

## ARTICLE

# Generation of a lentiviral vector producer cell clone for human Wiskott-Aldrich syndrome gene therapy

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We have developed a producer cell line that generates lentiviral vector particles of high titer. The vector encodes the Wiskott-Aldrich syndrome (WAS) protein. An insulator element has been added to the long terminal repeats of the integrated vector to limit proto-oncogene activation. The vector provides high-level, stable expression of WAS protein in transduced murine and human hematopoietic cells. We have also developed a monoclonal antibody specific for intracellular WAS protein. This antibody has been used to monitor expression in blood and bone marrow cells after transfer into lineage negative bone marrow cells from WAS mice and in a WAS negative human B-cell line. Persistent expression of the transgene has been observed in transduced murine cells 12–20 weeks following transplantation. The producer cell line and the specific monoclonal antibody will facilitate the development of a clinical protocol for gene transfer into WAS protein deficient stem cells.

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## INTRODUCTION

Wiskott-Aldrich syndrome (WAS) is an X-linked disorder characterized by a triad of clinical manifestations comprised of thrombocytopenia with small platelets, immunodeficiency reflected by recurrent infections and chronic eczema.<sup>1</sup> In addition to this classic triad, WAS patients are prone to develop autoimmune disorders and lymphoid malignancies, the latter often secondary to Epstein-Barr virus infection. Milder forms of WAS characterized predominantly by thrombocytopenia were recognized by virtue of a characteristic pattern of highly skewed X chromosome inactivation seen in maternal carriers<sup>2</sup> even before molecular cloning of the defective gene facilitated genotype–phenotype correlation.

The gene which is mutated in WAS patients was isolated by positional cloning in 1994.<sup>3</sup> It is composed of 12 individual exons spanning ~14 kb of genomic DNA. The most frequently used promoter is just upstream from exon 2. Mapping of the 5' end of mRNAs from the Jurkat hematopoietic cell line identified a minor transcript which originates from an alternative distal promoter ~6 kb up from the proximal cluster of predominant transcriptional start sites.<sup>4</sup> The minor transcript from the distal promoter includes a small, non-coding exon and an intron which is spliced to generate the same open reading frame that is present in the major transcript. This gene, which has been designated the WAS protein (WASp) gene, encodes a major 1.8 kb mRNA, which translates into a protein of 502 amino acids and a minor transcript from the distal promoter which is slightly larger. WASp gene expression is limited to hematopoietic cells<sup>3</sup> and it is expressed in cytoplasm throughout hematopoiesis in all lineages except erythroid cells.<sup>4–6</sup>

To date, two autologous gene therapy trials for WAS have been performed. The earliest WAS gene therapy trial used a  $\gamma$ -oncoretroviral vector which successfully elevated platelet counts in the blood to therapeutic levels and demonstrated functional correction of other blood lineages due to strong hWASp expression from the vector's intact enhancer containing long terminal repeat (LTR) in 9 of 10 patients.<sup>7</sup> Unfortunately, seven patients developed leukemia due to the insertions of the vector into proto-oncogenes such as LMO2 (ref. 7). A more recent WAS gene therapy trial used the endogenous WASp 1600 base pair (P1600) promoter to drive hWASp expression in the context of a third-generation lentiviral vector. While patients treated with this vector exhibited definite clinical improvement including eczema resolution and reduction in the number and severity of infections, platelet counts did not reach normal levels.<sup>8</sup>

We sought to develop a hWASp vector that provides strong and stable hWASp expression, but would also be less likely to activate proto-oncogenes upon vector integration. Work by the Rawlings laboratory has recently shown that a retroviral promoter in a lentiviral vector provides full correction of the WASp phenotype in WAS<sup>-</sup> mice but that the endogenous human promoter as a 1.6 kb fragment did not provide full correction either at the expression level or functionally.<sup>9</sup> We report the development of a full hWASp producer cell clone that generates lentiviral vector with a strong internal LTR promoter to drive hWASp expression at levels higher than the endogenous P1600, P500, or EF1 $\alpha$  promoters.<sup>10</sup> This producer clone also contains regions of the chicken hypersensitive site 4 (HS4) chromatin insulator in the vector's deleted U3 region to limit proto-oncogene activation.<sup>10</sup> The

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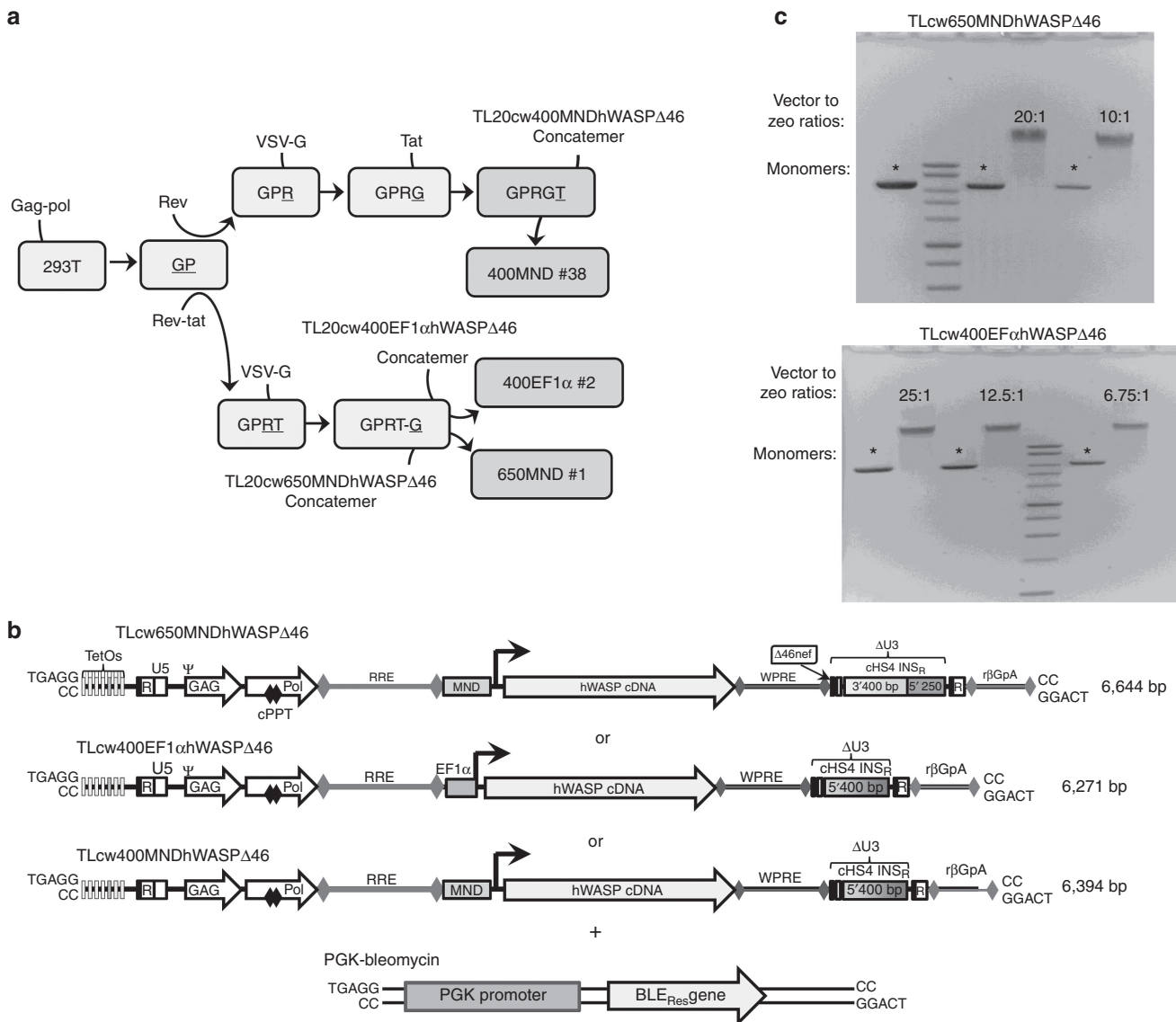
cHS4 650 insulator element was shown to significantly reduce LMO2 mRNA and protein expression *in vitro* in human Jurkat Lymphoid cells compared to an uninsulated proviral vector at two LMO2 insertion loci, which are identical to insertions that were identified in two patients who developed leukemia after receiving X-SCID gene therapy.<sup>10</sup> The generation of lentiviral producer cell clones in general will facilitate the manufacture of stable and predictable levels of vector (from batch to batch) in a GMP environment, without the additional costs or considerations that are involved in transient vector preparations, *e.g.*, production of highly pure and abundant plasmids, and their transfection into a helper line of interest.

**RESULTS**

**Derivation of hWASp producer clones**

Early in the developmental stage, it was not clear to us which packaging helper cell line (GPRGT and GPRT-G) developed at St.

Jude<sup>11</sup> would best support the generation of a producer cell clone that produced full-length lentiviral vector genome capable of transducing CD34<sup>+</sup> cells. Consequently, we tested both of these helper lines in conjunction with specific transfer vector concatemers (Figure 1a). Towards the goal of identifying a single hWASp producer clone that could be advanced for clinical manufacture, Bsu36I-digested hWASp transfer vector monomers were ligated at various ratios (6.25–25) relative to the bleomycin resistance cassette (Figure 1b). The monomeric transfer vectors displayed the expected band sizes (650MNDhWASpΔ46 = 6,644 bp, 400EF1αhWASpΔ46 = 6,271 bp, and 400MNDhWASpΔ46 = 6,349 bp), and after ligation, large molecular weight concatemers were generated (Figure 1c). To derive full producer clones, ligated concatemers were transfected into GPRGT (400MNDhWASpΔ46) or GPRT-G (650MNDhWASpΔ46 or 400EF1αhWASpΔ46) helper cells. Transfected cells were cultured in D10 selection medium for 12–14 days (Figure 2a). In general, most zeomycin-resistant cell clones that were picked for expansion



**Figure 1** Derivation of hWASp producer clones. **(a)** Helper lines used to generate hWASp producers. The helper lines GP, GPR, and GPRG have been described previously.<sup>11</sup> Helper lines GPRT and GPRT-G were generated by the St. Jude Vector Laboratory. **(b)** The pCL20cw based vectors have been previously described<sup>29</sup> and were cloned into the Tet-regulated backbone to generate the hWASp transfer vectors listed above. **(c)** The TL-based vectors were digested with Bsu36I and ligated at various ratios with a Bleomycin resistance cassette.

grew normally (only 2 of 81 cell clones failed to expand). Vector preparations were titered on HeLa cells using standard techniques (Materials and Methods). Based on unconcentrated HeLa cell titers of  $2.9 \times 10^7$ ,  $4.9 \times 10^7$ , and  $1.50 \times 10^8$  IU/ml for clones #38 (GPRGT 400MNDhWASp $\Delta$ 46, 25:1), #1 (GPRT-G 650MNDhWASp $\Delta$ 46, 20:1), and #2 (GPRT-G 400EF1 $\alpha$ hWASp $\Delta$ 46), respectively, and preliminary CD34<sup>+</sup> transduction experiments (data not shown), we generated a 10 vial research bank (of cryopreserved cells) for each producer clone to be used in subsequent animal and *in vitro* studies.

