Gene Correction of iPSCs from a Wiskott-Aldrich Syndrome Patient Normalizes the Lymphoid Developmental and Functional Defects

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SUMMARY

Wiskott-Aldrich syndrome (WAS) is an X-linked primary immunodeficiency disease caused by mutations in the gene encoding the WAS protein (WASp). Here, induced pluripotent stem cells (iPSCs) were derived from a WAS patient (WAS-iPSC) and the endogenous chromosomal WAS locus was targeted with a wtWAS-2A-eGFP transgene using zinc finger nucleases (ZFNs) to generate corrected WAS-iPSC (cWAS-iPSC). WASp and GFP were first expressed in the earliest CD34+CD43+CD45+ lineages examined. Whereas differentiation to non-lymphoid lineages was readily obtained from WAS-iPSCs, in vitro T lymphopoiesis represents a potential therapeutic approach for a variety of hematological disorders. Success in treating WAS via lentiviral-mediated gene delivery has recently been reported (Aiuti et al., 2013; Hacein-Bey Abina et al., 2015). Although no leukemogenic events were reported in up to 3 years following delivery of gene-modified CD34+ cells, it remains difficult to predict whether any of the unique integration sites (e.g., ∼10,000 per treated child in Aiuti et al. [2013]) will result in adverse consequences in the longer term as occurred in the original WAS retroviral gene-therapy trial (Braun et al., 2014). Thus, development of site-specific targeting strategies for treatment of WAS is warranted.

INTRODUCTION

Wiskott-Aldrich syndrome (WAS) is a severe X-linked primary immunodeficiency resulting from mutations in the WAS gene; WAS encodes a hematopoietic-specific and developmentally regulated cytoplasmic protein (WASp). WASp is a key regulator of the actin cytoskeleton, specifically regulating actin polymerization and formation of immunological synapses. Within the immune system, WASp deficiency results in well-documented functional defects in mature lymphocytes such as reduced antigen-specific proliferation of T cells and significantly reduced cytotoxic activity by natural killer (NK) cells when exposed to tumor cell lines (Orange et al., 2002).

Transplantation of hematopoietic stem cells (HSCs) represents a potential therapeutic approach for a variety of hematological disorders. Success in treating WAS via lentiviral-mediated gene delivery has recently been reported (Aiuti et al., 2013; Hacein-Bey Abina et al., 2015). Although no leukemogenic events were reported in up to 3 years following delivery of gene-modified CD34+ cells, it remains difficult to predict whether any of the unique integration sites (e.g., ∼10,000 per treated child in Aiuti et al. [2013]) will result in adverse consequences in the longer term as occurred in the original WAS retroviral gene-therapy trial (Braun et al., 2014). Thus, development of site-specific targeting strategies for treatment of WAS is warranted.

In this study, we wished to assess whether targeted gene editing of WASp-deficient induced pluripotent stem cells (iPSCs) would result in functional correction of the derived hematopoietic progeny. WAS can be caused by a diversity of mutations distributed across all 12 exons. To provide a gene correction solution potentially applicable to most, if not all, WAS patient cells, we used zinc finger nuclease (ZFN)-mediated, site-specific, homology-directed repair (HDR) to target the integration of a corrective WAS gene sequence into the endogenous WAS chromosomal locus. We hypothesized that utilizing the endogenous WAS promoter, the natural WAS chromatin environment, and transcription regulatory signals, would provide for a physiologically appropriate WAS transgene expression.
RESULTS

Derivation and Characterization of WAS-iPSCs
Skin fibroblasts were obtained from a WAS patient carrying the 1305 insG WAS mutation. This single-base-pair insertion in exon 10 of the WAS gene would be predicted to yield a WAS protein (WASp) frameshifted at amino acid 424, out-of-frame throughout the C-terminal VCA (verprolin homology, coﬁlin homology, acidic) domains critical for WASp-dependent actin polymerization and immunological synapse formation, and to conclude in a premature termination at position 493. Patients with the 1305 insG WAS mutation exhibit negligible WASp expression in hematopoietic cells, likely due to instability or degradation of the protein (Wada et al., 2003).

