WAS-promoter driven lentiviral vectors mimic closely the lop-sided WASP expression during megakaryocytic differentiation: a comparative study.

Pilar Muñoz, María Tristán-Manzano, Almudena Sánchez-Gilabert, Giorgia Santilli, Anne Galy, Adrian J. Thrasher, Francisco Martín

PII: S2329-0501(20)30191-1
DOI: https://doi.org/10.1016/j.omtm.2020.09.006
Reference: OMTM 553

To appear in: Molecular Therapy: Methods & Clinical Development

Received Date: 6 May 2020
Accepted Date: 11 September 2020

Please cite this article as: Muñoz P, Tristán-Manzano M, Sánchez-Gilabert A, Santilli G, Galy A, Thrasher AJ, Martin F, WAS-promoter driven lentiviral vectors mimic closely the lop-sided WASP expression during megakaryocytic differentiation: a comparative study., Molecular Therapy: Methods & Clinical Development (2020), doi: https://doi.org/10.1016/j.omtm.2020.09.006.

This is a PDF file of an article that has undergone enhancements after acceptance, such as the addition of a cover page and metadata, and formatting for readability, but it is not yet the definitive version of record. This version will undergo additional copyediting, typesetting and review before it is published in its final form, but we are providing this version to give early visibility of the article. Please note that, during the production process, errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

© 2020 The Author(s).
TITLE

WAS-promoter driven lentiviral vectors mimic closely the lop-sided WASP expression during megakaryocytic differentiation: a comparative study.

Pilar Muñoz1,2*, María Tristán-Manzano1*, Almudena Sánchez-Gilabert1,3*, Giorgia Santilli2, Anne Galy4, Adrian J. Thrasher2 and Francisco Martin1‡.

1 Genomic Medicine Department. GENYO, Centre for Genomics and Oncological Research, Pfizer-University of Granada-Andalusian Regional Government, Parque Tecnológico Ciencias de la Salud, Av. de la Ilustracion 114, 18016 Granada, Spain.
2 University College London (UCL) Great Ormond Street Institute of Child Health (ICH), 30 Guilford Street. WC1N 1EH. London, United Kingdom
3 Current address: Karuna Good Cells Technologies SL, C/ Cercas Bajas, 13 –BJ, Vitoria-Gasteiz, 01001, Álava, Spain
4 Integrare Research Unit UMR_S951, Génethon, INSERM, University Évry, EPHE, Évry, Université Paris-Saclay, 1 bis rue de l’Internationale, 91002 Évry, France.

* P.M., M.T.M and A.S.G share first authorship.

‡ Corresponding author: Francisco Martin: francisco.martin@genyo.es Genomic Medicine Department, GENYO, Centre for Genomics and Oncological Research, Pfizer-University of Granada-Andalusian Regional Government, Parque Tecnológico Ciencias de la Salud (PTS), Av. de la Ilustración 114, 18016 Granada, Spain. Phone: 34-958637103. FAX: 34-958637071
Running title: Physiological lentiviral vectors for gene therapy of Wiskott-Aldrich Syndrome

KEYWORDS: Wiskott-Aldrich syndrome (WAS), alternative WAS promoter, mouse model, hematopoietic stem progenitor cells (HSPCs), WAS knockout (WASKO) cellular models, megakaryocytic differentiation, lentiviral vectors, phenotypic rescue, WAS patients.
ABSTRACT

Transplant of gene-modified autologous hematopoietic progenitors cells has emerged as a new therapeutic approach for Wiskott-Aldrich syndrome (WAS), a primary immunodeficiency with microthrombocytopenia and abnormal lymphoid and myeloid functions. In spite of the clinical benefit obtained in the ongoing clinical trials, platelet restoration is suboptimal. The incomplete restoration of platelets in these patients can be explained either by a low number of corrected cells or by insufficient or inadequate WASP expression during megakaryocyte differentiation and/or in platelets. We therefore used in vitro models to study the endogenous WASP expression pattern during megakaryocytic differentiation and compared it with the expression profiles achieved by different therapeutic lentiviral vectors (LVs) driving WAS cDNA through different regions of WAS promoter. Our data showed that all WAS-promoter driven LVs mimic very closely the endogenous WAS expression kinetic during megakaryocytic differentiation. However, LVs harbouring the full-length WAS-proximal promoter (WW1.6) or a combination of the WAS alternative and proximal promoters (AW) had the best behaviour. Finally, all WAS-driven LVs restored WAS knockout (WASKO) mice phenotype and functional defects of hematopoietic stem progenitor cells (HSPCs) from a WAS patient with similar efficiency. In summary, our data back up the use of WW1.6 and AW LVs as physiological gene transfer tools for WAS therapy.
INTRODUCTION
Wiskott-Aldrich Syndrome (WAS) is a X-linked rare primary immunodeficiency (incidence 1 in \(10^5\) to 1 in \(10^6\) cases per live birth) (MIM no.301000) and makes up approximately 3% of all primary immunodeficiencies disorders. It is diagnosed early in the life and many patients with severe WAS do not survive past of 10 years without definitive treatment.\(^1\) The classical WAS phenotype is characterized by eczema, immunodeficiency, microthrombocytopenia, autoimmunity and malignancies.\(^2\) WAS is caused by mutations in the \(WAS\) gene \(^3\) (gene map locus \(Xp11.23-p11.22\)) that is expressed exclusively in hematopoietic cells and play important roles in signalling and actin cytoskeleton reorganization (reviewed in \(^4\)). Therefore, most hematopoietic cells are affected at different degrees, causing the phenotypic abnormalities observed in patients with WAS. Of all functional defects, increased bleeding due to low platelet (PLT) counts is the major challenge with up to 30% of WAS patients suffering life-threatening bleeding episodes.\(^5\)

The only curative treatments for WAS patients are allogeneic hematopoietic stem and progenitor cells (HSPCs) transplantation \(^6\) and autologous gene-modified HSPCs.\(^7\) Although HSPCs transplantation is the standard treatment procedure and is usually curative, the use of HLA-matched HSPCs is associated with acute morbidity and high incidence of long-term complications, although overall survival rates are continually improving.\(^8\)\(^\text{--}\)\(^10\) On the contrary, lentiviral-based gene therapy clinical trials observed limited toxicity and similar survival rates,\(^7\)\(^11\)\(^\text{--}\)\(^14\) making this treatment a rational alternative therapeutic option. In these trials, autologous CD34\(^+\) cells were genetically modified using a self-inactivating LV expressing \(WASP\) under a 1.6-kb fragment of the proximal promoter of the \(WAS\) gene.\(^15\) A general consensus arose from these clinical trials is that, although immune deficiency was corrected,
complete remission of microthrombocytopenia was more difficult to achieve.\textsuperscript{16} Of note, HSPC-transplantation is more effective than gene therapy in this aspect and is therefore a drawback to overcome in new WAS gene therapy products.\textsuperscript{16}

The reasons behind the low PLT recovery achieved with gene therapy in most WAS patients are unclear. It could be due to the absence of selective advantage of WASP-expressing PLTs,\textsuperscript{17} to the suboptimal WASP expression in megakaryocytes (MK), MK progenitors and/or PLTs\textsuperscript{14} or a combination of both factors. In this direction, our hypothesis is that a physiological expression of WASP would improve GT outcomes by preventing potential side effects of under-expression or overexpression of WASP along MK differentiation. The exact functions of WASP in PLTs remains largely unknown but there are strong evidences suggesting that it play a critical role regulating MK differentiation and PLTs formation by inhibiting these processes in the absence of the appropriated signals.\textsuperscript{18-20} This could sound contradictory with the thrombocytopenia found in WAS patients but is actually a potential explanation for it. Indeed, PLTs produced in the absence of WASP have not been developed properly and have reduce size, abnormal ultrastructure and surface markers that lead to their elimination in the spleen and other tissues. Therefore, in order to generate normal PLTs levels in WAS patients, we should, not only engraft with the appropriate levels of WASP-expressing HSPCSs, but also mimic very closely the physiological expression of WASP during MK development.

Different groups, including ours, have developed physiologically regulated LVs for the treatment of WAS using different fragments of the WAS-proximal-promoter to drive the expression of WAS cDNA.\textsuperscript{15,21} These studies showed hematopoietic-specific expression of the different WAS-promoter driven LVs that efficiently restored WASP defects in animal models.\textsuperscript{22,23} Later studies also showed improved safety of these LVs by avoiding WASP
expression in non-hematopoietic cells\textsuperscript{24} and reducing genotoxicity.\textsuperscript{25} These works lead to the approval of the clinical trials mentioned above using the 1.6kb WAS-proximal-promoter driven LVs.\textsuperscript{15} However, as referred to previously, in spite of the good clinical results, these trials showed suboptimal WASP expression in PLTs and suboptimal recovery of microthrombocytopenia in most patients. In an attempt to improve the behaviour of WAS-proximal-promoter driven LVs, our group generated AWE LVs\textsuperscript{26} that harboured regulatory fragments from the two WAS promoters described in the literature; the proximal\textsuperscript{27} and the alternative\textsuperscript{28}. The proximal promoter starts immediately upstream of the transcription start site (AC115618.3 Seqs 120189-121855) and the alternative promoter is located 6kb upstream (AC115618.3 Seq 114403-115000). The AWE LVs showed improved enhanced GFP (eGFP) expression in myeloid, megakaryocytic and B cell lineages compared to WAS-proximal-promoter driven LVs.\textsuperscript{26} However, since the WAS cDNA contain regulatory sequences that affect its expression pattern,\textsuperscript{29} it is fundamental to study the behaviour of the AW LVs backbone expressing WAS cDNA in order to determine if they achieve truly physiological expression. In this work we use \textit{in vitro} models to study whether WAS-promoter-driven LVs harbouring sequences from the alternative promoter could improve their therapeutic potential by mimicking closer the WASP expression pattern during megakaryocytic differentiation. Although WASP expression levels are well documented in all mature blood cells,\textsuperscript{30,31} the precise WASP expression profile in HSPCs along MKs differentiation and PLTs generation is mostly unknown. We therefore analysed first the WASP expression kinetic during MK differentiation and then compared it with the expression pattern of the different LVs driving the transgene through different regions of the WAS promoter, including the LV used in ongoing clinical trials for WAS. Our data showed that all WAS-promoter driven LVs mimic very closely the WASP endogenous expression kinetic during MK differentiation. We also studied potential therapeutic improvements of the AW-LVs compared with the WW1.6kb-
LVs, but could not find any significative differences.

