks, and the cells from four mice having the highest engraftment and GFP expression (based on a 16-week peripheral blood analysis) were each transplanted into two secondary recipients. Secondary recipients were analyzed at 28–36 weeks

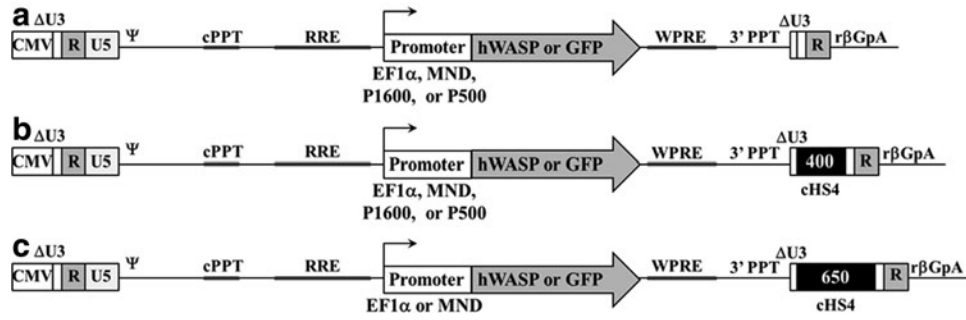


FIG. 1. Schematic of vectors used in this study. **(a)** Noninsulated vectors expressing hWASP cDNA or GFP from the EF1 α , P500, P1600, or MND promoters. **(b)** 400 bp cHS4 insulated vectors expressing hWASP cDNA or GFP from the EF1 α , P500, P1600, or MND promoters. **(c)** 650 bp insulated vectors expressing hWASP cDNA or GFP from the EF1 α or MND promoters. 3'PPT, 3' polypurine tract; β GpA, β -globin polyadenylation signal; cHS4, chicken hypersensitive site four; CMV, cytomegalovirus promoter; cPPT, central polypurine tract; hWASP, human Wiskott–Aldrich syndrome protein; RRE, Rev response element; WPRE, Woodchuck hepatitis post-transcriptional regulatory element.

post-transplant. The blood and BM were examined from both primary and secondary recipients for vector copy number (Supplementary Fig. S1; Supplementary Data are available online at www.liebertonline.com/humc) and for GFP expression (Fig. 2 and Supplementary Figs. S2 and S3) and for vector marking of methylcellulose culture colony forming units (CFU-C) (Supplementary Table S1). The inguinal, auxiliary, cervical, and mesenteric lymph nodes and spleen were pooled to examine expression in lymphoid tissues (Supplementary Fig. S4).

All of the promoters examined in this study did express in all relevant hematopoietic lineages. The details are provided

in Supplementary Data. The incorporation of insulators had a relatively minor impact on virus titers (<1 log drop with the 650 bp and no drop with the 400 bp; Supplementary Table S2). The effect of insulators on expression from the individual promoters, including the MND promoter, was modest. However, there were great differences in promoter activity. The endogenous WASp promoter fragments showed weak expression in the mouse model. In general, expression from the P500 and P1600 promoters was reduced in secondary transplants and raises concerns as to whether either of the promoters would provide sufficient expression for long-term correction. In addition, long-term