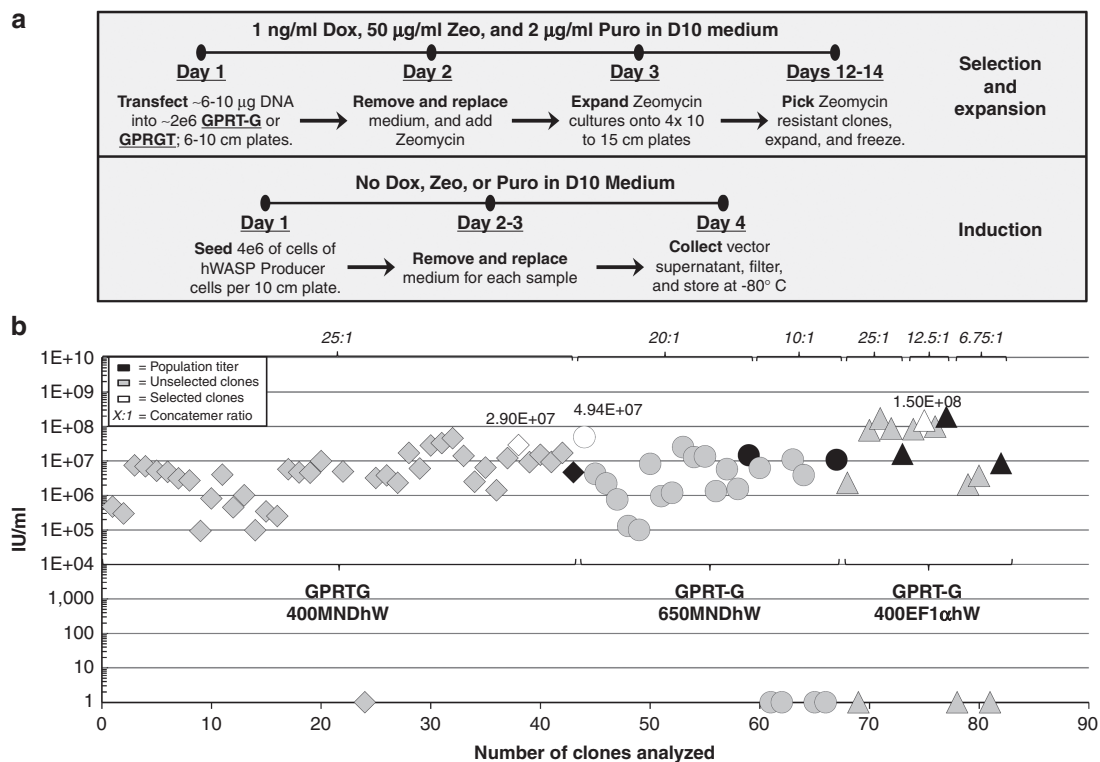
#### HeLa titers of hWASp producer clones

To assess vector production,  $\sim 4 \times 10^6$  producer cells from each clone were seeded onto a single 10-cm tissue culture plate in induction medium (no doxycycline) (Figure 2a). The HeLa titers for each group of producer clones are shown in Figure 2b. The MNDhWASp producers tended to achieve  $\sim 1$  to  $10 \times 10^6$  IU/ml of unconcentrated vector, while EF1 $\alpha$ hWASp producers tended to achieve levels of  $5 \times 10^7$  IU/ml. Of note, the doubling time of the 650MNDhWASp producer cell clone averaged 21.8 hours (Supplementary Figure S1), and its unconcentrated vector titers ( $\geq 1 \times 10^7$  IU/ml) remained stable for up to 8 weeks of continued passaging in culture (Supplementary Figure S2).

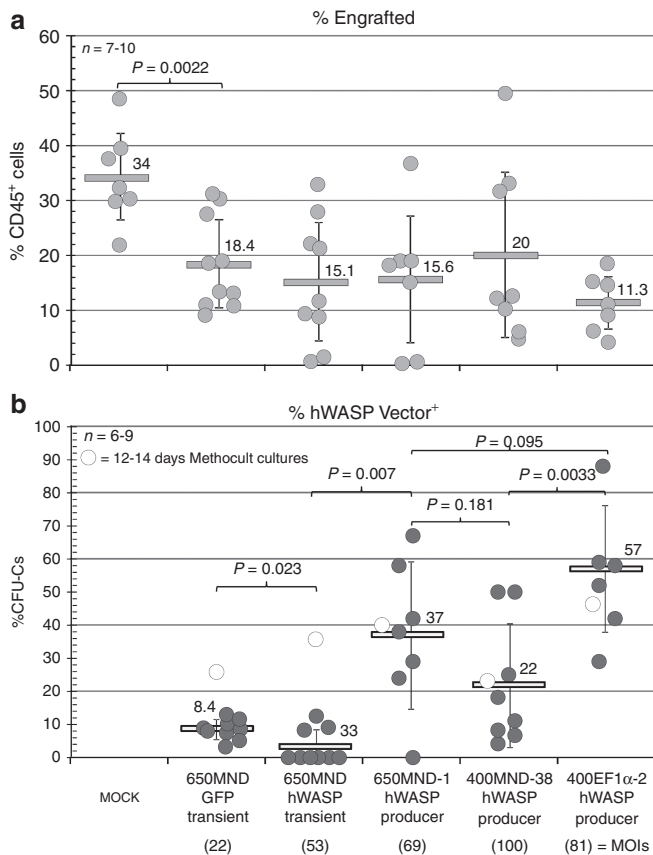
#### Human engraftment and transduction in nonobese diabetic severe combined immunodeficient (NSG) mice

We wanted to assess the transduction of human CD34<sup>+</sup> SCID repopulating cells (SRCs) using vector derived from our hWASp

producer clones and compare the results to vector prepared transiently. Equivalent amounts of vector (multiplicity of infection (MOI) of 150) were used to transduce human mobilized peripheral blood CD34<sup>+</sup> cells. On the same day, an aliquot of each vector was titrated again on HeLa cells. The MOIs based on this latter titration are shown in Figure 3. There were no observable differences in viability of the liquid Mock and vector-transduced CD34<sup>+</sup> cells (maintained for 14 days). Approximately 700,000 cells were transplanted into each NSG mouse by tail vein injection. Three months post-transplantation, mice were sacrificed and the levels of human CD45<sup>+</sup> engraftment and transduction were measured (Figure 3). While the CD45<sup>+</sup> engraftment levels among vector-transduced samples were not significantly different, the CD45<sup>+</sup> engraftment levels of these vector-treated groups as a whole (containing both transduced and untransduced cells) were significantly lower than the mock-transduced sample ( $P \leq 0.0022$ , Figure 3a), with the exception of the 400MNDhW group which was borderline ( $P = 0.052$ ). Treatment of CD34<sup>+</sup> cells with vector concentrated by ultracentrifugation from either transient transfections or producer inductions reduced human CD45<sup>+</sup> engraftment levels in NSG mouse BM approximately two- to threefold (ranging from 20 to 11.3% compared to 34% for the mock-transduced sample (Figure 3a). We next examined the levels of transduction for the transduced samples. Those transduced with transiently-derived vectors exhibited the lowest transduction levels where GFP and hWASp were 8.4 and 3.3%, respectively, while those transduced with producer-derived vectors exhibited significantly higher transduction levels—37, 22, and 57% for the 650MNDhWASp1, 400MND



**Figure 2** Derivation and titers of hWASp producer clones. **(a)** Derivation, selection, and expansion of hWASp producers. Ligated vector concatemers were transfected into the GPRG or GPRT-G helper lines,<sup>11</sup> cultured, and expanded in D10 medium containing Zeomycin to select for cell clones containing vector, puromycin to maintain GAG-POL expression, and Doxycycline to repress expression of the Tet transactivator, VSV-G envelope protein, human immunodeficiency virus Rev and Tat proteins, and the hWASp transfer vector genome. Clones that had undergone sufficient expansion for cell banking, were induced for vector production by removing all antibiotics and doxycycline from the medium. **(b)** Viral supernatants were collected on day 4 and titered on HeLa cells. The numbers above each grouping of clones indicate the ligated concatemer to bleomycin ratios that were used for each transfected cell population from which the isolated individual cell clones were derived.