RESULTS

WASP endogenous expression decreases during in vitro megakaryopoiesis and thrombopoiesis.

Since we want to mimic WASP endogenous expression during MK differentiation, we first established primary (figure 1) and immortalized (figure 2) cellular models to study WASP expression patterns through the MK differentiation process. MKs progenitors (CD34+CD41+), MKs (CD34-CD41+CD42+) and PLTs (CD41+CD42+FCS\textsuperscript{low}SSC\textsuperscript{low}) were routinely obtained from HSPCs (CD34+, CD41-CD42-) using the protocol depicted in figure S1a, where human CD34+ cells were incubated with stem cell factor (SCF), thrombopoietin (TPO) and ROCK inhibitor as described in M&M during 16 days. We could observe large cells that correlate with immature megakaryocytes (figure 1a, centre) that finally extend proplatelets (figure 1a, bottom) and also associated with the phenotypical acquisition of CD41a and CD42b megakaryocytic markers (figure 1b). In addition, PLTs derived from HSPCs were functional in response to thrombin (figure 1c, d) and expressed similar WASP protein levels as those PLTs isolated from peripheral blood of healthy donors (figure 1e), validating this cellular model to study MK differentiation. We then analysed WASP expression levels (figure 1f, 1g and 1h) in MKs progenitor, MKs and PLTs at days 4, 8, 11 y 16 of MK differentiation following gate strategy analysis represented in S1b Briefly, we first selected three different gates according to FSC and SSC (HPCs, MKs and PLTs) and then, we further gated 1) undifferentiated CD34+ cells; 2) MK early progenitors (CD34+CD41+ cells); 3) MK progenitors (SSC\textsuperscript{high} CD34+CD41+); 4) MKs (CD41+CD42+ SSC\textsuperscript{high}) and 5) PLTs (CD41+CD42+ SSC\textsuperscript{low}) as populations of interest. WASP expression was calculated as
the intensity ratio of WASP+ cells and background intensity of isotype control of the selected population (Fig S1b, M&M). Our data showed a clear WASP downregulation as the HSPCs differentiate to MKs progenitors (figure 1f, 1g and 1h; CD34+ versus CD34+CD41+ respectively), a slight WASP increment when MKs progenitors (CD34+CD41+ SSC\textsuperscript{high}) differentiate into early MKs (CD34+CD41+CD42+, day 4) and a gradual downregulation as the MKs mature into PLTs (days 8, 11, 16 and PLTs). Percentage of WASP+ cells showed the same described tendency but in a more dramatic manner.

We then validated these results in K562 and MEG-01 cell lines previously characterized to give rise to MK-like cells (figure 2a, 2b) as determined by the acquisition of CD41a and CD42b megakaryocytic markers (figure 2c, e). K562 cells differentiated with phorbol-myristate acetate (PMA) and MEG-01 cells with valproic acid (VPA) constitute classical models to study megakaryocytic differentiation, that mimic partially some megakaryocytic characteristics in terms of phenotype, maturation and function.\textsuperscript{32} Interestingly, WASP expression levels and percentage of positive cells were increased upon MK differentiation in K562 cells (figure 2d), while MEG-01 showed a clear downregulation along differentiation time (figure 2f). These data indicate that K562 cells behave like MK progenitors that differentiate into early MKs in the presence of PMA, while MEG-01 resemble early MKs that give rise to mature MKs and PLTs upon the addition of VPA.\textsuperscript{32} These two cell lines provide therefore additional cellular models to study LVs behaviour during MK differentiation.

\textbf{WAS-promoter driven LVs mimic WASP expression kinetics during MK differentiation and restored PLTs actin nodules formation.}

Once established the three different human models of MK differentiation and their WASP expression kinetics, we proceed to analyse the behaviour of different 2nd generation LVs
backbones expressing enhanced GFP reporter gene through different fragments of the WAS promoter (figure 3a and S2); the WE LV \(^{21}\) harbours the 500bp core of the WAS proximal promoter and the AWE \(^{26}\) and the cAWE (this work) contained additional sequences from the WAS alternative promoter. To analyse the physiological expression of these LVs during MK differentiation (figure 3b), K562 (figure 3c, S3a), MEG-01 (figure 3d, S3b) and HSPCs (figure 3e, S4) were transduced with the different LVs at MOI=1 for K562 and MEG.01 and at MOI=50 for HSPC, obtaining a similar efficacy of transduction (figure S5). Their eGFP expression kinetics (how the expression change related to the expression of non-differentiated cells, referred as fold expression and detailed in S3, S4) were compared to that of endogenous WASP (black bars in figures 3c, d, e), before and after MK differentiation. We observed similar behaviours of the three LVs and all followed WASP expression kinetic upon MK differentiation in K562 (figure 3c; upregulation), MEG-01 (figure 3d; downregulation) and HSPCs (figure 3e; downregulation). Interestingly, in the HSPCs model, the kinetic of transgene expression at different times of MK differentiation was also very similar (figure 3e).

We next analysed whether the physiological expression was maintained in the different WAS-promoter driven LVs expressing the WAS cDNA; WW, AWW and cAWW LVs (figure 4a). Here, we used homozygous WASKO-K562 previously generated by our laboratory \(^{20}\) (figures 4b-d) and HSPCs-WAS-null from a WAS patient (figures 4f-i). WASKO-K562 cells were transduced with the different therapeutic LVs at MOI=1 to reach similar transduction efficacies (as shown in figure S5). WASP expression levels were analysed in different populations (See figure S6 for details) after MK differentiation. Similarly to eGFP-LVs, all WASP-LVs increased WASP expression upon MK differentiation, measured as percentage of WASP+ cells (figure 4c) and as MeFI (figure 4d). However, the WW LVs showed higher WASP expression in resting K562 (figure 4d, WW) compared to endogenous WASP (figure...
4d, WT), AWW and cAWW LVs, indicating a more physiological behavior of the AWW and cAWW LVs.

Interestingly, AWW- and cAWW-transduced WAS patient-HSPCs also recovered a WASP expression kinetic more similar to healthy donor’s HSPCs compared to those transduced with the WW LVs (figures 4e and S7). Indeed, although the expression levels of WW LVs in undifferentiated CD34+ was closer to HD compared to AWW and cAWW (Figure 4e and 4f), the expression kinetic at early days of differentiation (day4) was different (figure 4e, red line). Nevertheless, WASP expression levels in PLTs derived from WAS patient-HSPCs transduced with the different LVs (MOI=50) were similarly rescued (figure 4f). In agreement with WASP expression levels, the restoration of actin nodules formation in contact to immobilized fibrinogen, which is severely compromised in patient’s PLTs, was also similar for all LVs (figures 4g, 4h).

**Phenotypic correction of WASKO mice after transplantation with WW- and AWW-transduced WASKO murine hematopoietic progenitor cells (mHSPCs).**

We next analysed the therapeutic efficacy of AWW- and WW- LVs in a mouse model of WAS, because cAWW-LV did not show significant improvement *in vitro* compared with the AWW-LVs. Lin- cells were isolated from bone marrow of WASKO mice (WASKO-mHSPCs) (see M&M) and transduced with AWW and WW LVs at MOI=100 . Both LVs achieved similar transduction efficiencies (lin-AWW=9.8 vcn/c; lin-WW=8.2 vcn/c) and expressed similar WASP levels in WASKO-mHSPCs (figure S8b ) and in their myeloid progeny (figure S8e, S8f ). Lethally irradiated WASKO mice (9.5Gy) were then injected with $3 \times 10^5$ AWW, WW and mock- WASKO-mHSPCs as well as with WT-mHSPCs (C57BL/6 mice). After 2 and 6 months of transplant, the side scatter (SSC) and forward scatter (FSC) of cells from peripheral blood of the transplanted mice were analysed (figure 5a, left). The non-
transduced transplanted mice showed higher number of granulocytes and lower number of lymphocytes meanwhile the WT, AWW and WW0.5-corrected mice showed a normal distribution of the population in peripheral blood. 6 months later, treated mice were sacrificed in order to analyse the ability of WW and AWW LVs of restoring phenotypic and functional defects (figure 5). Vector copy numbers per cell in spleen, bone marrow and blood of transplanted mice ranged from 0.1vcn/c to 15vcn/c, showing efficient repopulation of recipient mice with transduced AWW and WW lin-cells (figure S9a). Both LVs were equally efficient in restoring normal monocytes counts in a hemogram (Figure S9). More importantly, transplanted mice achieved equivalent ratios of B220+, Gr1+ and CD11b+ cells (figure 5a, right) as compared to wt mice, while WASKO mice presented reduced levels of B220+ cells and increased levels of Gr1+ and CD11b+ cells. Similarly, the efficacy of both LVs to rescue T cell responses (figure 5b, 5c), and PLTs counts (figure 5d) were equivalent. These experiments validate the efficacy of both WAS-promoter-driven LVs as tools for WAS gene therapy, but cannot differentiate any superiority.

Third generation WAS-driven LVs have improved physiological expression and restore functional defects in vitro and in vivo.