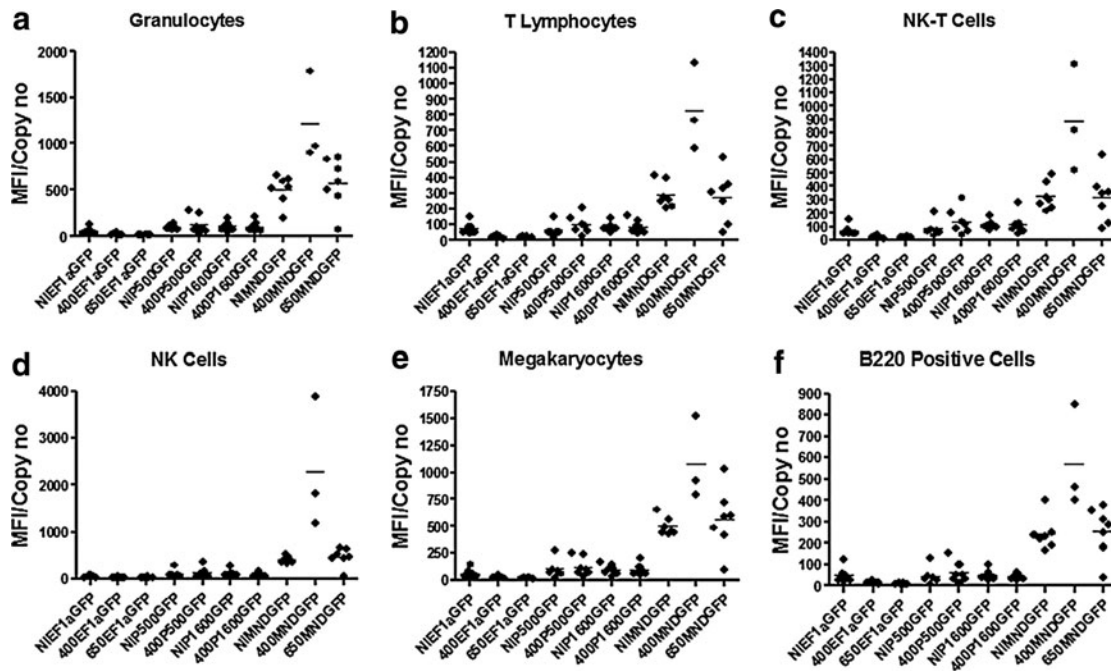
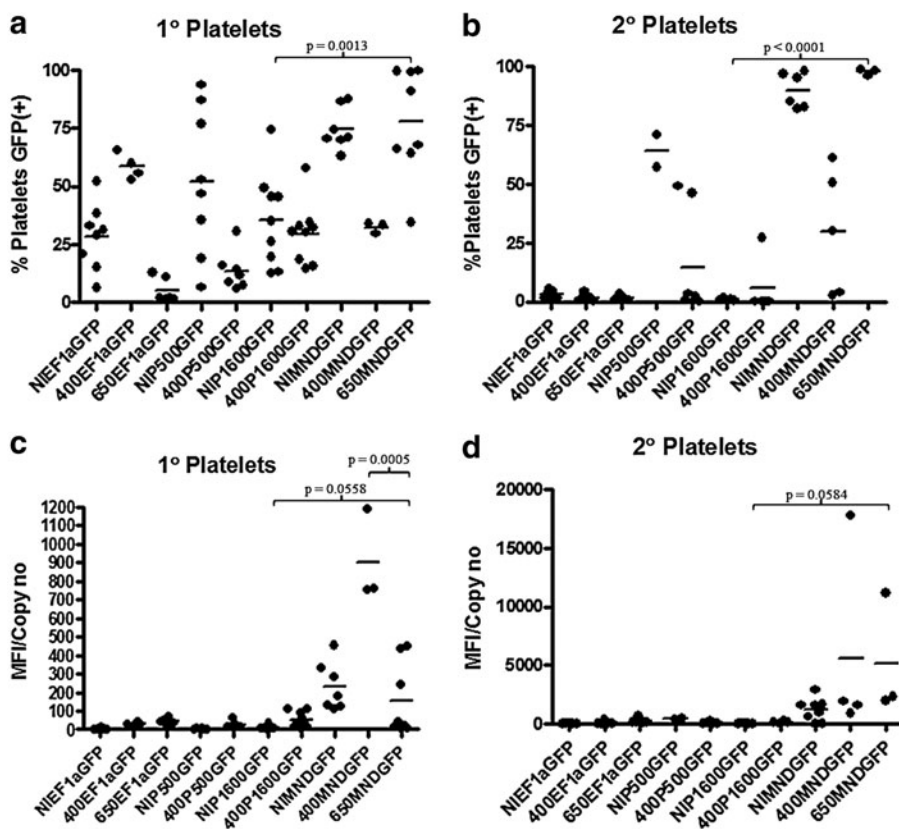