Following reprogramming, we veriﬁed the WAS 1305 insG mutation in WAS-iPSC clones, and conﬁrmed characteristic pluripotent stem cell antigen expression, a normal karyotype, and pluripotency (Figures S1A–S1D). Quantitative transcriptional proﬁling of WAS-iPSCs revealed a gene expression pattern highly similar to human embryonic stem cells (hESCs) (line WA09) (Figure S1E).

Endogenous Targeted Integration: WAS-iPSC Gene Correction
WAS-iPSCs were corrected via ZFN-mediated HDR as shown in Figure 1A. The targeting strategy was such that successful HDR-mediated targeted integration (TI) of the WAS exon 2–12 cDNA (WAS2–12) within intron 1 would result in normal transcriptional initiation at exon 1 (directed by the endogenous upstream transcriptional regulatory sequences); splicing from the splice donor at the end of exon 1 to the splice acceptor at the start of the WAS2–12 cDNA to yield the WAS-2A-GFP mRNA; the inclusion of GFP in the WAS2–12-2A-GFP cassette was to enable tracking of WASp-expressing cells. A loxP-ﬂanked pgk-puroTK-selectable cassette was inserted just downstream of the transgene sequences in order to permit puromycin-mediated selection of initial clones as well as subsequent ﬁxauridine (FIAU)-mediated selection of Cre-excised clones.

Successfully targeted puromycin-resistant clones were ﬁrst identiﬁed by PCR ampliﬁcation utilizing primers located outside the donor sequences and further conﬁrmed by DNA sequencing (data not shown). Southern blot analysis utilizing the pgk-puroTK sequences as probe, veriﬁed the intended TI within the WAS locus, and the absence of off-target integrations (Figure S2A). Transient expression of Cre-recombinase, followed by FIAU selection, was utilized to excise the pgk-puroTK-selection cassette. The corrected (cWAS) iPSCs, both prior to and following Cre-mediated excision of the selection cassette, retained a normal karyotype (Figure S2B) and pluripotency (Figure S2C). Comparative genomic hybridization (CGH) and whole-exome sequencing were performed on WAS and cWAS-iPSCs to determine whether the targeted correction methodology resulted in unanticipated changes to the chromosomal DNA. The results of these analyses are presented in Tables S1–S3. These data indicate that the cWAS-iPSCs were generated without any apparent deleterious mutation that could cloud the interpretation of further experiments.

Derivation and Characterization of Hematopoietic Progenitor Cells from WAS and cWAS-iPSCs
Since WASp is normally expressed in all hematopoietic cells including the CD34+ hematopoietic progenitor cells (HPCs), we examined HPCs derived from the patient WAS- or cWAS-iPSCs; WA01 or WA09 hESCs carrying wild-type (WT) WAS were used interchangeably as controls. Differentiation of iPSCs progressed from CD34+CD43– endothelial cells to CD34+CD43+ cells (CD43 is expressed early on hematopoietic cells) and finally to CD34+CD45+ HPCs, similar to cultures initiated by WAS- and cWAS-iPSCs, and highly similar to the patterns exhibited by control hESCs (Figure 1B). The absolute numbers of the speciﬁc cell populations (CD34+CD43–, CD34+CD43+, and CD34+CD43+CD45+) obtained per embryoid body (EB) demonstrates that the WAS 1305 insG mutation does not adversely affect the emergence/development of CD34+ HPCs from hemogenic endothelium when compared with cWAS (Figure 1C) (p = 0.502, not signiﬁcant [NS]). Further differentiation of WAS-derived CD34+CD43+CD45+/– HPC toward myeloid, erythroid, and megakaryocytic lineages revealed no major differentiation defects compared with cWAS or hESC (Figure 1D).

Restoration of WASp Expression in cWAS-iPSC-Derived Hematopoietic Cells
In order to assess restoration of WASp expression in the cWAS-iPSC-derived cells, we ﬁrst assayed GFP expression in the in vitro differentiation cultures. GFP expression, albeit at low levels, was clearly present in cWAS-iPSC-derived CD34+CD43+CD45– hematopoietic progenitors and in all hematopoietic lineages including myeloid, erythroid, and megakaryocytic lineages (Figure 2A). GFP expression was very weak to absent in cWAS-iPSC-derived CD34+KDR+CD43– endothelial cells (Figure 2A). There was no indication of differential GFP expression in CD34+KDR+CD43+ CD73– hemogenic versus CD34+KDR+CD43+CD73+ non-hemogenic endothelial cells (Figure S3A).