**Figure 3** Human engraftment and transduction in nonobese diabetic severe combined immunodeficient (NSG) mice. **(a)** 700,000 human mobilized peripheral blood CD34<sup>+</sup> cells were transduced with the vector (multiplicity of infection of ~80) derived from the hWASP producers listed, and transplanted into NSG mice. Twelve weeks posttransplantation, mice were sacrificed and bone marrow cells from tibias and femurs were isolated. The percentages of human CD45<sup>+</sup> cells engrafted are shown. Each dot represents one mouse. The *P* values listed are calculated based upon a two-tailed, heteroscedastic *T*-Test. The concentrated HeLa titers (IU/ml) determined by Southern blot analysis for the viruses prior to their use on CD34<sup>+</sup> cells were  $2.9 \times 10^9$  for transient GFP,  $1.4 \times 10^9$  for transient hWASP,  $5.9 \times 10^9$  for 650MNDhW producer,  $3.5 \times 10^9$  for 400MNDhW producer, and  $4.1 \times 10^9$  for 400EF1 $\alpha$ hW producers. Equivalent transducing units were used at the time of CD34<sup>+</sup> cell transduction based on the aforementioned titers. However, another HeLa titration was performed when the CD34<sup>+</sup> were transduced, the multiplicity of infections based on these titers are shown. **(b)** The percentage of human CFU-Cs that are hWASP vector positive, upon seeding 100,000 sorted human CD45<sup>+</sup> cells into methocult, and picking colonies 12–14 days postculture.

hWASP38, and 400EF1 $\alpha$ hWASP2 groups, respectively (Figure 3b). It is interesting that despite similar CD45<sup>+</sup> engraftment levels for the transient and producer-derived 650MNDhWASP vectors (15.1 and 15.6%, respectively) and CD34<sup>+</sup> progenitor transduction levels in methocultures (36 and 40%, respectively), the producer-derived vector transduced ~10-fold higher percentage of SRCs than transiently-derived vector (*cf.* 37 versus 3.3%) (Figure 3b). It may be argued that HeLa titers for the transiently derived vectors may have been skewed as residual plasmid DNA could have overestimated the infectious titer in preparations used to transduce human CD34<sup>+</sup> cells. While cytomegalovirus-containing plasmid sequences can be found in conditioned transient vector preparations at low levels ( $\leq 3$  fg/ml, via polymerase chain reaction (PCR), data not shown), it is our experience that contaminating plasmid

sequences are not detected in genomic HeLa DNA after 6 or 7 days of culture, where an initial seeding density of 100,000 cells undergoes >64-fold cell expansion (data not shown). Among producer-derived vectors, the 400EF1 $\alpha$ hWASP2 transduced sample exhibited the highest SRC transduction frequency (57%), followed by 650MNDhWASP1 (37%), then 400MNDhWASP38 (22%) (Figure 3b). These levels inversely correlated with CD45<sup>+</sup> engraftment.

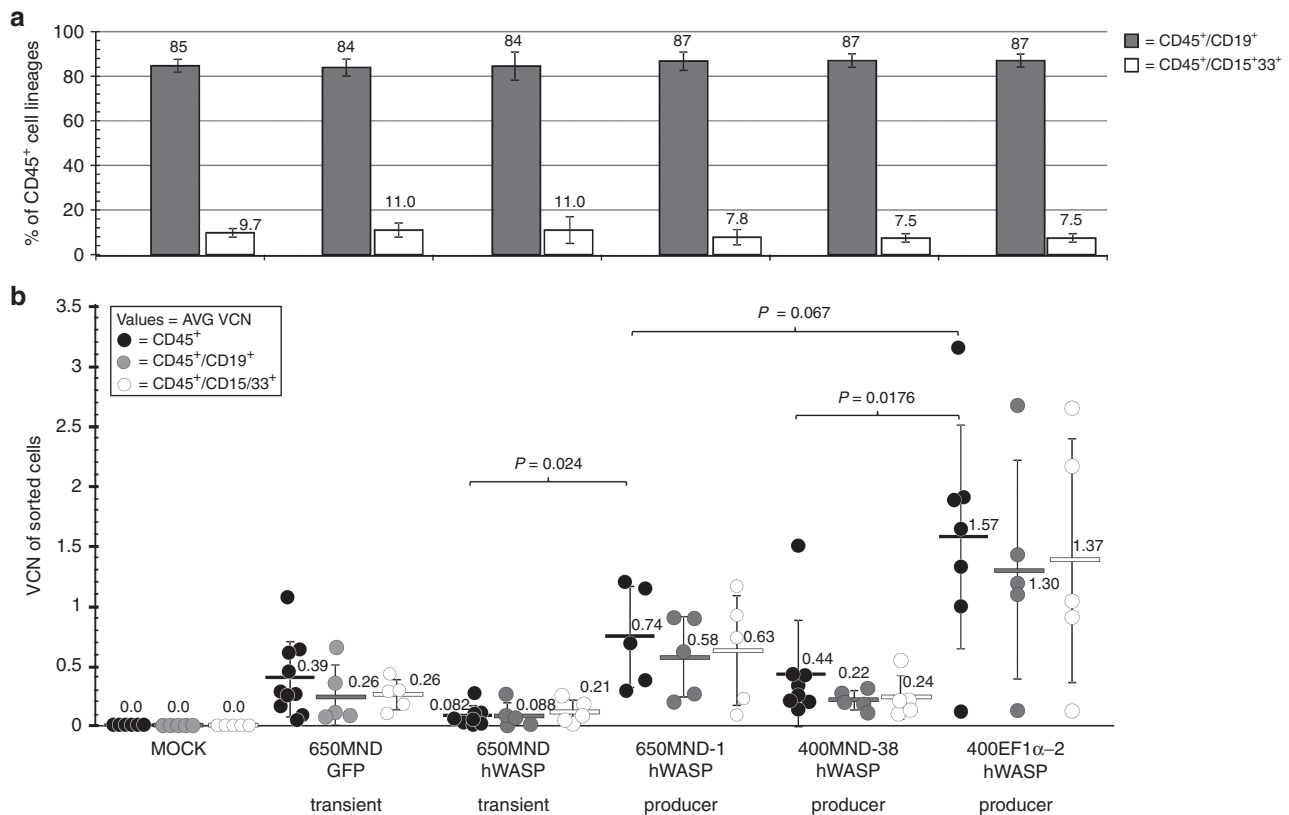
We also examined how small molecule treatments of CD34<sup>+</sup> cells during *ex vivo* cultures affected the engraftment and transduction of human CD45<sup>+</sup> cells with producer-derived vector in NSG mouse BM, as there is interest in the field of gene therapy to identify safe molecules that are able to increase both the transduction and engraftment levels of HSCs, particularly with respect to hematological disorders which have limited or no selective growth advantage upon transduction. Vector derived from the 650MNDhWASP producer cell clone was used in these studies, as this vector transduced SRCs better than the 400MNDhWASP producer-derived vector (Figure 3b), and previous work demonstrated that the MND promoter expressed hWASP at levels higher than the EF1 $\alpha$  promoter.<sup>9,10</sup> Ex527 (a specific inhibitor of SIRT1 (ref. 12)), NAM (a general SIRT1 inhibitor),<sup>12</sup> Rapamycin (mTOR inhibitor), CHIR98014 (a glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ), a serine/threonine kinase whose action regulates several cellular processes such as cell proliferation and apoptosis),<sup>13</sup> and SDF1 $\alpha$  (a cytokine which binds to the CXCR4, a receptor involved in chemotaxis) did not significantly increase the percentage of transduced SRCs in the NSG BM (Supplementary Figures S3b and S4b), but the CHIR98014-treated group did exhibit a higher percentage of transduced SRCs than the other small molecule-treated groups. This general inability to significantly increase the transduction of SRCs may be due to either suboptimal concentrations, time of addition, or duration. However, Ex527 and Rapamycin did significantly enhance human cell engraftment threefold (30 versus 9.9%, respectively, Supplementary Figure S3a), and twofold (24 versus 48%, respectively, Supplementary Figure S4a), over untreated control groups.

#### Lineage contribution and vector copy number (VCN) of engrafted human CD45<sup>+</sup> cells

We next examined the lineages of the engrafted human CD45<sup>+</sup> cells. The percentage of CD45<sup>+</sup> cells that were of lymphoid origin (CD45<sup>+</sup> CD19<sup>+</sup>) ranged from 84 to 87%, while those for myeloid cells ranged from 7.5 to 11% (Figure 4a). The VCNs in the lymphoid and myeloid cells tended to be  $\geq 2$ -fold lower for the transiently derived samples than for the samples transduced with producer-derived vector (Figure 4b). The 400EF1 $\alpha$ hWASP2 transduced lineages exhibited the highest VCN at 1.4, while the 650MNDhWASP1 and 400MNDhWASP38 lineages were 0.65 and 0.30, respectively.

#### Generation and specificity of hWASP C10.4 mAb

Until it is activated, the WAS protein exists in an autoinhibited conformation bound to its chaperone WIP<sup>14</sup> such that accessible binding sites for an antibody in an intact cell are limited. We found several antibodies that could detect denatured WAS protein for western blot analyses, but none were available to use for fluorescence-activated cell sorting (FACS). Zhu *et al.*<sup>15</sup> analyzed the antigenicity of three hydrophobic peptides from WASp and found that fragment 503 gave a strong, single band by western blot and functioned well in FACS analysis, using a rabbit polyclonal antibody raised against this peptide.<sup>16</sup> We attempted, without success, to use the 503 peptide



**Figure 4** Lineage contribution and vector copy number (VCN) of engrafted human CD45<sup>+</sup> cells. **(a)** The percentage of sorted human CD45<sup>+</sup> cells that were of myeloid (CD45<sup>+</sup>CD15<sup>+</sup>33<sup>+</sup>) or lymphoid (CD45<sup>+</sup>CD19<sup>+</sup>) origin, derived from NSG bone marrow 12 weeks post-transplantation. **(b)** VCN of the sorted lineages described above.

to immunize WASp<sup>-/-</sup> mice (no positive monoclonals) and rabbits (Rockland Immunochemicals, Limerick, PA, nonspecific binding). We found that the native WASp, produced as a secretory protein, gave better results as an antigen in WASp<sup>-/-</sup> mice to form specific antibodies against this protein. Thirty-nine clones reacted strongly to WASp by enzyme-linked immunosorbent assay. These clones were expanded, and their supernatants were purified and tested for their ability to recognize the native protein in human B-cell lysates. Only one clone, C10.4, was successful (Figure 5a). To assess the reactivity of mAb C10.4 to hWASp in murine cells, mWASp<sup>-/-</sup> lin<sup>-</sup> bone marrow cells were harvested, transduced with 650MNDhWASp1 producer-derived vector, and transplanted into WASp<sup>-/-</sup> mice. Twelve weeks later, peripheral blood was isolated and hWASp expression was measured using the antibody. Lin<sup>-</sup> 650MNDhWASp1 transduced cells clearly exhibited hWASp expression and a peak shift over WASp<sup>-/-</sup> cells (Figure 5b). We also measured hWASp expression in a WASp-human B-cell line, and found that transduced cells exhibited hWASp expression above untransduced cells (Figure 5c). These results demonstrated that the C10.4 antibody was able to specifically detect hWASp expression on a hWASp<sup>-</sup> background, following transduction with hWASp expression vectors.