FIG. 2. GFP expression in various lineages from primary bone marrow as determined by flow cytometry. **(a)** Granulocytes (CD45.1⁺/CD11b⁺/GR1⁺/GFP). **(b)** T lymphocytes (CD45.1⁺/CD3⁺/NK1.1⁻/GFP). **(c)** NK-T cells (CD45.1⁺/CD3⁺/NK1.1⁺/GFP). **(d)** NK cells (CD45.1⁺/CD3⁻/NK1.1⁺/GFP). **(e)** Megakaryocytes (CD45.1⁺/CD41⁺/GFP). **(f)** B220⁺ cells (CD45.1⁺/B220⁺/GFP). A two-tailed, heteroscedastic variance *t*-test was used to calculate *p*-values between the groups shown. Student's *t*-tests were used throughout to assess significance. *p*-Values less than or equal to 5% or 0.05 were deemed significant.

FIG. 3. Platelet analysis in transplant recipients. (a) GFP marking in the platelets of primary recipients. (b) GFP marking in the platelets of secondary recipients. (c) GFP expression per copy in the platelets of primary recipients. (d) GFP expression per copy in the platelets of secondary recipients. Copy number was determined by analysis of bone marrow.



expression in the platelets and megakaryocytes, needed to correct the thrombocytopenia defect, was not seen with the WASp gene promoters (Fig. 3 and Supplementary Table S3). Expression from EF1 α promoter was also very low in the mouse model, showing a lack of long-term expression in megakaryocytes and platelets. In contrast, expression from the MND promoter was much stronger and that expression level was maintained in all lineages 22 weeks after transplantation, even when challenged with a second repopulation/engraftment event and evaluated over 28–36 weeks after the secondary transplantation.

Vectors in which the WASp coding sequences were under the control of a retroviral promoter have been shown to correct human hematopoietic cell defects *in vitro* (Dewey *et al.*, 2006; Charrier *et al.*, 2007) and indeed have been used in the initial clinical trial in which WAS patients were shown to benefit from the gene transfer procedure (Boztug *et al.*, 2010). Our results contrast with those of Charrier *et al.* (2007), in which vectors using the endogenous promoters of the WASp gene were shown to give similar expression levels to that observed with vectors utilizing the EF1 α promoter or a γ -retroviral LTR. Most studies have been done in murine cells, which may account for some of the discrepancy compared with human cells. Recent studies have also shown that the EF1 α promoter gives normal levels of WASp from a vector in which the cDNA had been codon-optimized in both murine and human cells (Avedillo Diez *et al.*, 2011).

Production of hWASp in human B-cells. EBV immortalized B-cells were used to analyze hWASp expression in human B-cells *in vitro*. The cells were transduced with the

400EF1 α hWASp, 400P500hWASp, 400P1600hWASp, or 400MNDhWASp vector at an MOI=7.5 and hWASp expression was determined 21 days post-transduction by Western blotting. When copy number was taken into consideration, expression from 400MNDhWASp was higher than that from the other vectors (Fig. 4). Densitometry was performed and the density value of the WASp bands corrected for both the relative intensity of the actin band and also for the copy number as determined by polymerase chain reaction (PCR). An expression ratio is now provided in Figure 4 to allow the direct comparisons of the various vectors. Expression from 400EF1 α hWASp and 400P500hWASp was strong, but this reflected that the vector copy number was two- to three-fold higher. The cells transduced with the 400P1600hWASp vector had a comparable copy number to cells transduced with the 400MNDhWASp, but hWASp expression was much weaker with the WASp promoter. In a separate experiment, the 650MNDhWASp and 650EF1 α hWASp vectors were compared for expression in EBVWAS5-2 cells at low MOIs (0.5 and 1). Expression levels at both MOIs clearly showed that the 650MNDhWASp vector produced more hWASp per copy than the 650EF1 α hWASp vector (Fig. 4).