RT-PCR for WAS sequences was performed for the WAS- and cWAS-iPSC-derived CD34+CD43+ HPCs. The native WASp transcript including the 3′ UTR was present in
peripheral blood mononuclear cells (PBMCs) and in WAS- and hESC-derived blood cells, but not in cWAS-derived cells (Figure 2B). In contrast, cWAS-derived hematopoietic cells expressed the transgenic transcript that correlated with expression of the GFP transcript (Figure 2B). Sequencing confirmed that the WAS mRNA expressed by the cWAS CD34+CD43+ cells was of a WT sequence (i.e., not the mutant 1305 insG) (Figure 2C), and derived from the integrated WAS2-12 transgene (i.e., included a silent C > A substitution at position 995) (Figure 2C).

WASp expression in CD34+CD43+ cells was directly assessed by western blot and fluorescence-activated cell sorting (FACS) analysis. Western blot analysis confirmed the absence of WASp in WAS CD34+CD43+ progenitors but detectable WASp in cWAS CD34+CD43+ progenitors (Figure 2D). As expected, the cWAS-iPSC-derived progenitors,
in comparison with healthy PBMCs and hESC-derived progenitors, expressed a WASp of slightly higher molecular weight resulting from the additional 19 2A amino acids at the carboxyl end (Figure 2D). Anti-WASp immunostaining of CD34+CD43+ cells gave evidence for a low level of WASp expression in cWAS progenitors, consistent with the low GFP expression level (Figure S3B); this level of WASp expression was reproducible and just slightly less than that seen for hESC-derived progenitors. Together, these data demonstrate restoration of physiological expression levels of WASp in cWAS hematopoietic progenitors.
cWAS-iPSC-Derived Hematopoietic Progenitors Develop into NK Cells that Exhibit WASp-Dependent Function

WAS- and cWAS-ipSC-derived HPCs, as well as control hESC-derived progenitors, were further differentiated in culture conditions developed for NK cell differentiation. Whereas CD56⁺ NK cells were readily obtained from cWAS- and hESC-derived progenitors (evidenced by phenotypic CD56⁺CD7⁺ cells and CD56⁺CD94⁺ cells) (Figure 3A), WAS-derived progenitors yielded a significantly reduced number of NK cells (p = 0.007 when compared with cWAS) (Figures 3A and 3B). This deficiency in NK cell...
development for WAS progenitors was consistently observed using either OP9-DL1 (Figure 3A) or OP9-DL4 (data not shown) stromal cells to promote NK cell development. As expected for transgene-corrected cells, cWAS-derived NK cells expressed both GFP (Figure 3C) and the WAS transgene (Figure 3D). Restoration of WASp expression was confirmed both by FACS (Figure S4A) and western blot (Figure 3E).

We were able to expand the culture-generated NK cells including the few NK cells generated in the WAS-initiated cultures sufficiently to perform a functional analysis on these cells. WAS progenitor-derived NK cells exhibited significant functional deficits in comparison with both cWAS- and hESC-derived NK cells: upon stimulation with K562 cells, cWAS- and hESC-derived NK cells upregulated production of interferon-γ (IFNγ) and tumor necrosis factor alpha (TNFα); this response to stimulation was not exhibited by WAS-derived NK cells (Figure 3F). This indicates that the defect exhibited by WAS-iPSC-derived NK cells is functionally restored in transgene WASp-expressing NK cells. IFNγ and TNFα were increased in both WAS- and cWAS-derived NK cells on WASp-independent activation by phorbol myristate acetate/ionomycin (Figure S4B).