#### Human WASp expression in murine WASp<sup>-</sup> lineages 18 weeks post-transplantation

We next wanted to use the C10.4 mAb to measure hWASp expression in different murine lineages in WASp<sup>-/-</sup> mice that had been transduced with either 650MNDhWASp1 or 400EF1 $\alpha$ hWASp2. As shown in Figure 6, all murine lineages showed elevated hWASp MFI using the 650MNDhWASp1 vector, compared to the

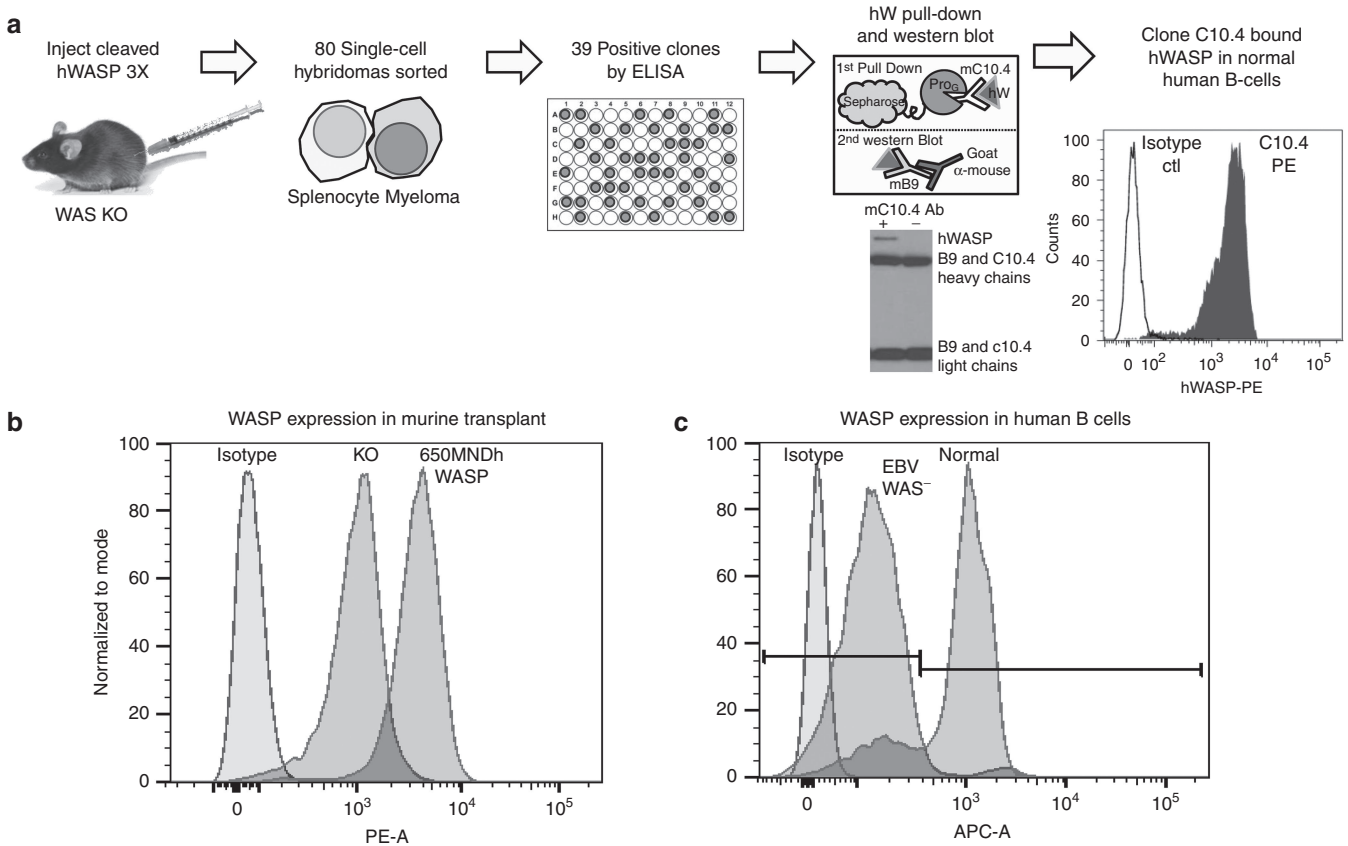
400EF1 $\alpha$ hWASp2 vector (~2–4 higher, normalized against VCN). These differences were statistically significant in B-cells, T-cells, and neutrophils ( $P \leq 0.02$ ), and approached significance in monocytes ( $P = 0.12$ ). This result showed that the C10.4 mAb could be used to detect hWASp in different murine lineages, and that the 650MNDhWASp1 vector expressed hWASp at ~3 $\times$  higher levels *in vivo* compared to the 400EF1 $\alpha$ hWASp2 vector, per VCN.

#### *In vitro* immortalization assay

Lastly, we examined the relative safety of the 650MNDhWASp1 and 400EF1 $\alpha$ hWASp2 producer vectors in an *in vitro* immortalization assay.<sup>17</sup> We found that both vectors yielded replating efficiencies/VCN that were not statistically different from mock-transduced cells ( $P \geq 0.52$ ), but were significantly less than the spleen focus forming virus (SFFV) positive control ( $P \leq 0.0039$ ), despite having almost two or four times more vector copies than SFFV samples, respectively (Figure 7). While not significant compared to mock samples ( $P = 0.11$ ), the uninsulated MND replating efficiencies were definitely trending above the 650MNDhWASp1 samples. This was similar to our previously observations using the LM02 activation assay, although the 650MND cassette in that more sensitive assay, yielded statistically significant lower LMO2 mRNA levels than the uninsulated MND cassette.<sup>10</sup>

## DISCUSSION

Our studies have yielded reagents, including a stable WASp vector producer clone with a vector genome which, when integrated, has insulators in both LTRs. Our monoclonal antibody reacts with intracellular WASp and thus provides a valuable reagent for monitoring

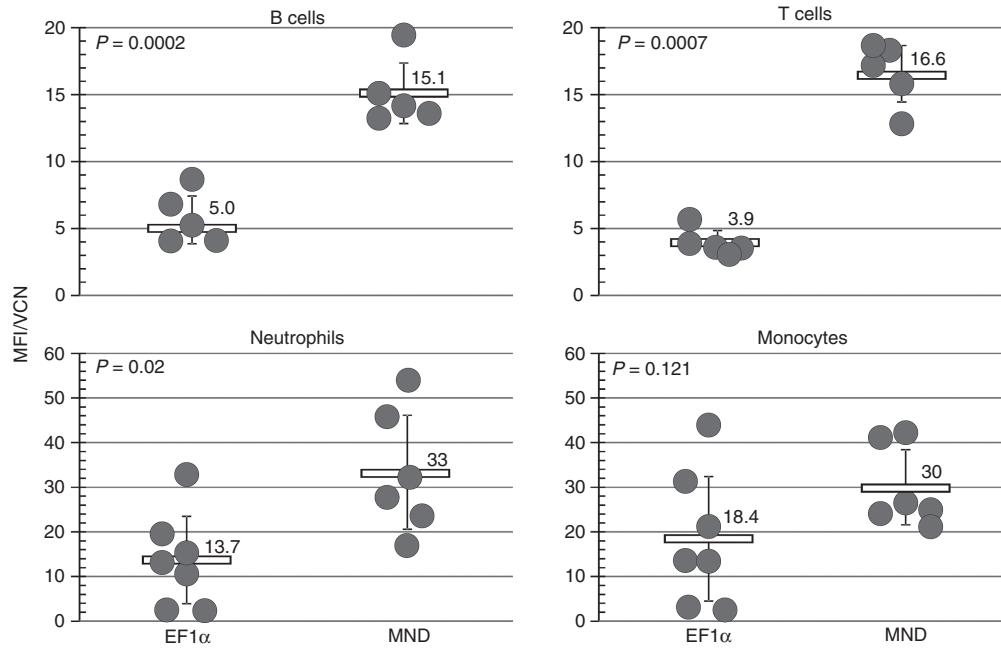


**Figure 5** Generation and specificity of hWASP C10.4 mAb. **(a)** Human WASp cDNA with a 6× His tag at the C-terminus was inserted into the pAcGP67-A transfer vector. The St. Jude Protein Production Core transfected and raised the full length hWASP secretory protein in the H5 cell line. The St. Jude Immunologic Reagent Facility injected hWASP and adjuvant 3× into a mouse. Murine splenocytes were isolated and fused with a murine myeloma cell line and cultured in HAT medium. Eighty single cell hybridomas were recovered by sorting, and secreted antibodies from said clones were screened by enzyme-linked immunosorbent assay on a 96-well plate coated with hWASP. Those testing positive were subsequently analyzed in a Protein G-sepharose pull-down assay to identify hybridoma clones whose antibodies recognized an epitope on natively expressed hWASP in hWASP<sup>+</sup> B-cell lysates. The specific reactivity of clone C10.4 against natively expressed hWASP is shown via flow cytometry. **(b)** The specific reactivity of hybridoma clone C10.4 antibody against WAS knock out murine hematopoietic stem cells transduced with hWASP vector, or **(c)** against human B cells that either express, or do not express hWASP protein.