We finally generated a third generation LV based on the AWW and investigated their behaviour during MK differentiation as well as their therapeutic activity in WASKO mice. We used the W1.6kb LV\textsuperscript{15} (figure 6a, top; this is the LV used in ongoing clinical trials for WAS gene therapy) as backbone to construct the third generation AW LV by replacing the full length (1.6kb) WAS proximal promoter by the chimeric WAS promoter (0.88kb) containing sequences from the proximal and alternative promoter (figure 6a and S2). WAS patient-HSPCs were transduced with WW1.6 and AW LVs at MOI=50 to achieve similar transduction efficacies (WW1.6 = 2.6±1.1vcn and AW= 3.1±1.6vcn and figure S5f).
Transduced cells were differentiated into MK and the different subpopulations analyzed for WAS expression (figures 6b and S9). As in second generation LVs, the WASP expression dropped during MK differentiation in both LVs (figure 6c). We could not find any differences between both LVs neither, in terms of expression kinetic (figure 6c) nor in PLT expression levels (figure 6d). However a comparison of the WASP expression levels of each vector with WASP expression in PLTs from healthy donor showed significant differences with PLTs derived from 1.6WW-transduced HPSCs, but not with PLTs derived from AW-transduced HPSCs, although more experiments would be necessary to demonstrate it. We next evaluated the functional restoration of MKs and PLTS derived from WW1.6- and AW-transduced WAS-HSPCs measuring PAC-1 expression in response to thrombin (figures 6e), the formation of actin nodules (figures 6f, 6g) or calcium uptake (figure S11). We found a very similar improvement with both LVs, restoring the response of MKs and PLTs to thrombin, as well as the ability of PLTs to form actin nodules per platelet. Similar finding were also observed for the restoration of podosomes formation and clustering on macrophages derived from WAS-HSPCs transduced with WW1.6- and AW LVs (Figure 6h-6k), which exhibited similar WASP expression in the CD33+CD14+ cells obtained in vitro (figure 6i). We finally analysed the therapeutic efficacy of WW1.6 and AW LVs in the WASKO mice model. WASKO-mHSPCs were isolated from bone marrow, transduced with both LVs and transplanted into irradiated WASKO mice (figure 6l). Mock-transduced WASKO-mHSPCs and WT-mHSPCs were injected in control mice. 7 months later, treated mice were sacrificed to analyse the ability of WW1.6kb- and AW0.88kb to restore functional defects. Both LVs were equally efficient in restoring spleen size (figure 6m) and PLTs counts (figure 6n).

**DISCUSSION**
GT has demonstrated to be a real therapeutic alternative for WAS patients. The current GT approaches for WAS used autologous HSPCs (CD34+) cells genetically modified using a 1.6 WAS-proximal-promoter driven LV and transplanted back into the patients. The clinical efficacy of these LVs has been clearly demonstrated since treated patients have shown reduction of infections and diminished severity of eczema as well as reduced frequency and severity of bleeding episodes. However, although the bleeding was reduced or completely corrected in most patients, microthrombocytopenia persisted and normalized PLT counts were not achieved in most patients. The reasons behind the low platelet recovery achieved with GT compared with the other immune functions are unclear. It was postulated that platelet recovery could correlate with the number of transduced HSPCs reinfused. In this case, the absence of a potent selective advantage in MK and MK progenitors expressing WASP could be a contributory factor in contrast to lymphocytic lineages. Other hypotheses are that the 1.6 WAS-LV achieved suboptimal levels of WASP expression per copy of integrated vector in MK, MK progenitors and/or PLTs. At the same level of HPC transduction, it may be possible to correct the function of lymphoid cells but not that of MK lineage cells. Furthermore, PLT with suboptimal WASP levels could undergo accelerated destruction in the spleen and in other organs.

An ideal GT vector for WAS should not only transduce HSPCs efficiently, but also mimic endogenous WASP expression during HSPCs differentiation to the different hematopoietic lineages. Our hypothesis is that physiological expression of WASP during MK differentiation and in PLTs would improve GT outcomes. The aim of this work was therefore to investigate WASP expression along MK differentiation and to define which therapeutic LV mimics closer this pattern.
Although WASP expression levels are well established in most mature blood cells,\(^{30,31}\) the WASP expression kinetics during MKs differentiation and thrombopoiesis is not largely studied. We used immortalized cellular models (K562 and MEG-01) as well primary HSPCs to investigate WASP expression kinetics during these processes. Our data showed an initial decrease in WASP expression from HSPCs to MK progenitors, a slight increase when they are differentiated into early MKs and a gradual downregulation as these MKs mature into PLTs. This WASP expression kinetic has never been described before and it could be of relevance for the normal MK differentiation and thrombopoiesis. We corroborated this expression pattern on two immortalized cell lines, K562 and MEG-01 that resemble different stages of MK differentiation. K562 has been described previously as a multipotent myeloid-MK cell line for the study of early events in MK differentiation,\(^{35,36}\) while MEG-01 cells are defined as an immature MK cell line able to differentiate into mature MKs and PLTs.\(^{37,38}\) Interestingly, upon MK differentiation, K562 cells mimicked the differentiation phase from MKs progenitors into early MKs, increasing WASP levels, while MEG-01 cells mimic the differentiation stage from early MKs into mature MKs and PLTs, reducing WASP expression. The exact role of these changes in WASP expression during MK differentiation is not clear but, since the absence of WASP increases PLTs production with abnormal phenotype,\(^{18-20}\) it could be relevant for GT strategies to mimic it. Therefore, our hypothesis is that WAS GT strategies should not only achieve good WASP expression levels on PLTs and MKs differentiated cells but also mimic the endogenous expression kinetic during MK differentiation.

Once we had established the physiological WASP expression pattern during megakaryopoiesis and thrombopoiesis, we analysed the behaviour of different LVs harbouring different fragments of the WAS proximal and alternative promoters. Our previous
data indicated that a LV driving eGFP through a WAS-promoter (AWE), containing a 386bp fragment of the alternative promoter immediately upstream the 500-bp-WAS-proximal promoter increased GFP expression in myeloid cells and mature megakaryocytes. The alternative promoter is located 6-kb upstream of the proximal promoter and contain several transcription factors binding sites (TFBS) shared with the proximal promoter (Sp-1, AP-2 c-Myb and EGR2) and others than are unique (C/EBP, CP1, Ets-2, and GCF). The differences found between both promoters, suggest that their activity may vary depending on the cell lineage and the state of differentiation or development. In particular, the presence of TFBS for C/EBP, CP1, c-Myb, Ets-2 and PU.1 suggest that the alternative promoter could play important roles in the myeloid and megakaryocytic lineages. However, the 386bp fragment of the alternative promoter does not include binding sites for C/EBP and CP1, required for higher expression levels in K562 cells. Based on these data, we generated new WAS-driven LVs (cAWE and cAWW) harboring the complete alternative promoter and the 500bp proximal promoter and compared their behavior with the WAS-proximal promoter LVs (WE and WW), the shortest version of the alternative promoter (386bp), and the 500bp proximal promoter (AWE and AWW). The different second-generation LVs backbones (W-, AW- and cAW-) were studied based on their ability to express eGFP and WAS cDNA during MK differentiation and analysing whether this expression followed the same kinetic as the endogenous WAS protein. Although all eGFP-expressing LVs mimicked WASP expression pattern along MK differentiation, the WE-LV was slightly weaker than AWE and cAWE LVs. Interestingly, the expression of WAS cDNA altered the physiological behavior of the 500-WAS-proximal promoter LVs (WW) at early stages of MK differentiation, while the AWW and the cAWW LVs still followed the WASP endogenous pattern. Our finding indicates that the 500bp-WAS proximal-promoter-driven LVs leads to a WASP overexpression at early stages of MK differentiation (CD34+CD41+CD41- to
CD34+CD41+CD42+), while the AW and cAWW achieve more physiological expression. A detailed analysis of WASP kinetic along MK differentiation revealed a more pronounced decay of WASP expression of the WAS-proximal promoter driven LVs compared to the WAS-proximal and alternative promoters and to endogenous WASP. However, we could not observe significant differences in the behaviour of AWW versus cAWW LVs. We therefore concluded that the insertion of regulatory sequences from the WAS alternative promoter into the 500pb proximal promoter improved physiological expression of therapeutic LVs during MK development.

In spite of their improved physiological expression, we could not detect significant differences between the second-generation WW and AWW LVs in term of functional correction, neither in animal models nor in WAS patient’s HSPCs. Importantly, our analysis showed that LVs expressing the WAS cDNA through the proximal and alternative promoter (in a second or third generation backbone) are able to: - restore actin nodules in PLTs (figures 4g and 4h), - normalize WAS mice blood populations upon transplantation with transduced WASKOLin- cells (Figure 5a, Figure S9), - improve T cell responses (Figure 5b and 5c), - improve PLTs count (Figure 5d and Figure 6n), - reduce spleen size (Figure 6m), - restore podosome clustering of macrophages derived from transduced WASnull-hHSPCs cells (new Figures 6h-k) and - restore calcium uptake dynamics after thrombin stimulation of PLTs derived from transduced WASnull-hHSPCs (figure S11).

Still, since the AWW LVs confer a more physiological expression profile during MK differentiation, we reasoned that it would be relevant to investigate the potential of a 3rd generation LVs harbouring the shorter version of the alternative and proximal promoter (AW) as another option to the existing therapeutic LV for WAS GT, the WW1.6kb, which drives the expression of WAS cDNA through a 1.6kb fragment of the WAS proximal promoter. Contrary to what was found with the LVs harbouring the 0.5kb proximal promoter,
the LV used in the on-going clinical trials for WAS, the WW1.6 LV, was as good as the AW mimicking the WASP endogenous pattern. We finally showed a very similar therapeutic efficacy of both 3rd generation LVs WW1.6 and AW in WAS patient-HSPCs as well as in WAS mice models. These data indicate that the thrombocytopenia defects found in WW1.6 LVs-treated patients is not due non-physiological WASP expression kinetics and favour the hypotheses of low numbers of engrafted WASP+HSPCs. In addition, we cannot completely exclude that WASP expression levels are inadequate for in vivo production of platelets in man, since our in vitro models may not fully model these steps.