cWAS-iPSC-Derived Progenitors Are Fully Competent in Generation of T Cells

We previously reported the ability to derive functional T cells from hESCs (Timmermans et al., 2009). Using this technique, EB-derived CD34+ cells from cWAS were differentiated along the T/NK lineage in OP9-DL1 cultures (Figure 4A). Besides CD7+CD33+ myeloid and CD7+CD56+ NK cells, CD7+CD5+ early T lineage committed precursors arise early in these cultures (day 14); these early T cell precursors differentiate (via CD8 and predominantly via CD4 single-positive intermediates) to CD5+CD4+CD8+ double-positive cells (days 19 and 33) and finally to CD3+CD1+CD4+CD8+ double-positive cells (day 33) (Figure 4A). Only few mature single-positive cells are seen in these cultures. In Figure 4B, representative plots are shown from a total of 12–14 independent experiments in which WA01, WAS, and cWAS were cultured and tested head-to-head: at day 14 CD5+CD7+ T precursor cells were generated in all three cultures. However, later, only few CD4+CD8+ double-positive precursors (day 28) and CD3+ cells (day 33) are generated in the WAS culture, whereas these cells are present in the control hESC WA01 and the cWAS cultures. This deficit in generation of double-positives and CD3+ T cells was not specific to a single WAS-iPSC clone, but was exhibited by two other mutant WAS clones examined. A summary of the data is shown in Figure 4C. Again the data show no significant difference in generation of CD5+CD7+ T lineage committed precursors between WAS and cWAS (p = 0.834, NS), although the difference with WA01 was significant (p = 0.016). The deficit of WAS relative to cWAS and WA01 was clearly evident in derivation of both CD4+CD8+ (p = 0.008 and p = 0.013, respectively) and CD3+ T cells (p = 0.002 and p = 0.05, respectively). Expression of WASp protein throughout T cell differentiation is evidenced by GFP expression in the various T lineage populations derived from the cWAS-iPSCs (Figure 4D). Thus, TI of the WAS transgene in WAS-iPSCs restored development of both CD4+CD8+ and CD3+ T cells.

DISCUSSION

In this study, we demonstrate the successful, sequence-specific correction of WAS-iPSCs via TI of a WAS transgene into the endogenous WAS locus. We chose to target integration of the WAS2.12 half-gene into intron 1 of the WAS locus with a view toward potentially providing correction for all WAS mutations; for exon 1 mutations, targeted iPSC clones also incorporating the donor WT exon 1 would be utilized.

This report provides proof-of-concept data for the potential utility of WASp-deficient iPSCs corrected using site-directed gene editing. Correcting the WAS gene mutations in patient-specific iPSCs versus primary HSCs, has two distinct advantages. The first potential advantage is the ability to comprehensively sequence the corrected iPSC clones to rule out any untoward genetic changes. As a first step toward this end, we compared the WAS and cWAS-iPSCs via whole-exome sequencing and CGH to identify potential consequences of the ZFN-mediated gene editing. Cre-mediated excision, and/or extended iPSC culture. Although some differences were observed (e.g., amplifications or deletions uniquely present in either WAS or cWAS in Table S1, non-synonymous coding variants in Table S2), we did not observe any generation of mutations that we could directly attribute to the ZFN-mediated gene editing. The second potential advantage is that transplantation of corrected iPSC-derived HSCs will result in patients receiving a genetically homogeneous population of corrected cells. Derivation of transplantable HSCs from hESCs/human iPSCs (hiPSCs) remains very inefficient and challenging (Kaufman, 2009; Slukvin, 2013). Although recent studies suggest strategies to improve generation of transplantable HSC from human pluripotent stem cells (Gori et al., 2015), these studies need to be confirmed with a demonstration of long-term, multi-lineage HSC engraftment. Non-random X-inactivation of the WAS gene in female carriers and somatic reversion suggest the possibility of a selective advantage in vivo for corrected blood cells at the level of T cell precursors, common lymphoid progenitor cells, and perhaps even at a more
primitive stage (Davis et al., 2008; Wengler et al., 1995). Thus, it may be sufficient to deliver to patients in-vitro-generated T cell precursors/progenitors from corrected iPSC.