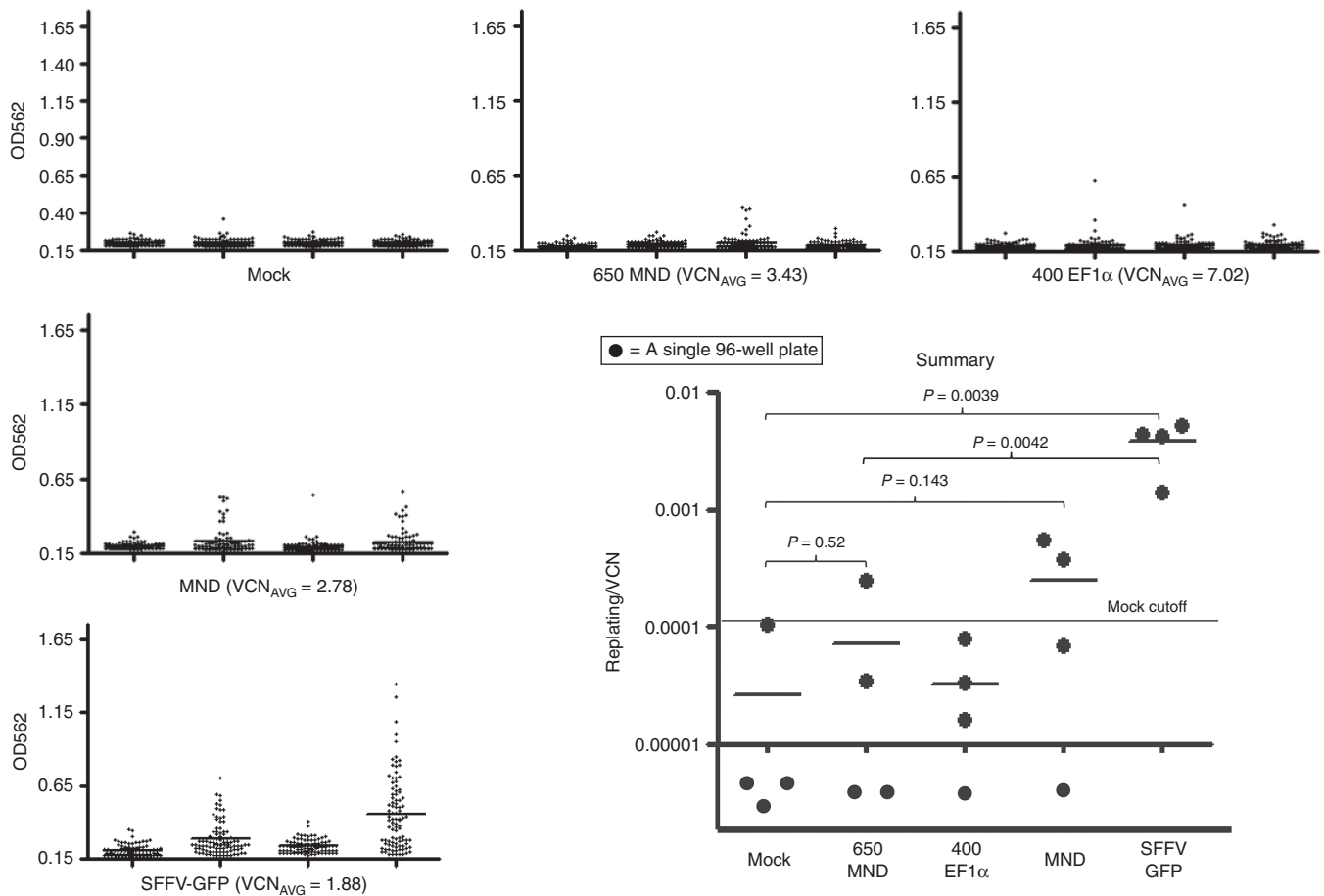
protein production following transfer into hematopoietic cells. Based on the human SRC transduction results, hWASP expression profiles in murine lineages, and safety profiles *in vitro* of the different producers tested, we believe the 650MNDhWASP1 vector producer best balances the ability to achieve reasonably high transduction levels and to express hWASP at therapeutic levels across hematopoietic lineages, particularly in megakaryocytes which produce platelets.<sup>10</sup> Consequently, we have now transferred the 650MNDhWASP1 producer clone to our GMP facility for clinical grade production of our lentiviral vector for WAS gene therapy.

Lentiviral vectors are able to transverse the nuclear membrane without cell mitosis and therefore have a greater potential to transduce nonmitotic cells, whereas  $\gamma$ -retroviral vectors which require cell division to access nuclear chromatin.<sup>18</sup> Most lentiviral vector systems intended for clinical use are based on human immunodeficiency virus (HIV).<sup>19</sup> Potentially safer lentiviral vectors having a self-inactivating design (SIN) in which the enhancer and promoter are eliminated from the U3 region of the 3' LTR in the plasmid vector construct have been proposed for clinical use.<sup>20</sup> Such SIN lentiviral vectors encoding WASp under the control of a constitutively active promoter or the native proximal WASp gene promoter partially correct functional defects in T-cells from WAS patients and the phenotype in WAS<sup>-</sup> mice.<sup>21–26</sup>

Insulator elements have the property of preventing interaction between enhancers and cellular promoters and also may have barrier function which prevents transgene silencing by chromatin condensation.<sup>27</sup> The most well-characterized insulator with barrier and enhancer blocking functions is a 1.2kb fragment which contains hypersensitive site 4 from the chicken  $\beta$ -globin locus (chS4).<sup>28</sup> We have found that the full 1.2kb fragment blocks complete reverse transcription in transduced cells thereby reducing functional titer.<sup>29</sup> The Malik lab has shown that a combination of a 5' 250bp segment, which has enhancer blocking and barrier activities, and a 3' 400bp segment of the 1.2kb insulator functions better than the 250bp core fragment or the 400bp fragments used in some of our vectors as a barrier element.<sup>30</sup> The Persons lab has shown that reversing the chS4 insulator improves expression by enhancing polyadenylation.<sup>31</sup> Gene silencing and/or variegation in expression have consistently been observed with cellular globin promoters in the absence of an insulator. Although less problematic for gene correction of SCID because of the very powerful selective advantage for gene-corrected cells, silencing or variegation from a cellular promoter has recently been observed in secondary murine transplant recipients in a model of X-linked agammaglobulinemia.<sup>32</sup> Because WASp is an abundant protein and the selective advantage of gene



**Figure 6** Human WASp expression in murine WAS<sup>-</sup> lineages, 18 weeks post-transplantation. Murine WAS<sup>-</sup> hematopoietic stem cells were transduced (two hits, multiplicity of infections of 25) with vector derived from two hWASp producer clones. Shown are hWASp<sup>+</sup> bone marrow singlets with VCN in B-cells, T-cells, neutrophils, and monocytes.



**Figure 7** *In vitro* immortalization assay. Murine hematopoietic stem cells were either mock or vector transduced as shown, seeded onto 96-well plates at a density of 100 cells/well. The OD562 replating efficiencies after three passages for each well on a 96-well plate are represented. Each cluster of small dots represents a 96-well plate, replicated four times. The average vector copy number (VCN) for each transduced sample is listed. A summary of the replating efficiencies for each sample (normalized per VCN) is shown.

corrected cells in WAS is less than in SCID, we evaluated the potential benefit on expression of adding an insulator element.

In this study, we observed that MNDhWASp producers generally achieved lower HeLa titers than the EF1 $\alpha$ hWASp producer clones. This difference may be due to elevated hWASp expression levels from the MND promoter. As hWASp is a key factor regulating the actin cytoskeleton,<sup>33</sup> overexpression of WASp upon vector induction may increase actin filaments numbers in the producer cell<sup>34</sup> and increase its rigidity, perhaps limiting particle release from the plasma membrane. We also found that the 650MNDhWASp1 producer derived vector transduced SRCs 10-fold higher than vector derived transiently. In a previous paper, we observed ~50% human CD34<sup>+</sup> transduction levels using unconcentrated transiently-derived GFP vector,<sup>35</sup> but concentrated GFP vector used in this study transduced only 8.4% SRCs (Figure 3b). A significant difference between these two studies was the introduction of an ultracentrifugation step to concentrate LV particles, which may have compromised particle integrity in some way. In any event, we did assume that equivalent transducing units (based on HeLa titer) were used, as it is our experience that a correlation exists between a vector's HeLa titer, and its ability to transduce human CD34<sup>+</sup> progenitors.

Recently, Wang *et al.*<sup>36</sup> found that a 10–20  $\mu$ g/ml rapamycin treatment of CD34<sup>+</sup> cells increased SRC transduction three- to fourfold over untreated controls, but observed no enhancement of CD45<sup>+</sup> engraftment. We found that a 50 nmol/l Rapamycin treatment of CD34<sup>+</sup> cells yielded a significant twofold increase in CD45<sup>+</sup> engraftment, but SRCs were transduced 17-fold less than the untreated control. The >200-fold rapamycin concentration may account for these observed differences. We also found that a higher MOI, multiple rounds of infection, and CHIR98014 tended to increase VCN and transduction, but negatively impacted SRC engraftment. In this regard, it is noteworthy that recent gene therapy clinical trials (LV-WAS) include a rest period between transductions (~12 hours) and possibly prior to transplantation (where cells are cultured a total of 60 hours *ex vivo*).<sup>37</sup> It may be that when CD34<sup>+</sup> cells are transduced at a high MOI, cell surface receptors important for homing and/or engraftment become internalized, and limit the ability of transduced cells to home or engraft. However, if transduced cells are rested, the cell surface receptors involved in homing/engraftment may be redeployed or newly synthesized.

Development of effective lentiviral vector mediated gene therapy for WAS would provide curative therapy for patients unable to undergo bone marrow transplantation and, if successful, an alternative for individuals for whom bone marrow transplantation carries a significant risk. We propose to achieve this goal by utilizing our lentiviral vector system<sup>29</sup> and by exploiting our colleagues' success in developing a novel packaging cell system and in subsequently deriving lentiviral vector producer clones by the novel concatamerization array transfection methodology,<sup>11</sup> as described below. Our focus on the evaluation of the safety of lentiviral vectors<sup>10,38–40</sup> renders us confident that we have developed a vector for WAS that is far safer than the  $\gamma$ -retroviral vectors utilized in past and on-going clinical trials for immunodeficiencies.