Production of hWASp in primary human WAS T cells. Expression in transduced WAS patient peripheral blood mononuclear cells (PBMCs) was determined using flow cytometry (Fig. 5). PBMCs were harvested from the blood of WAS patients and normal controls and underwent T-cell stimulation followed by transduction with either the 400EF1 α hWASp, NIP1600hWASp, or 400MNDhWASp

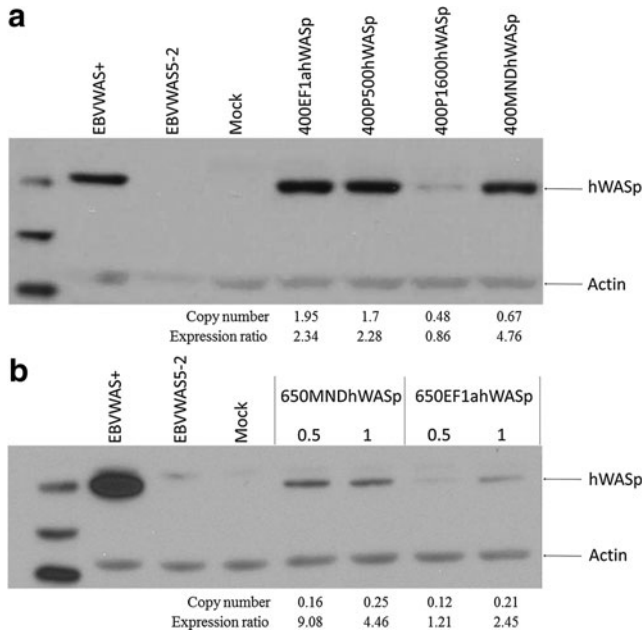


FIG. 4. hWASp expression in EBVWAS5-2 cells, a patient-derived WASp-negative B-cell line. **(a)** EBVWAS5-2 cells were transduced with either the 400EF1 α hWASp, 400P500hWASp, 400P1600hWASp, or 400MNDhWASp vectors at an MOI=7.5, and hWASp expression was determined 21 days post-transduction by Western blotting. **(b)** EBVWAS5-2 cells were transduced with the 650MNDhWASp or 650EF1 α hWASp vector at various MOIs, and hWASp expression was determined 21 days post-transduction by Western blotting. MOI, multiplicity of infection.

vector at an MOI=40. Transduction with the various vectors was approximately equivalent with a net over background at 7 days, ranging from 19% to 28%. Copy number was not performed at this early time point because of the likelihood of the presence of episomal genomes. hWASp expression was measured at 7 and 21 days post-transduction. NIP1600hWASp was included in this experiment as it is equivalent to a lentiviral vector currently in clinical trials for WAS gene therapy (Scaramuzza *et al.*, 2013). While all three samples showed hWASp expression at both time points, the proportion of cells expressing at a wild-type level of hWASp was greatest with the 400MNDhWASp vector (Fig. 5 and Supplementary Fig. S5). In a subsequent experiment, the noninsulated, 400 bp-insulated, and 650 bp-insulated versions of EF1 α and MND were compared under the same conditions. The presence/absence of an insulator had no effect on hWASp expression levels per cell, which is not surprising given the relatively short culture time (data not shown). As in the previous experiment, there were a higher proportion of cells expressing hWASp from the MND promoter compared with the EF1 α promoter.

Acute *in vivo* toxicity: No acute *in vivo* toxicity was observed in these studies other than the complications related to radiation and a transient period of myelosuppression in the murine model.

Biodistribution: Since repopulated hematopoietic stem cells were transduced, the biodistribution of the vector reflects the distribution of the progeny of transduced stem