Interestingly, this study of WASp-deficient iPSCs, in comparison with their corrected cWAS-iPSC counterparts, revealed not only the expected functional defects in certain iPSC-derived hematopoietic progeny (e.g., NK cells), but...
also identified potential consequences for WASp deficiency in T and NK development. There is prior evidence from both WAS patients and WAS knockout mice, that WASp deficiency may adversely affect the development of lymphoid cells. Relative to healthy individuals, WAS patients already at a very early age demonstrate reduced numbers of peripheral blood T lymphocytes, including naive T cells (Park et al., 2004). These findings led Park et al. to propose that WASp was important in the initial development and maturation of lymphocytes. Studies of WAS knockout mice (Snapper et al., 1998; Zhang et al., 1999) have also identified a significantly reduced number of peripheral blood T lymphocytes. Importantly, Zhang et al. (1999) demonstrated that WASp deficiency adversely affected the development of immature thymocytes from the CD4<sup>+</sup>CD8<sup>-</sup> double-negative (DN) to the CD4<sup>+</sup>CD8<sup>+</sup> double-positive (DP) stage. However, a significant thymic developmental defect was not identified in the Snapper et al. WAS knockout mouse strain. These latter results were consistent with an in vivo WAS<sup>+</sup> competitive model indicating minimal advantage for WASp-expressing cells either in the thymus during transition from DN to DP T cells, or in the spleen for NK cells (Westerberg et al., 2008). Importantly, WAS knockout mice expressing a WASp ΔVCA transgene exhibited severe impairment in thymopoiesis, with a clear impairment in differentiation of CD4<sup>+</sup>CD8<sup>-</sup> thymocytes (Zhang et al., 2002).

We note that this study was limited to iPSCs derived from one WAS patient and one WAS genotype (1305 insG). The WAS 1305 insG mutation is predicted to produce a WASp lacking the C-terminal VCA domain. The aforementioned publication by Zhang et al. (2002) suggested the possibility that enforced expression of WASp ΔVCA functions in a dominant-negative manner to suppress successful thymic T cell development. Although we were not able to detect residual expression of the mutant frameshifted, truncated WASp in WAS-iPSC-derived HPCs, it remains a possibility that it exists at a very low level, and thus contributed to our observed defective in vitro T cell development. The majority of the experiments directly compared one corrected WAS-iPSC clone with the mutant WAS-iPSC clone from which it was derived; to convince ourselves that the inability to generate T cells was not a clone-specific defect but rather was due to non-functional WASp, we verified the T cell development deficiency with two other WAS-iPSC clones derived from the same donor fibroblasts (Figure 4C). Both the WAS<sub>2,12</sub> transgene and the GFP reporter distinguished the cWAS-iPSCs from the WAS-iPSCs. It is conceivable that GFP expression, albeit at a low level, could have affected the quantitative comparison between cWAS and WAS. However, we note that the statistically significant defects observed for NK and T cell development were quite specific, not seen for example in derivation of endothelial cells, hematopoietic progenitors (CD34<sup>+</sup>CD43<sup>+</sup> or CD34<sup>+</sup>CD43<sup>+</sup>CD45<sup>+</sup>), or CD5<sup>+</sup>CD7<sup>+</sup> T cell precursors.

Somatic revertant mosaicism is a frequent finding in WAS, identifiable in up to 10% of patients, with WASp-expressing cells arising in T, B, and/or NK cell lineages. The identification of revertant WASp-expressing T cells in WAS patients carrying either the 1305 insG or the closely related 1305 delG mutation (also encoding WASp ΔVCA) is a frequent finding (Lutskiy et al., 2008; Wada et al., 2003). The revertant genotypes in these patients re-establish expression of a WASp including the VCA domains. These data indicate that restoration of expression of a WASp including the VCA domain, whether by intentional gene correction (as done in the present study) or by reversion, confers a strong selective advantage to T cells. It is generally accepted that a strong selective advantage for revertant cells operates at the level of mature peripheral T cells in their transition, on exposure to antigen, from naive to memory T cells. Our data, together with results from some, but not all, of the WAS knockout mouse models summarized above, suggest that an additional selective advantage for corrected cells possibly exists at a much earlier stage, during T cell development in the thymus. This latter possibility, consistent with a polyclonal T cell receptor (TCR) repertoire exhibited by revertant WAS cells having the same revertant genotype (Wada et al., 2003), would have implications both for somatic reversion and for transplantation of corrected WAS-iPSC-derived T cell precursors.