## MATERIALS AND METHODS

### Mice

Housing and care of the animals were in accordance with a protocol approved by the Institutional Animal Care and Use Committee. Eight- to 12-week-old female NOD/LtSz-IL2R  $\gamma$ <sub>c</sub> (NSG) mice (Jackson Lab, Sacramento, CA; strain no. 005557) were used for human CD34<sup>+</sup> transplantation studies. Twenty-four hours prior to transplantation, mice were injected intraperitoneally with 35 mg/kg busulfan (BUSULFEX; PDL BioPharma, Redwood City,

CA). Twelve to 13 weeks post-transplantation, NSG mice were sacrificed and bone marrow cells were collected from tibias and femurs in Dulbecco's phosphate-buffered saline (DPBS) (Corning; CellGro, Herndon, VA; cat. no. 21-013-CV), supplemented with 2% heat inactivated fetal calf serum (HI-FCS) (Thermo Scientific; HyClone, Logan, UT; cat. no. SH30396.03). Six- to 8-week-old female WAS<sup>-/-</sup> and male WAS<sup>-</sup> mice derived on a C57Bl/6 strain as described previously<sup>41</sup> were used to compare strength of WASp expression using different promoters.

### Cells

Adherent human embryonic kidney 293T and HeLa cells were cultured at 37 °C 5% CO<sub>2</sub> in Dulbecco's modified Eagle's medium (Corning; CellGro; cat. no. 10-013-CV), supplemented with 2 mmol/l glutamine (Life Technologies; Gibco, Grand Island, NY; cat. no. 25030) designated hereafter as 1 $\times$  Glut or 2 mmol/l L-Alanyl-L-Glutamine (Corning; CellGro; cat. no. 25-015-Cl), and 50 IU/ml penicillin G/50 mg/ml streptomycin designated hereafter as 1 $\times$  P/S (Life Technologies; Gibco; cat. no. 15070), and 10% FCS (with or without heat in-activation) henceforth designated as D10 medium. The lentiviral helper cell lines GP, GPR, and GPRT have been described previously.<sup>11</sup> The GPRT-G cell line was generated essentially as described previously for the GPRG helper cell line. The 5G1 cell clone (derived from the GPRT-G population) was selected for expansion, and used as the helper line to derive the 650MNDhWASp-1 and 400EF1 $\alpha$ hWASp-2 producer clones. The 400MNDhWASp-38 producer clone was derived from the GPRG helper cell line. Cells were cultured in 10-cm diameter tissue culture treated plates and were passaged as needed by removal of D10 medium by aspiration, washing the cells once with 10 ml DPBS, then trypsinizing cells for 5 minutes using 1 ml Trypsin-versene (Lonza, Walkersville, MD; cat. no. 17-161E) at room temperature.

The protocol on which the CD34<sup>+</sup> cells were obtained was approved by the St. Jude Children's Research Hospital Institutional Review Board and the studies were conducted in accordance with the Declaration of Helsinki. Volunteers gave written informed consent and were compensated for their time and expenses. Human CD34<sup>+</sup> cells derived from peripheral blood were mobilized with granulocyte colony stimulating factor (G-CSF) 4 days before apheresis, and CD34<sup>+</sup> cells were purified ( $\geq$ 95% purity) in the Human Applications Laboratory at St. Jude Children's Research Hospital using the CliniMACS device from Miltenyi Biotech (Auburn, CA) according to the manufacturer's protocol. Human donor CD34<sup>+</sup> cells were aliquoted and frozen, and later used in preliminary transduction experiments. For transplant experiments involving small molecules, frozen G-CSF mobilized normal peripheral blood human CD34<sup>+</sup> cells were purchased from Key Biologics LLC (Memphis, TN), and cultured at 37 °C 5% CO<sub>2</sub> in X-VIVO 10 medium (Lonza; cat. no. 04-743D), supplemented with 100 ng/ml each of SCF (Peprotech, Rocky Hill, NJ; cat. no. 300-07), TPO (Peprotech; cat. no. 300-18), and Flt-3 Ligand (Peprotech; cat. no. 300-19), and 1 $\times$ P/S, henceforth designated as CD34<sup>+</sup> culture medium.

Lineage depleted (Lin<sup>-</sup>) murine WASp<sup>-</sup> cells were obtained by harvesting bone marrow cells from tibias and femurs of WASp<sup>-/-</sup> and WASp<sup>-</sup> mice,<sup>41</sup> and recovering lineage depleted cells using a commercially available kit (Miltenyi Biotech; cat. no. 130-092-211). The cells were collected by centrifugation at 1,500 rpm for 10 minutes at 4 °C, then resuspended in StemSpan SFEM medium (Stem Cell Technologies; Vancouver, BC, Canada; cat. no. 09650), containing 10 ng/ml mSCF (Peprotech; cat. no. 250-03), 20 ng/ml mTPO (Peprotech, NJ; cat. no. 315-14), 20 ng/ml mIGF-II (R&D Systems, Minneapolis, MN; cat. no. 792-MG), 10 ng/ml hFGF (Peprotech; cat. no. 100-17A), 10  $\mu$ g/ml Heparin (Sigma, St Louis, MO; cat. no. H3149), 1 $\times$ Glut, and 1 $\times$  phosphate-buffered saline (PBS), henceforth designated as Lin<sup>-</sup> culture medium. For transduction, 7.5  $\mu$ g/ml polybrene (Sigma; cat. no. H9268) was added to the Lin<sup>-</sup> culture medium.

### Plasmids

pCAG4 RTR2, pCAG-kGP1.1R, pCAG VSV-G have been previously described.<sup>10</sup> The pCL20cw400EF1 $\alpha$ hWASp $\Delta$ 46, pCL20cw400MNDhWASp $\Delta$ 46, and pCL20cw650MNDhWASp $\Delta$ 46 have also been previously described.<sup>10</sup> The SFFV-GFP transfer vector plasmid was originally obtained from Chris Baum, and was kindly provided by the Derek Persons' lab. The pEQPAM3-E plasmid was generated by Elio Vanin and has been previously described.<sup>10</sup> The TL20i4r3MSCVYFP and pLT-PGK-Ble plasmids have also been previously described.<sup>11</sup> The TL20cw400EF1 $\alpha$ hWASp $\Delta$ 46, TL20cw400MNDhWASp $\Delta$ 46, and TL20cw650MNDhWASp $\Delta$ 46 plasmids were generated by ligating the BstBI-PspXI vector-containing fragments (3,050, 3,173, and 3,423 bp,



respectively) to the BstBI-PspXI  $\beta$ -lactamase resistance cassette fragment (5,987 bp) of TL20i4r3M3CVFP.

### Derivation of lentiviral hWASp producer clones

The TL20cw400EF1 $\alpha$ hWASp $\Delta$ 46, TL20cw400MNDhWASp $\Delta$ 46, and TL20cw650MNDhWASp $\Delta$ 46 plasmids were digested with Bsu36I (6,271, 6,394, and 6,644 bp, respectively) and ligated to Bsu36I digested, bleomycin-resistance containing fragment of pLT-PGK-Ble (1,152 bp) at various ratios (ranging from 25:1 to 6.75:1). For the generation of the 650MND hWASp-1 and 400EF1 $\alpha$ hWASp-2 vector producer cells, ~6–10  $\mu$ g of ligated concatemeric DNA was calcium phosphate transfected onto GPRT-G helper cells on a 60 mm diameter tissue culture treated plate which contained  $1.1 \times 10^6$  cells 1 day before. For the generation of the 400MND hWASp-38 producer, ~2.45  $\mu$ g of ligated concatemeric DNA was calcium phosphate transfected into GPRG cells on a 35 mm diameter tissue culture treated plate which 1 day before contained  $5 \times 10^5$  cells. The cells were cultured in D10 without 1 $\times$  P/S, but supplemented with 50  $\mu$ g/ml Zeocin (Life Technologies; Invitrogen; #R250-01), 2  $\mu$ g/ml Puromycin (Life Technologies; Invitrogen; #P9620), and 1 ng/ml Doxycycline (Clontech/Takara Bio USA Mountain View, CA; cat. no. 631311), hereafter designated D10 selection medium. After 2 days in culture, cells were trypsinized and seeded onto 10 cm diameter tissue culture treated plates, and passaged every 2–3 days with fresh D10 selection medium for a total of 12–14 days. Zeocin-resistant cells were trypsinized and seeded onto 96-well tissue culture treated plates at a density of ~1 cell per 2 wells. Zeocin-resistant cell clones were cultured and expanded to prepare a cell freeze and concomitantly to examine vector production for all hWASp producer clones, via cell induction and subsequent titration of producer supernatants on HeLa cells.

### Vector production and titration

The generation of transiently derived lentiviral vector preparations containing the pCL20cw backbone has been described previously specifically for pCL20cw650MNDhWASp $\Delta$ 46 (ref. 10). Briefly,  $4 \times 10^6$  293T cells were seeded onto 10-cm diameter tissue culture-treated plates on Day 1. On Day 2, the medium overlaying each plate of cells was removed, and 10 ml of fresh D10 medium was added to each plate. The cells were transfected with 1 ml of transfection solution (10  $\mu$ g pCL20cw650MNDhWASp $\Delta$ 46 or pCL20cw650MNDGFP $\Delta$ 46, 6  $\mu$ g pCAGGPI.1R, 2  $\mu$ g pCAGGS-VSVG, 2  $\mu$ g pCAG4RTR2, 125 mmol/l CaCl<sub>2</sub>, 25 mmol/l HEPES, 140 mmol/l NaCl, 0.74 mmol/l Na<sub>2</sub>HPO<sub>4</sub>, pH 7.05), then incubated overnight at 37 $^\circ$  C. On Day 3, the supernatants overlaying each cell plate were aspirated and replaced with 10 ml fresh D10 medium. On Day 4, the conditioned supernatants were collected, filtered through a 0.22 micron filter, transferred to a 30 ml polyallomer tube (Beckman Coulter, Indianapolis, IN, cat# 358126), underlaid with 5 ml of a 20% sucrose solution prepared in PBS, then centrifuged for 90 minutes at 25,000 rpm. The supernatants were aspirated and the vector pellets were resuspended in ~0.25 ml unconcentrated conditioned medium to obtain ~100 $\times$ -fold concentrated vector.