In summary, in this work we have identify the WASP expression kinetic during MK differentiation showing an initial decrease in WASP expression as HSPCs differentiated into MK progenitors, a slight increase when progenitors differentiate into early MKs and a continuous downregulation as these MKs mature into PLTs. Based in this data, we have shown that the 500bp WAS-proximal promoter-driven LVs improved their physiological behaviour after inclusion of regulatory sequences from the alternative promoter. However, the WW1.6 LV was as good as the AW mimicking the WASP expression pattern and also restoring functional defects of WAS patient’s HSPCs and WASKO mice models. Altogether, our data indicated that the WW1.6 and AW LVs are able to mimic WASP endogenous expression patterns during MK differentiation. We propose to use the AW LVs for a clinical trial for WAS gene therapy in order to investigate potential therapeutic benefits over the WW1.6 LVs.
MATERIAL AND METHODS

Cells

293T Cells (CRL11268; American Type Culture Collection; Rockville, MD) were maintained in Dulbecco's Modified Eagle's Medium (DMEM, ThermoFisher Scientific, Waltham, MA USA) with GlutaMAX™ supplemented with 10% Fetal Bovine Serum heat inactivated (FBS, Sigma-Aldrich, St. Louis, Missouri, USA) and antibiotics. The human cell line K562 (lymphoblast from bone marrow chronic myelogenous leukemia, CML) was obtained from ATCC (CCL-243), and maintained in RPMI media (Invitrogen), supplemented with 10% FBS at 5% CO₂ and 37º C. Autologous CD34+ cells were collected from mobilized peripheral blood (after cryopreservation of an unmodified back up stem cell harvest). The WAS patient carried the WAS mutation: c.58C>T p.(Gln20*). Cells were grown for 6 days in StemSpan media (StemCell Technologies, Vancouver, Canada) supplemented with 1% penicillin/streptomycin, 100ng/ml stem cell factor (SCF), 100 ng/ml Flt-3L, 20 ng/ml TPO, 20ng/ml IL-6 (all from Peprotech, (NJ, USA), 1μM StemRegenin 1 (SR1) (Cayman Chemical, MI, USA), 500 nM UM729 or UM171 (StemCell Technologies) and 10μM 16,16-dimethyl Prostaglandin E₂ (dmPGE2) (Cayman Chemical).

Bone marrow of C57BL/6J and B6.129S6-Was<sup>tm1Sbs</sup>/J mice were harvested from the femurs and tibias and lineage negative progenitors (lin-) were isolated with magnetic beads using the lineage cell depletion kit (130-090-858, MACS, MiltenyiBiotec, Germany) following manufacturer’s instructions. 1x10⁶ cells/ml were culture in StemSpan media (StemCell Technologies) supplemented with 1% penicillin/streptomycin, 1% glutamine, 100 ng/ml murine stem cell factor, 20ng/ml mFlt-3L, 20 ng/ml mIL3 and 20 ng/ml mIL6 (Peprotech).

Human platelets were obtained by centrifugation of peripheral blood at 200g for 20 min. The supernatants were recovered and centrifuged at 1000 x g 10 min in the presence of
prostacyclin I2 (0.1 µg/ml, Abcam ab120912). The pellet was resuspended in modified Tyrode’s buffer (150 mM NaCl, 2.9 mM KCl, 12 mM NaHCO₃, 0.1% glucose, 0.1% BSA, 5mM HEPES, 1 mM CaCl₂ and 1mM MgCl₂, pH 7.3) containing 0.1 µg/ml prostacyclin.

**Animals**

C57BL/6J and B6.129S6-Was<sup>tm1Sbs</sup>/J mice colonies were already established at UCL’s animal facility (original stock obtained from The Jackson Laboratories (USA). NOD.Cg-Prkdc<sup>scid</sup> Il2rg<sup>tm1Wjl</sup>/SzJ mice were obtained from The Jackson Laboratories (USA)). All animals were handled in strict accordance with good animal practice as defined by UK Home Office Animal Welfare Legislation, and all animal work was approved by the Institutional Research Ethics Committee (Institute of Child Health, University College London, UK) and performed under project licenses number 70/7024 and 2557.

**LV plasmids constructs**

The WW and WE carry a 0.5kb fragment of the WAS proximal promoter (AC115618.3 Seq 121356-121851) driving the expression of human WAS<sup>21</sup> and eGFP cDNAs (respectively). The AWW and AWE were engineered by inserting a 0.38kb fragment of the WAS alternative promoter<sup>22</sup> immediately upstream of the 0.5kb WAS proximal promoter in the WW vector<sup>26</sup> (AC115618.3: Seq 114605-114991 - 17nt fragment containing the EcoRI site - Seq 121356-121851) driving the expression of human WAS and eGFP (respectively). The cAWE contains the complete alternative promoter (0.69 kb) upstream the 500bp proximal promoter (AC115618.3: Seq 114403-115000 - 17nt fragment containing the EcoRI site - Seq 121356-121851) for the expression of eGFP. cAWW plasmid was obtained after insertion of WAS cDNA in cAWE backbone (instead eGFP sequence) (restriction enzymes BamHI and KpnI)
(New England Biolabs), by standard cloning techniques. All these LVs share the self-inactivated (SIN) lentiviral backbone described by Zuffery et al.\textsuperscript{43}

For the construction of the 3\textsuperscript{rd} generation AW LVs, we used the WW1.6 plasmid\textsuperscript{15} (kindly provided by Génétion) as backbone and replaced the ClaI/BstXI fragment containing the 1.6kb WAS proximal promoter (\texttt{AC115618.3 Seq 120189-121855}), by the ClaI/BstXI fragment from the AWW LV\textsuperscript{26} harboring the WAS alternative and proximal promoter (0.38+0.5kb) (\texttt{AC115618.3: Seq 114605-114991 - 17nt fragment containing the EcoRI site - Seq 121356-121851}).

**LVs production, titration and Multiplicity of infection (MOI)**

LVs particles were produced by polyethylenimine (PEI) (Sigma Aldrich, ref 408727) or lipoD293 (SignaGen Laboratories, Gainthersburg, MD, USA) as previously described.\textsuperscript{44} Briefly, for 2\textsuperscript{nd} generation LVs 293T packaging cells were transfected with packaging (pCMV\texttt{ΔR8.91}), envelope (pMD2.G) (\texttt{http://www.addgene.org/Didier_Trono/}) (Naldini, Blomer et al. 1996) and the desired vector plasmids (WW, WE, AWW, AWE, cAWW or cAWE). The 3\textsuperscript{rd} generation LVs were obtained by four plasmids transfection: CTE (gag/pol), pMD2.G (envelope), pRev plasmids and vector plasmid (AW and WW1.6kb). The producer cells were cultured for 48 and 72 hours and the viral supernatants were collected at those times and filtered through 0.45 μm filter (Stericup and Steritop sterile filters, Merck Millipore, Massachusetts, USA). The viral particles were then concentrated by ultracentrifugation in a Sorvall Discovery SE ultracentrifuge (Kendro Laboratory, Weaverville, USA) at 40.000 rpm, 2 hours at 4º C and the viral pellets were resuspended in StemSpan media (StemCell Technologies) for 1 hour on ice, aliquoted and immediately frozen at -80º C.
Viral titers (transduction units [TU] per millilitre) were calculated using quantitative PCR. Briefly, 293T cells were transduced with serial diluted amounts of LV. Genomic DNA was isolated (10^5 cells equivalent to 0.6 μg of genomic DNA) and the copy number of LV integrated was measured using a standard curve (from 10^2 to 10^7 copies) of plasmid DNA. Titre obtained for the therapeutic LVs were: WW= 7.91x10^7 - 6.35x10^9 TU/ml; AWW=1.58x10^8 - 8.67x10^9 TU/ml; cAWW=1.07x10^8 - 7.24x10^9 TU/ml; WW1.6kb=1.13x10^9 - 3.47x10^9 TU/ml; AW= 6.52x10^8 - 3.31x10^9 TU/ml.

Immortalized cell lines and primary human HSPCs were incubated with the different viral supernatants for 5h at 37ºC 5% CO₂. Transduction levels of the cells were determined by flow cytometry 48h-72h after transduction.

A different MOI was established for the different cell types. K562 and MEG-01 were transduced at MOI =1, human HSPCs at MOI=50 and mice HSPCs at MOI=100

**Megakaryocytic differentiation of K562 and MEG-01 cell lines**

10^5 cells K562 cells/ml were plated on a 6-well-plate and incubated with 30 nM of PMA (phorbol 12-myristate 13-acetate, Sigma-Aldrich) during 96h without media exchange. DMSO was added as negative control of differentiation. 10^5 cells MEG-01 cells/ml were incubated with 2 mM of VPA (valproic acid, Sigma-Aldrich) during 21 days. Media was changed every 2-3 days with fresh VPA. Cells were detached with TrypLE (5 min, 37ºC) for flow cytometry analysis.

**Megakaryocytic differentiation of HSPCs (CD34+)**

2.5x10^5 cells/well were seeded in a 24 well plate in a final volume of 1ml of StemSpan SFEM (StemCell™Technologies) supplemented with thrombopoietin (TPO) (50 ng/ml) and Stem
Cell Factor (SCF) (5ng/ml) and added to the cells on days 0, 4, 8, 11 and 16. The concentration of the cells was always kept below 1x10^6 cells/ml. Inhibitor of the kinase ROCK (iROCK) was added to the medium previously described at day 8 of differentiation to a final concentration of 10µM. Cells were harvested at different time points for staining with anti-human CD34-PECy7, anti-human CD41a-PE and anti-human CD42b-APC (all from eBioscience) and analysed by flow cytometry.