### EXPERIMENTAL PROCEDURES

#### iPSC Generation and Characterization

The WAS fibroblast cell line GM01598 (NIGMS Human Genetic Cell Repository, Coriell Institute for Medical Research) was transduced with pMXs retroviruses expressing OCT4, SOX2, KLF4, NANOG, and c-MYC. iPSC colonies were subsequently identified based on morphology and live-cell staining for Tra-1-60 and Tra-1-81. Pluripotency was assayed by teratoma formation; animal experimentation was overseen by the University of Texas Health Science at Houston Animal Welfare Committee.

#### ZFN-Mediated Correction

ZFN plasmids (pVax15755 and pVax15724) targeted WAS intron 1 sequences 5'-CCT TTG GGC CCA tga ctG TCA TGA GGC AGg AAG GAC-3' (spacer sequences in lower case). iPSCs were nucleofected with ZFNs together with a donor construct which included wt WASex2-12 cDNA linked to a GFP reporter via 2A peptide sequences, the bovine growth hormone pA sequences, followed by a loxP-flanked puro-TK selection cassette.

#### Generation of Hematopoietic Progenitor Cells

hESC and hiPSC were dissociated using TrypLE Select and resuspended in APEL medium supplemented with BMP-4, VEGF, SCF,
and Y-27632. 5,000–7,000 cells were transferred into one well of a 96-well plate and centrifuged at 480 × g. Following 3–4 days of differentiation, individual EBs were transferred onto OP9 stromal cells and cultured for an additional 8 days in the presence of the cytokines above.

Generation of NK Cells
Sorted CD34+CD43+ progenitors derived from each cell line were seeded at 3 × 10^3 to 5 × 10^4 cells per well onto 24-well plates pre-coated with live OP9-DL1. Cells were maintained in NK cell differentiation medium including 15% heat-inactivated human AB serum, SCF, IL-7, IL-15, Flt3 ligand, and IL-3 (first week only). Cells were cultured in this system for 28–32 days.

T Cell Differentiation
The T cell protocol was based on the protocol described by Timmermans et al. (2009) with minor modifications. Briefly, day 12 spin EBs were dissociated and cells were resuspended in minimum essential medium alpha supplemented with 20% fetal bovine serum, SCF, Flt3 ligand, and rhIL-7. Cells were transferred onto sub-confluent OP9-DL1 feeder layers. Every 5–7 days, cells were transferred on a fresh OP9-DL1 monolayer, for up to 6 weeks. Flow cytometric analysis was performed on days 14, 21, 28, and 33, plus or minus 2 days.