To induce vector production from hWASp producer cell clones, cells were seeded onto a 10-cm diameter tissue culture-treated plate at a density of  $4\text{--}5 \times 10^6$  cells per plate in D10 (no Doxycycline, Puromycin, or Zeocin). Each day, the medium overlaying the cells was removed and replaced with fresh D10 medium to wash out doxycycline and maximize vector component expression. On day 4, the vector-containing supernatants were collected (~25 ml per conical ultracentrifuge tube (Beckman Coulter, Brea, CA; cat. no. 358126)) and ultracentrifuged (25,000 rpm, 4 $^\circ$  C, 1.5 hours) through a 5 ml 20% sucrose cushion (w/v) in DPBS. The supernatants were aspirated and the vector pellets were resuspended in ~250  $\mu$ l (per conical tube) conditioned D10 medium (containing unconcentrated vector supernatant). Resuspended vector pellets were pooled and aliquoted into cryovials for storage at  $-80^\circ$  C. Vector titers were determined by diluting a concentrated vector stock 100-fold in D10, and using 2, 10, and 50  $\mu$ l of diluted vector to transduce 100,000 HeLa cells, using 7.5  $\mu$ g/ml polybrene. Transduced HeLa cells were cultured for 6–7 days prior to genomic DNA isolation using a commercially available kit (Qiagen, Valencia, CA; cat. no. 158388). Eight to 10  $\mu$ g of HeLa genomic DNA was digested with *Bgl*III and resolved on a 1% agarose gel for Southern analyses. Titers were determined by quantitating band intensities of WPRE-containing fragments with a phosphorimager (GE Healthcare, Pittsburgh, PA; Molecular Dynamics Storm 860), relative to WPRE-containing fragments derived from a lentiviral GFP vector of known titer.

### Small molecules used in CD34<sup>+</sup> culture

Nicotinamide (NAM) (Fisher Scientific, Acros Organics, NJ; #128271000) was solubilized in water and used at a final concentration of 5 mmol/l throughout

the entire culture of CD34<sup>+</sup> cells, including prestimulation and transduction. NAM and EX527 are inhibitors of Sirtuin 1 (SIRT1), an important epigenetic regulator governing the expansion and engraftment of hematopoietic progenitors.<sup>12</sup> Ex527 (Santa Cruz Biotechnology, Dallas, TX; sc-203044) was solubilized in DMSO and used at a 50  $\mu$ mol/l final concentration throughout the entire CD34<sup>+</sup> *ex vivo* culture. CHIR98014 (Santa Cruz Biotechnology; sc-207421) is an inhibitor of glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ), a critical regulator of stem cell function.<sup>42</sup> GSK3 $\beta$  inhibitors have been previously shown to enhance the engraftment of myeloid, lymphoid, and primitive stem cell compartments.<sup>13</sup> CHIR98014 was added at a concentration of 0.5  $\mu$ mol/l final to CD34<sup>+</sup> cells during an overnight transduction with lentiviral vector, 24 hours before transplantation into NSG mice.<sup>43</sup> Stromal derived growth factor 1 $\alpha$  (SDF1 $\alpha$ ), a ligand to the CXCR4 receptor is a growth factor important for the homing and engraftment of hematopoietic stem cells.<sup>44</sup>

### Transduction of human CD34<sup>+</sup> and murine lin<sup>-</sup> cells

Frozen human CD34<sup>+</sup> cells were gently thawed in a 37 $^\circ$  C water bath until the last ice crystal was still visible and carefully placed in a 15-ml conical tube. Two milliliters of CD34<sup>+</sup> culture medium were slowly added to the thawed cell suspension dropwise over the course of 2 minutes. One milliliter aliquots of CD34<sup>+</sup> culture medium was slowly added to the cells and gently vortexed between 1 ml additions until a total of 8 ml of CD34<sup>+</sup> culture medium had been added to the cells. The 15-ml conical tube was laid on its side to minimize cell clumping and allow cryopreserved cells to acclimate at room temperature for ~30 minutes. After acclimation, the cells were collected by centrifugation and resuspended in 10-ml fresh CD34<sup>+</sup> culture medium in untreated 10-cm diameter tissue culture plates (500,000 cells/ml) and cultured at 37 $^\circ$  C 5% CO<sub>2</sub> to undergo a 24-hour prestimulation. After prestimulation, the cells were collected by centrifugation and plated on Retronectin (Clontech/Takara Bio USA; cat. no. T100B) coated plates at a density of  $1 \times 10^6$  cells/ml in 10 ml of CD34<sup>+</sup> culture medium. Cells were transduced once with concentrated virus using MOIs ranging from 80 to ~200 overnight (~14 hours), with 0.4  $\mu$ g/ml protamine sulfate (St. Jude Pharmacy). For cells undergoing a second transduction, previously transduced cells were collected by centrifugation and resuspended in 10-ml fresh CD34<sup>+</sup> culture medium (per 10-cm plate) and rested at 37 $^\circ$  C for ~9 hours, before a second overnight transduction (~14 hours) was performed. Mock- and vector-transduced CD34<sup>+</sup> cells were recovered from retronectin-coated plates using cell dissociation buffer (Sigma; cat. no. C5789), collected by centrifugation, and resuspended in injection buffer (DPBS supplemented with 1 $\times$  P/S and 2% HI-FCS). Five hundred thousand to 700,000 CD34<sup>+</sup> cells (in 300  $\mu$ l) were transplanted into NSG mice via tail vein injection.

Murine lin<sup>-</sup> cells were transduced twice at an MOI of 25 per transduction. Briefly, lin<sup>-</sup> cells were prestimulated in untreated tissue culture plates for 30 hours in Lin<sup>-</sup> culture medium at 37 $^\circ$  C 5% CO<sub>2</sub>. The next day, cells were collected by centrifugation and seeded onto retronectin-coated culture plates at a density of  $1 \times 10^6$  cells/ml and transduced with virus overnight for 14 hours. The next day, the cells were collected and seeded back onto the retronectin-coated plates at a density of  $2 \times 10^6$  cells/ml and transduced a second time with virus for 4 hours. The cells were collected by centrifugation, washed, and resuspended in injection buffer. Approximately 500,000 cells in 200  $\mu$ l were injected per mouse.

### Human CD45<sup>+</sup> and murine CD45.2 lineage staining

Twelve to 13 weeks post-transplantation, NSG mice were sacrificed and bone marrow cells from tibias and femurs were collected into DPBS, supplemented with 2% HI-FCS. Samples from individual mice were prepared as single-cell suspensions and passed through a nylon cell strainer (BD Biosciences, San Jose, CA; Ref. no. 352360) to remove debris and then stained with the following monoclonal antibodies per the manufacturer's instructions: allophycocyanin-conjugated antihuman CD45 (BD Biosciences; Clone HI30 cat. no. 555485), phycoerythrin-Cy7-conjugated antihuman CD19 (BD Biosciences; Clone SJ25C1 ca. no. 557835 phycoerythrin-conjugated antihuman CD15 (BD Biosciences; Clone HI98 cat. no. 555402), and phycoerythrin-conjugated antihuman CD33 (Dako; Carpinteria, CA; Clone WM-54 Code no. R0745). Dead cells which stained with 4'-6-diamidino-2-phenylindole were excluded from the analysis. This four-color flow cytometric analysis was conducted using a FACS Aria (BD Biosciences). Human CD45<sup>+</sup> cells stained for CD19<sup>+</sup> (lymphoid) or CD15/33<sup>+</sup> (myeloid) lineages were sorted and collected in MACS Buffer (Miltenyi Biotech; cat. no. 130-091-221).

Twenty weeks post-transplantation, female WASp<sup>-/-</sup> and male WASp<sup>-</sup> mice were sacrificed and BM cells were analyzed. Approximately  $1 \times 10^6$  BM cells were stained at a ratio of 1:50 with the following antibodies: CD45.1-APC-Cy

7 (BD Biosciences; cat. no. 560579) for murine cells, CD3-APC (BD Biosciences; cat. no. 553066) for T-cells, B220-FITC (BD Biosciences; cat. no. 553974) for B-cells, CD11b-APC (BD Biosciences; cat. no. 561690) and Ly-6G-PECy7 (BioLegend, San Diego, CA; cat. no. 127618) for neutrophils, and CD11b-APC and Ly-6G-PECy7 (BioLegend; cat. no. 128006) for monocytes. All murine BM cells were analyzed on a FACS LSR II D machine, using the BD FACS Diva software (BD Biosciences).

### Generation of recombinant hWASP

The St. Jude Protein Production Core generated a construct of full length hWASP gene using the pFASTBAC vector (Invitrogen, Camarillo, CA; cat. no. 10584-027) with a C-terminal His tag for cytosolic expression. An additional construct of the hWASP full length gene was made in the secretory pACGP67 vector (BD Biosciences; cat. no. 554756) also using a C-terminal His tag. All the constructs were sequence verified. The pFASTBAC constructs were processed following the BAC-to-BAC protocol (Invitrogen). The pACGP67 construct was processed using the protocol from BD biosciences. A P1 virus stock was generated in both the systems and the protein expression was verified by anti-His Western blot. A small scale expression of hWASP protein was made in High Five cells (Invitrogen; cat. no. B885-02) using baculoviruses from both constructs. The cells were harvested 72 hours postinfection and the pellets and supernatants further processed for purification. A metal affinity chromatographic screen was performed for soluble protein using 1 ml HiTrap HP chelating column (GE Life Sciences, Piscataway, NJ cat. no. 17-0408-01). The protein from the cell lysates of the pFASTBAC construct did not bind to the nickel column and further expression using this system was stopped. The protein secreted using baculovirus made with pACGP67 encoding the hWASP gene bound to the nickel column and this construct was selected for large scale expression and purification. The remaining P1 baculovirus stock from the transfection of the pACGP67 construct was amplified for two rounds into a high titer viral stock. 20 l of High Five cells were infected with the baculovirus and the culture supernatant was clarified by centrifugation followed by tangential flow filtration and diafiltration. The resulting diafiltrate was loaded to a metal affinity column and hWASP was eluted from the affinity column using an imidazole gradient. The hWASP-containing fractions were further purified using size exclusion chromatography then flash frozen and stored in liquid nitrogen.