**Activation assay: Calcium flux**

Megakaryocytes (MKs) and platelets (PLTs)-derived from HSPCs were washed at 900g during 5 min and resuspended in Tyrode’s Buffer (TB, Sigma). Cells were stained with CD42b-APC during 20 min at RT and washed with TB. Then, cells were incubated with calcium-eFluor514 (eBioscience) at a final concentration of 5µM during 40 min at 37ºC. Cells were washed in TB and acquired on a FACsCanto II flow cytometer during 30 seconds to establish basal levels (T=0). Then, thrombin (2 units/ml) were added and acquired immediately during 90 seconds.

**Activation assay: Binding of PAC-1**

MKs and PLTs from HSPCs were centrifuged at 900g, washed and resuspended in Tyrode’s buffer (TB) with calcium and magnesium. Cells were activated with thrombin (2 units/ml) during 10 minutes at RT and stained anti CD42b-APC and anti PAC-1-FITC (all from eBiosciences) and incubated during 25 minutes at RT. The cell suspension was washed with TB, fixed with 0.25% PFA in TB and acquired on a FACSCanto II cytometer.

**Actin nodule formation assay**
MKs and PLTs derived from HSPCs were harvested and washed in Tyrode’s Buffer in the presence of prostacyclin I2 (Abcam, Cambridge, UK). Cells were seeded in chamber slides (Lab-Tek™ II Chamber Slide™, ThermoFisher) pre-treated with fibrinogen (10 g/ml, Sigma Aldrich), and let them adhere during 30 minutes at 37º C without stimuli. Chambers were washed briefly with PBS and fixed with 10% formalin for 10 min RT. After 3 washes with PBS, fixed cells were treated with NH₄Cl 50 mM for 10 min for quenching residual fluorescence, washed 3 times and permeabilized with 0.1% Triton-100x during 10 min. After washing, cells were incubated with rhodamine-phalloidin (1:200, ThermoFisher) during 30 min, washed and mounted with Prolong Gold Antifade Reagent (ThermoFisher). Images were acquired on a Confocal Laser Zeiss LSM 710 microscopy and analysed with ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA). Actin nodules per platelet were counted of WT (31 images), WAS-patient (6), WW (17), AWW (26), cAWW (22), WW1.6 (4), AW (9) of three independent experiments.

**Myeloid differentiation of HSPCs (CD34+) cells.**

Briefly, 50 000 of HSPCs were cultured in a low-attachment 24-well plates with StemSpan (STEMCELL Technologies) supplemented with FTL3-L (50 ng/ml), SCF (200 ng/ml), IL-3 (10 ng/ml), IL-6 (50 ng/ml) and M-CSF (50 ng/ml) (all from Peprotech) during 21 days. Media was renewed every 2-3 days. Monocytic differentiation was determined by the increment of FSC and SSC and expression of CD33-PE and CD14-APC (all from eBiosciences) by flow cytometry.

**Podosomes immunostaining.**

200,000 HSPCs derived-macrophages were cultured overnight over Lab-Tek II Chamber Slide (ThermoFisher Scientific) coated with 10 µg/ml fibronectin (ThermoFisher Scientific).
Day after, cells were fixed with 4% formaldehyde, permeabilized with 0.1% Triton X-100 in PBS and blocked with PBS+1% BSA. Then, cells were incubated with Phalloidin-Alexa Fluor 568 (ThermoFisher Scientific) (20 min) to detect F-actin, wash and incubated with anti-vinculin antibody (hVIN-1) (Sigma-Aldrich, Missouri, USA) (20 min). After washing, the cells were incubated with a goat anti-mouse IgG conjugated to Alexa Fluor 488 (Invitrogen), DAPI and they were placed on slide with mounting media (Prolong Gold, ThermoFisher Scientific). Images were captured on a Zeiss LSM 710 inverted confocal microscope (Zeiss, Oberkochen, Germany). Images were processed in Fiji-ImageJ program (Maryland, USA). Clusters of podosomes were defined as a zone of at least 20 podosomes.

**Purification, transduction and transplantation of lineage negative (lin-) cells.**

Bone marrow of C57BL/6J and B6.129S6-Was^tm1Sbs/J mice were harvested from the femurs and tibias and lineage negative progenitors (lin-) were isolated with magnetic beads using the lineage cell depletion kit (130-090-858, MACS, MiltenyiBiotec, Germany) following manufacturer’s instructions. 1x10^6 cells/ml were cultured in StemSpan media (StemCell Technologies) supplemented with 1% FCS (Invitrogen), 1% penicillin/streptomycin, 100 ng/ml murine stem cell factor, 20ng/ml mFlt-3L, 20 ng/ml mIL3 and 20 ng/ml mIL6. Isolated murine lin- cells were transduced for 16-18 hours with the different LVs (MOI=100). A sample was retained and further cultured for 72 hours to determine transduction efficiency (integrations per cell), *in vitro* differentiation into myeloid or dendritic cells and for transplantation into B6.129S6-Was^tm1Sbs/J mice. WT, non-transduced and transduced WASKO lin- cells (3x10^5-1x10^6), were inoculated intravenously into lethally irradiated mice (split dose of 9.5 Gy). Animals were sacrificed by using CO2 inhalation and cervical dislocation 3-7 months after transplants. Percentage of myeloid populations and expression of WASP was analysed.
**LV integrations in transplanted WASKO mice**

Genomic DNA from spleen of transplanted WASKO mice was extracted using the DNeasy blood and tissue kit (Qiagen, Hilden, Germany). The quantitative PCR was performed in a CFX96 TouchTM Real-Time PCR Detection System (Biorad, California, USA). To amplify the vector, we used sequences annealing the human WAS described by Charrier et al\(^45\) and HIV-psi sequences (forward: 5’-CAGGACTCGGCTTGCTGAAG-3’, reverse: 5’-TCCCCCGCTTAAATCTGACG-3’and probe: 5’FAM-CGCACGGAAGAGGCGAGG TAMRA3’). Titin was used as endogenous two-copy gene control (forward: 5’-AAAACGAGCAGTGACGTGAGC-3’, reverse: 5’-TTCAGTCATGCTGCTAGCGC-3’ and probe: 5’FAMTGCACGGAAGCGTCTCAGTC-3’TAMRA). Serially diluted plasmid DNA containing the relevant sequences was used as a standard curve, with all measurements performed in duplicate.

**Proliferation and IL-2 production of murine T cells**

Murine T cells were purified from spleen with CD90.2 Microbeads isolation kit (Miltenyi). For TCR stimulation, 96 well plates were coated with 1μg/ml α-CD3e (functional grade purified) (Affymetrix eBioscience). CD90.2\(^+\) cells were preincubated with CFSE dye (CellTrace™ CFSE Cell Proliferation Kit, ThermoFisher Scientific) and plated in triplicate for 5 days in RPMI supplemented with 10% FCS and 1% penicillin/streptomycin. At day 3, supernatant from each well was save for IL-2 analysis (DuoSet Elisa mouse IL-2, R&D System, Minneapolis, USA) following manufacturer’s instructions. At day 5, proliferation was measured by flow cytometry in a CyAn™ ADP analyser (Beckman Coulter) and the analysis was performance with the software FlowJO (Oregon, USA).
**Flow cytometry analysis**

Murine cells from *in vitro* experiments and those harvested from engrafted mice were fixed with 4% formaldehyde, wash and permeabilised with methanol (on ice) for 1 hour. After that, the cells were resuspended in PBS+0.1% Triton X-100 +3% BSA and blocked with purified anti-mouse CD16/32 (10μg/ml) (Biolegend) and 5% normal goat serum. The cell suspension was stained intracellularly with monoclonal antibody anti-human/mouse WASP (F8) (Santa Cruz Biotechnology) and purified mouse IgG2a isotype control (Biolegend) in PBS+0.1% Triton X-100 +3% BSA buffer. After washing, a secondary antibody goat anti-mouse IgG2a-FITC conjugate (adsorbed against human immunoglobulins) (SouthernBiotech, Birmingham, USA) was used. The antibodies used for surface staining were: Brilliant Violet-421 anti-mouse/human CD45R/B220, Brilliant Violet-421 anti-mouse CD3, PE anti-mouse/human CD11b, Brilliant Violet-421 anti-mouse Ly6G/Ly6C (Gr1) (all from Biolegend) and APC mouse anti-mouse CD45.2 (BD Pharmingen). Samples were analysed using a FACS LSRII flow cytometer (Beckton Dickinson, NJ, USA) and the analysis software FlowJo.

For counting murine platelets, blood was collected in tubes containing 20 μl of heparin and measured in the Sysmex XE-5000™ Automated Hematology System (Sysmex Corporation, Japan).