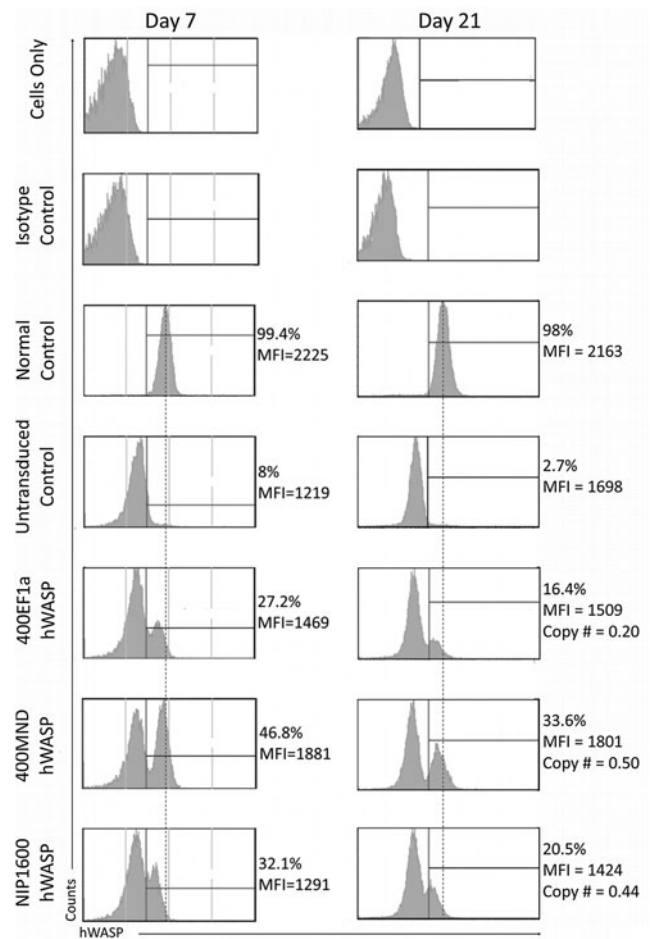


FIG. 5. hWASp expression from 400MNDhWASp, 400EF1 α hWASp, or NIP1600hWASp in patient peripheral blood mononuclear cells. Cells were prestimulated with T cell activation beads and transduced with virus at an MOI of 40. hWASp expression was determined at 7 and 21 days by intracellular staining for hWASp and flow cytometry.

cells. Thus, vector sequences and expression were found in the BM, spleen, and lymph nodes as predicted. No formal biodistribution studies were performed.

Genotoxicity: We utilized Jurkat cell lines having a targeted integration site within the promoter (Zhou *et al.*, 2012) or the first intron (Ryu *et al.*, 2008; Zhou *et al.*, 2010) of the *LM2* gene to test our vectors for the potential for proto-oncogene activation. The targeted integrations were obtained by homologous recombination of an LTR-GFP or mCherry vector with LoxP sites followed by Cre-mediated cassette exchange (Ryu *et al.*, 2008; Zhou *et al.*, 2010; Zhou *et al.*, 2012). The methodology for deriving the new clones with an insertion upstream from the distal promoter is shown in Supplementary Figure S6. As shown in Figure 6a and Supplementary Figure S7a, the vector without the insulator having the MND promoter (NIMNDhWASp) activated the *LM2* gene in several clones although the level of activation was significantly lower than that observed with clones containing the original LTR fluorescent targeting cassette (G27e). Addition of the 400 or 650 bp insulator elements in combination with the MND promoter reduced proto-oncogene