### Derivation of hWASP-specific C10.4 mAb

The methodology employed to generate the C10.4 mAb essentially follows the protocol given by Wang *et al.*<sup>45</sup> The St. Jude Immunologic Reagent Facility immunized WAS<sup>-/-</sup> mice i.p. with KLH-conjugated WAS peptide 503 or full-length WAS protein (20 µg) in equal volumes of complete Freund's adjuvant (200 µl total per mouse). Mice were boosted twice by injection of KLH-conjugated peptide or protein in incomplete Freund's adjuvant at 3-week intervals. Mice were bled for screening purposes prior to immunization and at every boost. Three months later, 72 hours after the last boost, the mice which demonstrated the highest response by enzyme-linked immunosorbent assay to WAS protein were euthanized. Splenocytes and lymph node cells were collected, processed to make a single cell suspension, mixed with X63-Ag-8.653 mouse myeloma cells at a ratio of 5:1, pelleted, and incubated with 37 °C prewarmed polyethylene glycol 1,500 (PEG 1,500; Sigma; cat. no. 81210). The cell mix was slowly and gently resuspended in 37 °C prewarmed HBSS buffer. After gentle agitation for 3 minutes, the cell mix was pelleted, resuspended in compete tumor medium (CTM), and plated in 96-well plates at 10<sup>5</sup> splenocytes per well. After a 24-hour incubation, CTM containing Hypoxanthine Aminopterin Thymidine (HAT, Invitrogen; cat. no. 21060-017) was added to select hybrid clones. Plates were incubated at 37 °C in a tissue culture incubator until visible colonies were present in the wells. The supernatants were screened by enzyme-linked immunosorbent assay, and the positive wells were subcloned by single-cell sorting. Nine single cell hybridomas were recovered from the peptide immunization and eighty from the protein immunization. Clones were expanded and secreted antibodies from said clones were again screened by enzyme-linked immunosorbent assay. No peptide clones were positive, but the 39 positive clones from the protein immunization were subsequently analyzed in a Protein G- pull-down assay to identify monoclonal antibodies which could recognize the native protein of normal human B-cells. Five micrograms of purified antibody were incubated with B-cell lysate and mixed with 50 µl Protein G-Sepharose (Invitrogen; cat. no. 10-1241). The mixture was pelleted and the antibody eluted with 0.1 mol/l Glycine solution, pH 2.7, neutralized with 1 mol/l TRIS, pH 8.0. After centrifugation, the supernatant was removed, denatured, and run on a gel for western blot. hWASP captured by the various antibodies being screened

was detected by WASP antibody B-9 that is capable of detecting hWASP in a western blot (Santa Cruz Biotechnology; cat.no. sc-13139).

For intracellular flow cytometric staining purposes, the C10.4 mAb was conjugated with PE (AbD Serotec, Kidlington, Oxon, UK; cat. no. LNK023RPE), or APC (AbD Serotec; cat. no. LNK032APC). Approximately 1e6 cells were centrifuged and resuspended in 100 µl PBS + 2% FBS, and transferred to a well of a 96-well plate. Another 100 µl PBS + 2%FBS was added to each well of cells and centrifuged at 1,500 rpm for 3 minutes. The 96-well plate was inverted sharply to decant the overlaying supernatants. The cells were washed once more with 200 µl PBS +2% FBS, then 100 µl of anti-human CD32 Fc blocker (Fisher Scientific, Pittsburgh, PA cat# NC0273319) was added to each well, and incubated on ice for 10 minutes. Upon the addition of 150 µl PBS to each well, the cells were centrifuged and sharply inverted as described above, then washed again with 200 µl PBS per well. The cells were fixed with 200 µl of a 1% formaldehyde solution in PBS and incubated at room temperature for 20 minutes. After removing the overlaying supernatant by centrifugation and sharp inversion, the cells were washed with 200 µl PBS. Two hundred µl of Perm/Wash Buffer (BD Biosciences; cat# 557885) was added to each well and incubated for 10 minutes at room temperature. After centrifugation and sharp inversion to remove the supernatants, anti-hWASP antibody (conjugated with PE at 1 mg/ml, then used at a 1:3,000 dilution) in Perm/Wash Buffer was added in a final volume of 50 µl, and was incubated on ice for 30 minutes. After adding 150 µl of PBS to each well, the plate was centrifuged, the supernatants removed via sharp inversion, then each cell sample was resuspended in 200 µl PBS +2%FBS, filtered through a 35 micron filter, and analyzed by FACS. hWASP mean fluorescence intensity (MFI) was normalized by dividing the MFI for each mouse by the VCN obtained for each lineage.

### Assessing human CD45<sup>+</sup> and murine CD45.2 transduction

Two hundred thousand human CD45<sup>+</sup> cells from NSG bone marrow (harvested 12–13 weeks post-transplantation) were sorted for two different DNA analyses. For colony PCR, 100,000 sorted CD45<sup>+</sup> cells were seeded into 3 ml of methocellulose (Stem Cell Technologies; cat.no. 0444) containing human cytokines, and two 1 ml cultures in 35-mm diameter plates were maintained for 12–14 days at 37 °C 5% CO<sub>2</sub>. Colony forming units that arose from individual human cells (CFU-Cs) were picked and DNA was prepared for colony PCR. CFU-Cs that were transduced with hWASP vector were scored positive by PCR when amplification of both endogenous and vector-derived hWASP DNA (exons 3–6) was observed. It was possible to discriminate amplification of endogenous hWASP DNA from vector-derived hWASPcDNA, as endogenous hWASP contains 302bp of intronic sequence that the vector-derived hWASP does not. Using hWASP specific primers Ex3-s (5' CTGTGCTGGCTGCTCTGGGAAC 3') and Ex6-as (5' CCTTGGTCTCCACCTGGATGCA 3'), in a Phusion (New England Biolabs, Ipswich, MA; cat. no. M0531L) PCR reaction (98 °C for 90 seconds, then 36 cycles of a 10-second 98 °C denaturation, and 30 seconds 72 °C annealing/elongation, followed by a 5-minute 72 °C hold), the endogenous hWASP product was 585bp, and the vector-derived hWASP product was 283bp. Alternatively, CFU-Cs were scored hWASP<sup>+</sup> in subsequent NSG transplantation studies when both HIV GAG and human RNaseP were detected from CFU-C DNA by qPCR (see below).

### qPCR

For human VCN analyses, a 20× HIV GAG probe was prepared; forward primer (18 µmol/l 5' GGAGCTAGAACGATTGCGAGTTA 3'); reverse primer (18 µmol/l 5' GGTGTAGCTGTCCAGTATTGTGTC 3') primers; and probe (5 µmol/l of 6FAM-CAGCCTTCTGATGTTTCTAACAGGCCAGG-NFQ-MGB, synthesized by Applied Biosystems, Foster City, CA), and used with TaqMan fast universal PCR master mix (Applied Biosystems; part no. 4352042) in qPCR reactions (95 °C for 20 seconds, then 40 cycles of a 1sec 95 °C denaturation, and 20 seconds 60 °C annealing/elongation, followed by a 5-minute 72 °C hold). Human RNaseP probe (Applied Biosystems; part no.4401631) was included in the qPCR reactions (*i.e.*, a duplex qPCR reaction with HIV GAG probe) to quantitate the relative VCNs between cell samples using the  $\Delta\Delta C_t$  method against a 1 copy lentiviral Jurkat cell control.

For murine VCN analyses, 20× HIV GAG and 20× murine glyceraldehyde 3-phosphate dehydrogenase (Applied Biosystems; part. no. 4351309) were used in singleplex qPCR reactions, and VCNs were determined by using a standard curve generated from murine mammary gland tumor (MGT) cell (ATCC, Manassas, VA; cat. no.CRL-6468) genomic DNA that contained 1 to 7 vector genomes per cell clone.<sup>10</sup>

For murine VCN analyses pertaining to the *in vitro* immortalization assay (described below), the specific probes/primers used to target the lentiviral backbone, GFP, and murine  $\beta$ -actin (an internal control), along with relevant single copy control human and murine cell lines used to generate relative standard curves for VCN quantification have been described previously.<sup>10</sup>

### *In vitro* immortalization assay

The protocol essentially follows that developed by Modlich *et al.*<sup>17</sup>, but differs in the following manner: (i) Iscove's modified Dulbecco's medium was used throughout the entire culture, instead of StemSpan HS2000, (ii) four vector transductions (days 3–6) were performed instead of two (days 4–5), and (iii) culture volumes increased 0.25 ml each day, for days 5, 6, 7, and 8, instead of 0.5 ml on day 5. Briefly, Lin<sup>-</sup> BM cells from C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME) were prestimulated for 2 days in Iscove's modified Dulbecco's medium (Corning; CellGro; cat. no. 15-016-CV) containing 50 ng/ml murine SCF, 100 ng/ml hFlt-3 ligand, 100 ng/ml hIL-11 (PeproTech; cat. no. 200-11), 10 ng/ml mL-3 (PeproTech; cat. no. 213-13), 10% fetal calf serum, 1 $\times$  P/S, and 1 $\times$  Glut (hereafter called IMDM10 medium) at a density of  $\sim 5 \times 10^5$  cells/ml. One hundred thousand cells were transduced on days 3, 4, 5, and 6 at an MOI of 1 to 10 per transduction by spinoculation (30 minutes centrifugation at 4 °C onto Retronectin-coated suspension culture dishes preloaded with virus). For each vector or vector dose, four replicate transductions were performed in four different wells of a 24-well tissue culture plate. On the first day of transduction, 100,000 cells were seeded onto wells in 500- $\mu$ l medium per sample. On subsequent transduction days, the culture medium was increased by 250  $\mu$ l, such that the culture volume per sample was 1.25 ml by day 6. On day 10, (4 days after the last transduction), VCN was determined by qPCR to normalize replating frequencies. After transduction, BM cells were expanded and cultured for 2 weeks in IMDM10 medium. Cell densities were adjusted to  $5 \times 10^5$  cells/ml every 3 days. After 14 days of culture, BM cells were seeded onto 96-well plates at densities of 100 cells per well. Two weeks later, the cell viability was determined by the adding tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to the wells. Wells that contained actively growing cells tested positive colorimetrically at an OD of 562 nm, whereby the unreduced MTT dye (yellow in color) was reduced by NAD(P)H-dependent cellular oxidoreductase enzymes to form formazan, which is purple in color. Cell replating frequencies were calculated based on Poisson statistics, using L-Calc software (Stem Cell Technologies).

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