Human primary cells were stained with anti-CD34-PE-Cy7, anti-CD41a-PE and CD42b-APC (all from eBiosciences) and cells lines with anti-CD41a-PE and CD42b-APC to evaluate megakaryocytic differentiation. Cells were incubated during 30 min at 4°C, washed with PBS 300g 5 min prior to acquisition. For intracellular WASP determination, 10⁵ cells were washed and fixed with 2% PFA during 20 min RT. Permeabilization was performed with Saponin 0.2% (Sigma-Aldrich) in PBS+3% BSA (Sigma-Aldrich) and blocking with FcR Blocking (Miltenyi Biotech) and 5% of normal goat serum (Abcam). Cells were then incubated 1h on
ice with anti-WASP (1:50, EP2541Y, Abcam) or rabbit IgG isotype control (Abcam). Secondary goat anti-rabbit-IgG-FITC (AB6717, Abcam) was added at 1:1000 during 40 min on ice. Acquisition was performed on a FACSCanto II (BD Bioscience) cytometer. Data were analysed with FlowJo (Tree Star) and FACsDiva (BD Bioscience) Software. Gates and analysis strategy for WASP and eGFP expression are shown in figures S1, S3, S4, S5a. Briefly, WASP expression is referred as MeFI of WASP+ population/ MeFI of isotype control- or WASKO- population and ‘fold expression’ uses as control the expression of non-differentiated cells (CD41-CD42- or CD34+, depending on the model):

\[
\text{Expression} = \frac{\text{WASP MeFI of WASP + cells in selected population}}{\text{WASP MeFI of IsC in total selected population}}
\]

\[
\text{Fold expression} = \frac{\text{Expression in selected population}}{\text{Expression in undifferentiated population}}
\]

**Statistical analysis**

Statistical comparisons were performed with GraphPad Prism Software (San Diego, CA, USA). Non-parametric test (Mann-Whitney test), two-tailed P value (statistical significance was defined as a P value <0.05), 2 way-Anova, Bonferroni Post-Test and unpaired t-test were used. All data are expressed as mean ± SEM.
ACKNOWLEDGMENTS

This study was funded by the Spanish ISCIII Health Research Fund and the European Regional Development Fund (FEDER) through research grants PI12/01097, PI15/02015 and PI18/00337 (F.M.). The CECEyU and CSyF of the Junta de Andalucía FEDER/European Cohesion Fund (FSE) for Andalusia provided the following research grants: 2016000073391-TRA, 2016000073332-TRA, PI-57069 and PAIDI-Bio326 (F.M.) and PI-0407/2012 (P.M).

P.M. was supported by the European Union through grant agreement nº 329284 (PEOPLE MARIE CURIE ACTIONS, Intra-European Fellowship (IEF), Call: FP7-PEOPLE-2012-IEF) and by ISCIII through a postdoctoral fellowship “Sara Borrell” (CD09/00200). M.T.M. is funded by MCI through a fellowship FPU16/05467. A.G. is supported by funds from AFM/Telethon.

We thank Dr. Claire Booth for providing patient samples and Ailsa Greppy for her technical support with the animals (Western Labs, ICH-UCL). Some figures were created with Biorender.com.

AUTHOR CONTRIBUTIONS

P.M, M.T.M and A.S.G: Experimental design, collection and/or assembly of data, data analysis and interpretation, manuscript writing and final approval of manuscript. G.S: discussion and final approval of manuscript. A.G: contributed reagents, discussion and final approval of manuscript. A.J.T: financial support, discussion and final approval of manuscript. F.M: Conception and design, financial support, data analysis and interpretation, manuscript writing and final approval of manuscript.

CONFLICTS OF INTEREST

No conflicts of interest
REFERENCES

1. Imai, K., Morio, T., Zhu, Y., Jin, Y., Itoh, S., Kajiwara, M. et al. (2004) Clinical course of patients with WASP gene mutations. Blood 103, 456-464
2. Thrasher, A.J. & Kinnon, C. (2000) The Wiskott-Aldrich syndrome. Clin.Exp.Immunol. 120, 2-9
3. Derry, J.M., Ochs, H.D. & Francke, U. (1994) Isolation of a novel gene mutated in Wiskott-Aldrich syndrome. Cell 78, 635-644
4. Blundell, M.P., Worth, A., Bouma, G. & Thrasher, A.J. (2010) The Wiskott-Aldrich syndrome: The actin cytoskeleton and immune cell function. Dis Markers 29, 157-175
5. Ochs, H.D. & Thrasher, A.J. (2006) The Wiskott-Aldrich syndrome. J Allergy Clin Immunol 117, 725-738; quiz 739
6. Parkman, R., Rappeport, J., Geha, R., Belli, J., Cassady, R., Levey, R. et al. (1978) Complete correction of the Wiskott-Aldrich syndrome by allogeneic bone-marrow transplantation. N Engl J Med 298, 921-927
7. Aiuti, A., Biasco, L., Scaramuzza, S., Ferrua, F., Cicalese, M.P., Baricordi, C. et al. (2013) Lentiviral hematopoietic stem cell gene therapy in patients with Wiskott-Aldrich syndrome. Science 341, 1233151
8. Burroughs L, P.A., Brazauskas R, Liu X, Griffith LM, Ochs HD, Bleesing J, Edwards S, Dvorak CC, Chaudhury S, Prockop S, Quinones R, Goldman F, Quigg T, Chandrakasan S, Smith AR, Parikh SH, Dávila Saldaña BJ, Thakar MS, Phelan R, Shenoy S, Forbes LR, Martinez CA, Chellapandian D, Shereck E, Miller H, Kapoor N, Barnum JL, Chong H, Shyr D, Chen K, Abu-Arja RF, Shah A, Weinacht K, Moore TB, Joshi A, DeSantes K, Gillio AP, Cuvelier GDE, Keller MD, Rozmus J, Torgerson TR, Pulipher MA, Haddad E, Sullivan K, Logan BR, Kohn DB, Puck JM, Notarangelo LD, Pai SY, Rawlings D, Cowan MJ. (2020) Excellent Outcomes Following Hematopoietic Cell Transplantation for Wiskott-Aldrich Syndrome: A PIDTC Report. Blood Epub ahead of print
9. Elfeky, R.A., Furtado-Silva, J.M., Chiesa, R., Rao, K., Amrolia, P., Lucchini, G. et al. (2018) One hundred percent survival after transplantation of 34 patients with Wiskott-Aldrich syndrome over 20 years. J Allergy Clin Immunol 142, 1654-1656 e1657
10. Moratto, D., Gilliani, S., Bonfim, C., Mazzolari, E., Fischer, A., Ochs, H.D. et al. (2011) Long-term outcome and lineage-specific chimerism in 194 patients with Wiskott-Aldrich syndrome treated by hematopoietic cell transplantation in the period 1980-2009: an international collaborative study. Blood 118, 1675-1684
11. Hacein-Bey Abina, S., Gaspar, H.B., Blondeau, J., Caccavelli, L., Charrier, S., Buckland, K. et al. (2015) Outcomes following gene therapy in patients with severe Wiskott-Aldrich syndrome. JAMA 313, 1550-1563
12. Morris, E.C., Fox, T., Chakraverty, R., Tendeiro, R., Snell, K., Rivat, C. et al. (2017) Gene therapy for Wiskott-Aldrich syndrome in a severely affected adult. Blood 130, 1327-1335
13. Ferrua, F., Cicalese, M.P., Galimberti, S., Giannelli, S., Dionisio, F., Barzaghi, F. et al. (2019) Lentiviral haemopoietic stem/progenitor cell gene therapy for treatment of Wiskott-Aldrich syndrome: interim results of a non-randomised, open-label, phase 1/2 clinical study. Lancet Haematol 6, e239-e253
14. Sereni, L., Castiello, M.C., Di Silvestre, D., Della Valle, P., Brombin, C., Ferrua, F. et al. (2019) Lentiviral gene therapy corrects platelet phenotype and function in patients with Wiskott-Aldrich syndrome. *J Allergy Clin Immunol* **144**, 825-838
15. Dupre, L., Trifari, S., Follenzi, A., Marangoni, F., Lain de Lera, T., Bernad, A. et al. (2004) Lentiviral vector-mediated gene transfer in T cells from Wiskott-Aldrich syndrome patients leads to functional correction. *Mol Ther* **10**, 903-915
16. Fischer, A. (2019) Platelets are the Achilles' heel of Wiskott-Aldrich syndrome. *J Allergy Clin Immunol* **144**, 668-670
17. Candotti, F. (2019) Gene therapy for Wiskott-Aldrich syndrome: here to stay. *Lancet Haematol* **6**, e230-e231
18. Sabri, S., Foudi, A., Boukour, S., Franc, B., Charrier, S., Jandrot-Perrus, M. et al. (2006) Deficiency in the Wiskott-Aldrich protein induces premature proplatelet formation and platelet production in the bone marrow compartment. *Blood* **108**, 134-140
19. Toscano, M.G., Munoz, P., Sanchez-Gilabert, A., Cobo, M., Benabdellah, K., Anderson, P. et al. (2016) Absence of WASp Enhances Hematopoietic and Megakaryocytic Differentiation in a Human Embryonic Stem Cell Model. *Mol Ther* **24**, 342-353
20. Toscano, M.G., Anderson, P., Munoz, P., Lucena, G., Cobo, M., Benabdellah, K. et al. (2013) Use of zinc-finger nucleases to knock out the WAS gene in K562 cells: a human cellular model for Wiskott-Aldrich syndrome. *Dis Model Mech* **6**, 544-554
21. Martin, F., Toscano, M.G., Blundell, M., Frecha, C., Srivastava, G.K., Santamaria, M. et al. (2005) Lentiviral vectors transcriptionally targeted to hematopoietic cells by WASP gene proximal promoter sequences. *Gene Ther* **12**, 715-723
22. Dupre, L., Marangoni, F., Scaramuzzza, S., Trifari, S., Hernandez, R.J., Aiuti, A. et al. (2006) Efficacy of gene therapy for Wiskott-Aldrich syndrome using a WAS promoter/cDNA-containing lentiviral vector and nonlethal irradiation. *Hum Gene Ther* **17**, 303-313
23. Bosticardo, M., Draghici, E., Schena, F., Sauer, A.V., Fontana, E., Castiello, M.C. et al. (2011) Lentiviral-mediated gene therapy leads to improvement of B-cell functionality in a murine model of Wiskott-Aldrich syndrome. *J Allergy Clin Immunol* **127**, 1376-1384 e1375
24. Toscano, M.G., Frecha, C., Benabdellah, K., Cobo, M., Blundell, M., Thrasher, A.J. et al. (2008) Hematopoietic-specific lentiviral vectors circumvent cellular toxicity due to ectopic expression of Wiskott-Aldrich syndrome protein. *Hum Gene Ther* **19**, 179-197
25. Cesana, D., Ranzani, M., Volpin, M., Bartholomae, C., Duros, C., Artus, A. et al. (2014) Uncovering and dissecting the genotoxicity of self-inactivating lentiviral vectors in vivo. *Mol Ther* **22**, 774-785
26. Frecha, C., Toscano, M.G., Costa, C., Saez-Lara, M.J., Cosset, F.L., Verhoeyen, E. et al. (2008) Improved lentiviral vectors for Wiskott-Aldrich syndrome gene therapy mimic endogenous expression profiles throughout haematopoiesis. *Gene Ther* **15**, 930-941
27. Petrella, A., Doti, I., Agosti, V., Giarrusso, P.C., Vitale, D., Bond, H.M. et al. (1998) A 5‘ regulatory sequence containing two Ets motifs controls the expression of the Wiskott-Aldrich syndrome protein (WASP) gene in human hematopoietic cells. *Blood* **91**, 4554-4560.
28. Hagemann, T.L. & Kwan, S.P. (1999) The identification and characterization of two promoters and the complete genomic sequence for the Wiskott-Aldrich syndrome gene. *Biochem Biophys Res Commun* **256**, 104-109
29. Toscano, M.G., Benabdellah, K., Munoz, P., Frecha, C., Cobo, M. & Martin, F. (2009) Was cDNA sequences modulate transgene expression of was promoter-driven lentiviral vectors. *Hum Gene Ther* **20**, 1279-1290