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Supplemental Information

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Figure S1. Characterization of mutant WAS iPSCs. (related to Figure 1). A. DNA sequence of PCR amplicon spanning the WAS exon 10 region from normal iPSC gDNA and WAS iPSC gDNA. The WAS iPSC gDNA includes the 1305 ins G mutation. B. WAS iPSCs express antigens characteristic of human ES cells. Expression of Tra-1-81, non-specific alkaline phosphatase, OCT-4, and SSEA-4 by WAS iPSCs. Scale bars = 200 µm. C. WAS iPSCs exhibit normal male karyotype. D. Trichrome staining of teratomas formed by WAS iPSCs in immunodeficient mice; the four panels include examples of endoderm (Gland), ectoderm (Neuronal rosette, Pigmented cells), and mesoderm (Cartilage). Scale bars = 200 µm. E. Expression profile of WAS iPSCs. The transcriptional RT2 Profiler PCR array data, comparing mutant WAS iPSCs vs. WA09 hESCs, were analyzed by the ΔCt method. The black line represents fold changes ($2^{(-\Delta Ct)}$) of 1 and pink lines indicate a three-fold change in gene expression.
Figure S2. Characterization of corrected cWAS iPSCs. (related to Figure 1). A. Southern blot analysis of targeted endogenous integration. The schematic shows the expected genomic organization of a successfully targeted WAS locus including the WAS-2A-GFP transgene together with the pgk-puroTK selection cassette. The probe contains pgk-puroTK sequences. A unique 16.1 kbp hybridizing band is expected for ScaI digestion of a correctly modified clone, and a 3.8 kbp hybridizing band for PvuII digestion. The expected bands are present in a cWAS iPSC clone but absent in the original WAS iPSCs. B. cWAS iPSCs exhibit normal male karyotype. C. Teratoma formation by cWAS iPSCs. Hematoxylin and eosin staining of teratomas formed by cWAS iPSCs in immunodeficient mice; the four panels include examples of endoderm (Gland), ectoderm (Neuronal cells, Squamous epithelium), and mesoderm (Cartilage). Scale bars = 200 µm.
Figure S3. Expression of the WAS-2A-GFP transgene in cWAS iPSC-derived cells. (related to Figure 2).
A. Assessment of GFP transgene expression in cWAS iPSC-derived endothelial cells. Expression in CD34+CD43-KDR+CD73+ non-hemogenic and CD34+CD43-KDR+CD73- hemogenic cell populations are shown.
B. Assessment of WASp expression in iPSC and ESC (WA09)-derived CD34+CD43+ cells. R_{MFI} (the ratio of the Mean Fluorescence Intensity [MFI] for anti-WASp stained hESC- and cWAS-derived cells to the MFI for anti-WASp stained WAS-derived cells) is shown.
Figure S4. In vitro derivation and functional assessment of CD56+ NK cells from WAS and cWAS iPSCs. (related to Figure 3). A. Assessment of WASp expression in iPSC and ESC (WA09)-derived CD56+ cells. $R_{\text{MFI}}$ (the ratio of the Mean Fluorescence Intensity [MFI] for anti-WASp stained hESC- and cWAS-derived cells to the MFI for anti-WASp stained WAS-derived cells) is shown. B. Functional assessment of IFN-γ and TNF-α expression in NK cells. Shown is the intracellular expression IFN-γ (top panels) and TNF-α (bottom panels) by WAS and cWAS iPSC-derived CD56+ cells, either unstimulated or stimulated by exposure PMA/Ionomycin. WA01 ESC-derived CD56+ cells are shown as control. Indicated is the percentage of NK cells expressing either IFN-γ or TNF-α upon stimulation.
Table S1. Comparative Genomic Hybridization Analysis of mutant and corrected WAS iPSCs. (related to Figure 1)

| Class | Chr | Cytoband | Size(bp) | Start  | Stop   | Type | p-Value / LOH Score | WAS | cWAS |
|-------|-----|----------|----------|--------|--------|------|---------------------|-----|------|
| CGH   | 1   | q21.1    | 143611   | 145,655,992 | 145,799,602 | DEL  | 3.95E-16            |     |      |
| CGH   | 2   | p11.2    | 159607   | 89,141,608  | 89,301,214  | AMP  | 2.03E-39            |     |      |
| CGH   | 3   | q13.31   | 184015   | 116,691,055 | 116,875,069 | DEL  | 6.25E-24            |     |      |
| CGH   | 4   | q13.2    | 90702    | 69,392,576  | 69,483,277  | AMP  | 3.23E-15            |     |      |
| CGH   | 6   | p25.3    | 102973   | 259,318     | 362,290     | DEL  | 1.38E-34            |     |      |
| SNP   | 7   | p21.3 - p21.2 | 2480335 | 11,664,329 | 14,144,663 | LOH  | 6.642396            |     |      |
| CGH   | 8   | p11.22   | 122621   | 39,258,894  | 39,381,514  | AMP  | NA                  |     |      |
| CGH   | 8   | p12      | 412790   | 32,196,359  | 32,609,148  | DEL  | NA                  |     |      |
| CGH   | 8   | p22      | 58286    | 15,952,011  | 16,010,296  | AMP  | 5.42E-15            |     |      |
| CGH   | 8   | p23.1    | 583097   | 7,169,490   | 7,752,586   | DEL  | 2.26E-23            |     |      |
| CGH   | 8   | q11.2    | 112557   | 45,218,780  | 45,331,336  | AMP  | NA                  |     |      |
| CGH   | 11  | p15.5    | 485      | 2,016,675   | 2,017,159   | AMP  | 6.40E-31            |     |      |
| CGH   | 14  | q32.33   | 132778   | 106,670,876 | 107,243,421 | AMP  | NA                  |     |      |
| CGH   | 14  | q32.33   | 321250   | 106,636,701 | 106,957,950 | AMP  | 3.17E-24            |     |      |
| CGH   | 15  | q11.1 - q11.2 | 2077055 | 20,481,702 | 22,558,756 | AMP  | 1.73E-14            |     |      |
| CGH   | 16  | p11.2    | 1838301  | 31,934,834  | 33,773,134  | DEL  | 1.31E-12            |     |      |
| CGH   | 17  | q21.31   | 171922   | 23,056,562  | 23,228,483  | AMP  | NA                  |     |      |
| CGH   | X   | p11.23   | 61836    | 48,317,353  | 48,379,190  | AMP  | NA                  |     |      |
| CGH   | X   | p11.23   | 551      | 48,759,648  | 48,760,198  | AMP  | 6.35E-14            |     |      |
| CGH   | X   | p22.33   | 2630127  | 60,701      | 2,690,827   | AMP  | NA                  |     |      |
| CGH   | X   | q28      | 184649   | 152,987,955 | 153,172,603 | AMP  | NA                  |     |      |
| CGH   | X   | q28      | 206750   | 153,576,890 | 153,783,639 | AMP  | NA                  |     |      |
| SNP   | 11  | q12.3 - q13.3 | 6883527 | 62,032,068 | 68,915,594 | LOH  | 6.3685884            |     |      |
| CGH   | 22  | q13.31   | 206671   | 46,997,715  | 47,204,385  | AMP  | 1.67E-16            |     |      |