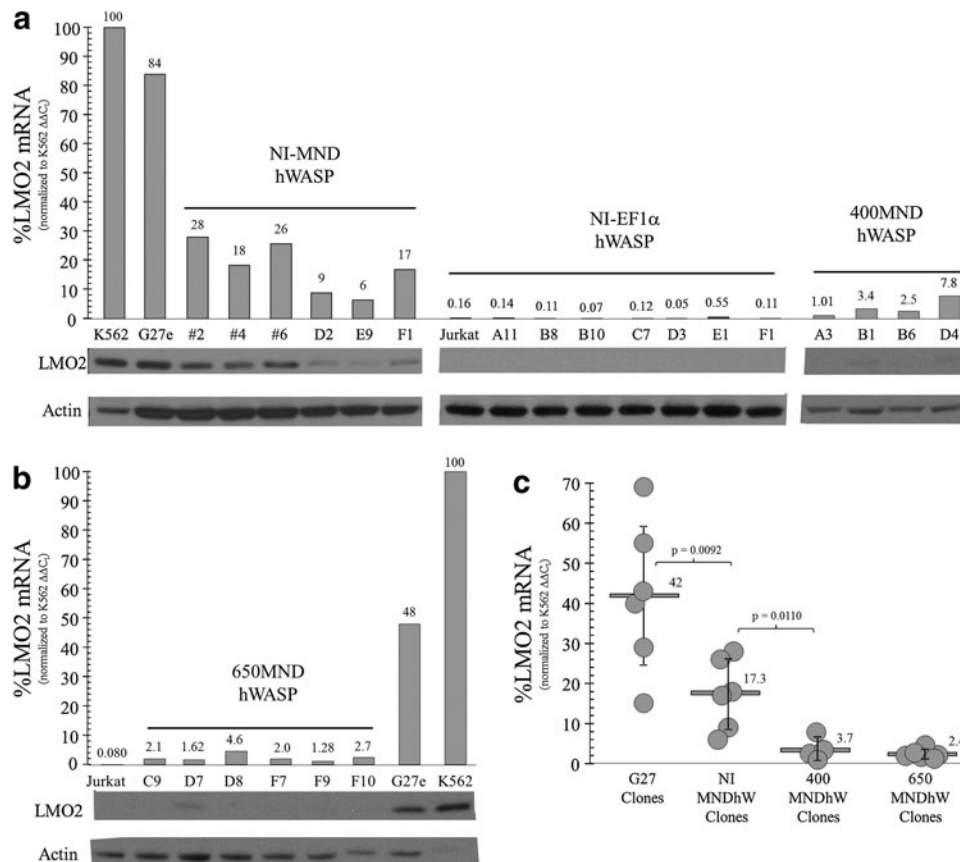


FIG. 6. The *CHS4* insulator diminishes *LMO2* activation in proviral MNDhWASP cell clones with the vector-integrated upstream of Exon 1. **(a)** Jurkat cell clones were screened by polymerase chain reaction (PCR) to identify CRE-mediated, MNDhWASP exchange clones, in an anti-sense orientation, upstream of Exon 1 of *LMO2*, identical to the site mapped for an X-SCID patient containing a γ_c retroviral insertion (Hacein-Bey-Abina *et al.*, 2003). G27e was one of six AAV-targeted clones that contained an MFG mCherry expression cassette, anti-sense to *LMO2*, and was used for all CRE-mediated cassette exchange reactions. Positive exchange clones were identified by PCR, expanded, and validated by Southern blot. *LMO2* mRNA (quantitative real-time [qRT]-PCR) and protein (Western blot) were measured, relative to that in K562 cells (positive control) and Jurkat cells (negative control). NI-MNDhWASP and NI-EF1 α hWASP exchange clones served as relevant, proviral positive and negative controls, respectively, to assess the effect of the *CHS4* 400 bp insulator on *LMO2* activation for 400MNDhWASP exchange clones. **(b)** *LMO2* mRNA and protein levels of CRE-mediated 650MNDhWASP exchange clones. **(c)** The relative amount of *LMO2* mRNA levels for all CRE-mediated, proviral MNDhWASP exchange clones, as assessed by qRT-PCR. Six independently targeted, *LMO2*-MFG mCherry G27 clones, including subclone G27e, were used as positive controls to assess the ability of *CHS4* insulator elements to diminish *LMO2* activation in MNDhWASP clones containing a strong retroviral LTR promoter, upstream of Exon 1. *LMO2*, LIM domain only two; PCR, polymerase chain reaction.

activation compared with MND vector without an insulator, with statistical significance with both vectors (Fig. 6b and c, and Supplementary Fig. S7b and c). The 650 bp insulator was somewhat more effective than the 400 bp insulator fragment in reducing *LMO2* activation compared with the non-insulated vector (Fig. 6 and Supplementary Fig. S7).

Conclusions

The characteristics of an ideal lentiviral vector for WAS gene therapy are as follows: Expression at a therapeutic level at a low copy number, long-term maintenance of expression in all hematopoietic lineages (except erythrocytes) and insulation to prevent transactivation of nearby genes. On the basis of the data presented here, the 650MNDhWASP vector meets these criteria and is undergoing further testing and development for clinical application.

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Author Disclosure Statement

No competing financial interests exist.

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