30. Stewart, D.M., Treiber-Held, S., Kurman, C.C., Facchetti, F., Notarangelo, L.D. & Nelson, D.L. (1996) Studies of the expression of the Wiskott-Aldrich syndrome protein. *J Clin Invest* **97**, 2627-2634

31. Parolini, O., Berardelli, S., Riedl, E., Bello-Fernandez, C., Strobl, H., Majdic, O. et al. (1997) Expression of Wiskott-Aldrich syndrome protein (WASP) gene during hematopoietic differentiation. *Blood* **90**, 70-75.

32. Dhenge, A., Kuhikar, R., Kale, V. & Limaye, L. (2019) Regulation of differentiation of MEG01 to megakaryocytes and platelet-like particles by Valproic acid through Notch3 mediated actin polymerization. *Platelets* **30**, 780-795

33. Poulter, N.S., Pollitt, A.Y., Davies, A., Malinova, D., Nash, G.B., Hannon, M.J. et al. (2015) Platelet actin nodules are podosome-like structures dependent on Wiskott-Aldrich syndrome protein and ARP2/3 complex. *Nat Commun* **6**, 7254

34. Snapper, S.B., Rosen, F.S., Mizoguchi, E., Cohen, P., Khan, W., Liu, C.H. et al. (1998) Wiskott-Aldrich syndrome protein-deficient mice reveal a role for WASP in T but not B cell activation. *Immunity* **9**, 81-91

35. Gewirtz, A.M., Burger, D., Rado, T.A., Benz, E.J., Jr. & Hoffman, R. (1982) Constitutive expression of platelet glycoproteins by the human leukemia cell line K562. *Blood* **60**, 785-789

36. Tabilio, A., Pelicci, P.G., Vinci, G., Mannoni, P., Civin, C.I., Vainchenker, W. et al. (1983) Myeloid and megakaryocytic properties of K-562 cell lines. *Cancer Res* **43**, 4569-4574

37. Ogura, M., Morishima, Y., Ohno, R., Kato, Y., Hirabayashi, N., Nagura, H. et al. (1985) Establishment of a novel human megakaryoblastic leukemia cell line, MEG-01, with positive Philadelphia chromosome. *Blood* **66**, 1384-1392

38. Takeuchi, K., Ogura, M., Saito, H., Satoh, M. & Takeuchi, M. (1991) Production of platelet-like particles by a human megakaryoblastic leukemia cell line (MEG-01). *Exp Cell Res* **193**, 223-226

39. Hagemann, T.L., Mares, D. & Kwan, S. (2000) Gene regulation of Wiskott-Aldrich syndrome protein and the homolog of the Drosophila Su(var)3-9: WASP and SUV39H1. two adjacent genes at Xp11.23. *Biochim Biophys Acta* **1493**, 368-372

40. Garcia, P., Berlanga, O., Vlegopoulos, A., Vyas, P. & Frampton, J. (2011) c-Myb and GATA-1 alternate dominant roles during megakaryocyte differentiation. *J Thromb Haemost* **9**, 1572-1581

41. Postigo, A.A., Sheppard, A.M., Mucenski, M.L. & Dean, D.C. (1997) c-Myb and Ets proteins synergize to overcome transcriptional repression by ZEB. *EMBO J* **16**, 3924-3934

42. Reddy, M.A., Yang, B.S., Yue, X., Barnett, C.J., Ross, I.L., Sweet, M.J. et al. (1994) Opposing actions of c-ets/PU.1 and c-myb protooncogene products in regulating the macrophage-specific promoters of the human and mouse colony-stimulating factor-1 receptor (c-fms) genes. *J Exp Med* **180**, 2309-2319

43. Zufferey, R., Dull, T., Mandel, R.J., Bukovsky, A., Quiroz, D., Naldini, L. et al. (1998) Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery. *J Virol* **72**, 9873-9880.

44. Benabdellah, K., Munoz, P., Cobo, M., Gutierrez-Guerrero, A., Sanchez-Hernandez, S., Garcia-Perez, A. et al. (2016) Lent-On-Plus Lentiviral vectors for conditional expression in human stem cells. *Sci Rep* **6**, 37289
45. Charrier, S., Stockholm, D., Seye, K., Opolon, P., Taveau, M., Gross, D.A. et al. (2005) A lentiviral vector encoding the human Wiskott-Aldrich syndrome protein corrects immune and cytoskeletal defects in WASP knockout mice. Gene Ther 12, 597-606
FIGURES TITLES AND LEGENDS

Figure 1. WASP endogenous expression decreases during *in vitro* megakaryopoiesis and thrombopoiesis.

a) Representative bright-field images of hHSPCs before (top panel, 0-4 days) and after 4-8 days (middle panel, MKs are indicated with white arrows) or 11-16 days (bottom panel, MKs releasing proplatelets, white arrows) of MK differentiation with TPO, SCF w/o iROCK as indicated in M&M. b) Representative plots showing changes in morphology (FSC$_{\text{high}}$ SSC$_{\text{high}}$) and appearance of megakaryocytic markers (CD41a and CD42b) of HSPCs cells cultured with the MK differentiation media. c) Mature MKs (FSC$_{\text{high}}$ SSC$_{\text{high}}$ CD42$^+$ cells) respond to thrombin by increasing intracellular calcium uptake (N=3). d) Expression of PAC-1 activation marker in response to thrombin in obtained MKs (FSC$_{\text{high}}$ SSC$_{\text{high}}$ CD42$^+$ cells) and PLTs (FSC$_{\text{low}}$ SSC$_{\text{low}}$ CD42$^+$ cells) (N=3). e) Representative histograms showing WASP expression levels of PLTs (FSC$_{\text{low}}$ SSC$_{\text{low}}$ CD41$^+$CD42$^+$) obtained from peripheral blood (PB, top left) and HSPCs-derived (bottom left). WASP expression (related to isotype control) (right) is represented as mean ± SEM (non-parametric Mann-Whitney, two-tailed). f) Representative dot-plots of endogenous WASP expression kinetics upon MK differentiation *in vitro*. Grey populations in each plot display the isotype control (IsC) staining and coloured population the WASP staining (N=7) g) Analysis of WASP expression, depicted as MeFI of WASP+ population/ MeFI of isotype control, in the different populations during MK differentiation. CD34+ indicated HSPCs at day 0 (CD34$^+$CD41$^-$CD42$^-$), MK progenitors (CD34$^+$CD41$^+$ SSC$_{\text{low}}$ and SSC$_{\text{high}}$) at days 4-8, mature MKs (CD34-CD41+CD42$^+$) during 4 to 16 days of differentiation, and PLTs (CD41+CD42+FCS$_{\text{low}}$ SSC$_{\text{low}}$) obtained at day 16. h) Percentage of WASP positive cells in the above described populations. Represented data are...
mean ± SEM. *, p<0.05; **, p<0.01; ***, p<0.001 (Non-parametric Mann-Whitney, two-tailed, compared to CD34\(^+\) expression levels) (N=7).

**Figure 2.** Immortalized “MK-like” cell lines are relevant models to study WASP expression during MK differentiation.

a) Scheme of megakaryocytic differentiation protocol of K562 cells (top) and associated morphological changes after 96h of PMA incubation. b) Diagram of MK differentiation protocol of MEG-01 cells (top) and morphological changes after VPA addition. c) Representative FACs plots of CD41a and CD42b megakaryocytic markers of K562 cells in absence or presence of PMA. d) Percentage of WASP+ K562 cells and WASP expression w/o PMA treatment, analysed in total population (N=4). e) Phenotypic changes of MEG-01 cells after VPA treatment expressing CD41a and CD42b markers. f) Percentage of WASP+ MEG-01 cells and WASP expression at 0, 7, 14 and 21 days of VPA-megakaryocytic differentiation (N=3). Represented data are mean ± SEM, non-parametric Mann-Whitney test, two-tailed. **, p<0.01; ***, p<0.001.

**Figure 3.** eGFP-LVs expression driven by WAS-promoter mimic endogenous WAS protein expression kinetics during megakaryocytic differentiation.

a) Schematic representation of the 2nd generation LVs used in this study expressing enhanced GFP (eGFP). WE\(^{21}\) harbours the 500bp core of the WAS proximal promoter (AC115618.3 Seq 121356-121851), while AWE\(^{26}\) (AC115618.3: Seq 114605-114991 - 17bp - Seq 121356-121851) and cAWE (AC115618.3: Seq 114403-115000 - 17bp- Seq 121356-121851) contain an additional 386bp (Alt) and 597bp (complete Alt) fragments of the WAS alternative promoter respectively. b) Experimental protocols for the comparison of WASP endogenous levels with eGFP expression given by the different LVs during MK differentiation in the
different models. c) Fold expression of endogenous WASP (black bars) and eGFP (coloured bars) in K562 cells transduced with WE, AWE and cAWE LVs (MOI=1) are related to the expression levels of CD41\(^{-}\)CD42\(^{-}\) cells in PMA+ condition (non-parametric Mann-Whitney test, *, p<0.05) (N=4). d) WASP and eGFP fold expression at day 7 and 14 of MK differentiation of MEG-01 cells treated with VPA (non-parametric Mann-Whitney test, *, p<0.05, **, p<0.01) related to undifferentiated cells (N=4). e) WASP and eGFP fold expression in human CD34+, CD41\(^{+}\) SSC\(^{\text{low}}\), CD41\(^{+}\) SSC\(^{\text{high}}\) and CD41\(^{+}\)CD42\(^{+}\) cells derived from HSPCs (endogenous WASP, black bars; eGFP, coloured bars) at 4, 8, 11 and 16 days after in vitro MK differentiation (N=10). Non-parametric Mann-Whitney test, two-tailed (#, p<0.05, ##, p<0.01, ###, p<0.001) and ***, 2-way-Anova, Bonferroni Post- Test compared to WT. All the data are represented as mean ± SEM.

Figure 4. WAS-promoter driven LVs harbouring sequences from the alternative promoter rescue WASP expression kinetic in human cellular models of WAS. a) Schematic representation of the 2\(^{nd}\) generation LVs expressing WAS cDNA. WW\(^{21}\) harbours the 500bp core of the WAS proximal promoter, AWW\(^{26}\) and cAWW (this manuscript) LVs contain an additional 386bp and 600bp fragment of the WAS alternative promoter respectively (see Figure S2 for details). b) Experimental diagram of K562 WASKO transduced with WAS-LVs (MOI = 1) and differentiated to MKs-like cells with PMA stimulation. c) y d) Graphs showing the percentage of WASP+ cells (c) and WASP expression levels (d) in WT K562 cells (black bars) and in WASKO K562 cells transduced with WW (grey bars), AWW (blue bars) and cAWW (green bars) after MK differentiation with PMA in CD41-CD42- and CD41+ populations (N=7). e) Graph showing relative WASP expression in HD-CD34+ cells and WAS patient cells transduced with WW (grey), AWW (blue) and cAWW (green) (MOI=50) at 4, 8, 11, 16 days of MK in vitro differentiation.
f) Graph showing WASP expression levels of PLTs-derived from WAS patient’s HSPCs transduced with WW, AWW, cAWW related to WT-driven WASP expression.
g) Representative confocal images of actin nodule formation of platelets derived from healthy donors-CD34+ cells (left), WAS patient cells (second left) and WAS patient cells transduced with WW (middle), AWW (second-right) and cAWW (right). Several nodules are indicated with white arrows.
h) Quantification of actin nodule per PTL counted in images from g). Photographs analysed: HD=31, WAS=6, WW=17, AWW=26, cAWW=22). Non-parametric Mann-Whitney test, two-tailed.

Figure 5. WW- and AWW- transduced WASKO mHSPCs rescue phenotypic defects of WASKO mice.

a) Restoration of the hematopoietic profile in mice transplanted with WW and AWW-transduced WASKO mHSPCs (MOI=100; WW-mHSPCs=8.2vcn/c and AWW=9.8vcn/c). Side and forward scatter (left) of cells from peripheral blood of the transplanted mice 2 (left-up) and 6 (left-down) months after transplant. The non-transduced transplanted mice showed a higher number of granulocytes and a lower number of lymphocytes meanwhile the WT mHSPCs and WASKO mHSPCs AWW and WW-corrected mice showed a normal distribution of the population in peripheral blood. 6 months post-transplant (right graph), mice were sacrificed and peripheral blood analysed for vcn/v (see Figure S9), CD45.2 (donor cells) and lineage markers: CD3 (T cells), CD220 (B cells), Gr1+ (granulocytes) and CD11b (myeloid cells). Mice per group: WT=2, NT=2, AWW=3, WW0.5=3. Statistic unpaired t-test.

* p<0.05. Data represent mean ± SEM. (b, c, d) Phenotypic rescue of WASKO mice after transplantation with WW and AWW- transduced WASKO mHSPCs (MOI=100; WW-mHSPCs=8vcn/c and AWW=6vcn/c). Mice were sacrificed after 3 months and T cell
proliferation assay (CFSE) (b) and IL-2 production (c) were measured 5 days or 3 days, respectively, post-stimulation with anti-CD3 (1μg/ml) by ELISA. One tail Mann-Whitney test. Data represent mean ± SEM (WT:N=2, NTKO:N=2, AWW:N=5, WW:N=4). ** p<0.01 * p<0.05 d) Number of platelets in blood of transplanted mice compared with wild-type (WT) and non-transduced (WT=2, NT=1, AWW=4, WW0.5=3). Copies/cell in spleen: AWW: 1.9±1.1 cc, WW0.5= 1.8±0.4 cc. Data represent mean ± SEM.

Figure 6. Third generation WAS-driven LVs mimic endogenous WASP expression kinetics during MK differentiation and restore functional defects in vitro and in vivo.
a) 3rd generation LVs used for the study. WW1.6 referred the current clinical vector hWW1.6 that includes the complete WAS 1.6kb proximal promoter\textsuperscript{15} (AC115618.3 Seq 120189-121855) and AW that includes and alternative promoter previously described (AC115618.3: Seq 114605-114991 - 17bp - Seq 121356-121851).\textsuperscript{26} b) HSPCs from a WAS patient were transduced with the 3rd generation LVs at MOI=50 (WW1.6=2,61±1,13 vcn/c and AW=3,16±1,61 vcn/c) and WASP expression and functional restoring were analysed after in vitro MK-differentiation. c) Relative WASP expression of HD, WAS_WW1.6 and WAS_AW in the CD41\textsuperscript{+}CD42\textsuperscript{+} gated population at days 4, 8, 11 and 16 of MK-differentiation. Represented data are mean ± SEM, Mann-Whitney, two tails, ***p<0.001 (N=5). d) WASP relative expression levels in PLTs (CD41\textsuperscript{+}CD42\textsuperscript{+} FSC\textsuperscript{low} SSC\textsuperscript{low}) derived from HD, WAS_WW1.6 and WAS_AW HSPCs compared to those of HD-derived PLTs. * p<0.05 (N=4). e) PAC-1 expression of gated MKs (CD42\textsuperscript{+} FSC\textsuperscript{high} SSC\textsuperscript{high}) (left) and PLTs (CD42\textsuperscript{+} FSC\textsuperscript{low} SSC\textsuperscript{low}) (right) from HD, WAS, WAS_WW1.6 and WAS_AW-derived HSPCs. Represented data are mean ± SEM, Mann-Whitney, two tails, *p<0.05 (N=3). f) Confocal images of actin nodules in platelets derived from HSPCs. White arrows indicate examples of actin nodules. g) Quantification of actin nodules/platelet in the transduced cells Photograph
analysed; **p<0.01, ***p<0.001 HD=31, WAS=6; WW1.6=5, AW=9. Mann-Whitney, two-tails. h) Schematic diagram showing the procedure to study podosome restoration in monocytes derived from WAS patient’s HPSCs. i) Histograms showing CD14 expression after monocytic differentiation (DIF+) in the CD33+ population (left). WASP expression (right) was determined in the CD33+CD14+ population derived from healthy (black bars), WAS-patient cells (set as control, not shown) and WAS-patient cells transduced with WW1.6 (light-blue bars) and AW (dark-blue bars) LVs at the end of the differentiation (day 20). Non-parametric Mann-Whitney T test, non-significant (WT: N=3; WW1.6 and AW: N=4). j) Graph showing the percentage of cells with podosomes in a cluster structure (composed for at least 20 podosomes). Photographs from 3 different experiments were analyzed: HD (18 pictures, 338 cells), WAS patient (12 pictures, 195 cells), WW1.6 (12 pictures, 110), AW (14 pictures, 198 cells). Non-parametric Mann-Whitney T test two-tails, **, p<0.05. k) Representative images of podosomes in clusters from confocal microscopy. Cells obtained in vitro were incubated overnight onto fibronectin-coated chambers at 37°C and nuclei (DAPI, blue), actin (red) and vinculin (green) were stained. l) Schematic diagram of transplant procedure of WASKO mice with AW- and WW1.6 lin-WASKO transduced cells (lin-AW= 5vcn/c, lin- WW1.6= 8vcn/c). m) Graphs showing spleen size (longitudinal size measured in centimeters) of WT and WASKO mice (mice 10-12 weeks old, left graph) and WASKO mice transplanted with AW- and WW1.6- transduced lin-WASKO cells (18-20 weeks old, right graph). n) Graph showing PLTs counts in WT and WASKO mice as well as in WASKO mice transplanted with lin-WASKO cells transduced with AW- and WW1.6-LVs. Vector copies analysed in spleen: AW= 1.03 ± 0.31 vcn/c and WW1.6kb= 0.58 ± 0.30 vcn/c.
In this work Martin et al have uncovered the lop-sided WASP expression kinetic during human MK differentiation showing that WAS-promoter-driven LVs follow closely this expression pattern. Based on these analyses, the authors propose a new clinical trial with the AW LVs incorporating additional regulatory sequences from the alternative WAS promoter.