The positions of Amplifications (AMP), Deletions (DEL), or Loss of Heterozygosity (LOH) identified in WAS and/or cWAS iPSCs are indicated. Shading indicates presence of the AMP/DEL/LOH in the respective iPSC line.
Table S2. Non-Synonymous Coding Variants (NSCVs) Identified in ZFN-Corrected iPSCs. (related to Figure 1)

WAS ZFN Target Sequence: 
CCTTTGGGCCCAtgactGTTCATGGCAAGGAAGGAC

| Gene_ID | Chr | Position | Variant Type | Ref | Mut   | AA Change | Variant DNA sequence                  |
|---------|-----|----------|--------------|-----|-------|-----------|---------------------------------------|
| ROBO4   | 11  | 124766196| snp          | G   | T     | p.L193M   | cttctgtcttgccatcaTcagggacccccccggacacct |
| CNTNAP5 | 2   | 124783244| snp          | G   | C     | p.R6P     | ggaatggcattttaccacGctgaccagcgttttgacct |
| FLG     | 1   | 152276459| snp          | C   | T     | p.D3635N  | gcctgagtgctggagctgtTgctgactgtgggtggtgg |
| KBTBD6  | 13  | 41704927 | snp          | C   | T     | p.R574H   | tcttttccacgtgggtgTgagaggtgtagcaacaaat  |

NSCVs found in cWAS iPSCs consist of single base pair substitutions (snp) with the reference (Ref) changed to Mut. Mut changes are in bold and capitalized shown with surrounding sequence under Variant DNA Sequence. Shown above are the WAS ZFN recognition sequences with ZFN-L/ZFN-R sequences in capitals and the spacer sequences in lowercase.
| SELEX Score | Chrom. | Target Site                                                                 | Mismatch | Gene         |
|-------------|--------|------------------------------------------------------------------------------|----------|--------------|
| 15724 homodimer |        | ACCTaTGGGCCACACTTTTGGGCCCAgtGtG                                              | 4        | MAPK12       |
| 5.82E-11     | chr 22 | GCCTcTGGaaCccACCTGAGGGCCCAAAGGG                                               | 4        | LRBA         |
| 2.75E-11     | chr 4  | CaCaTTGGGCCaAAATACAGGGgaCAAAGGG                                               | 5        | LINC00471    |
| 2.74E-11     | chr 2  | AGgCaTtGCTGaCagATcACAGCTGCTGGGCCAAAAGGT                                      | 7        | RP11-433J8.1 |
| 15755 homodimer | NONE  |                                                                               |          |              |
| 15724-15755 heterodimer |     | AGgCaTtGCTGaCagATcACAGCTGCTGGGCCAAAAGGT                                      | 7        | ZNF496       |
| 1.93E-14     | chr 14 | AacCtTgTCTGctTcATgcCAGCTCTAGGtCCCAAAGGG                                      | 9        |              |
| 9.10E-15     | chr 1  |                                                                               |          |              |
Supplemental Experimental Procedures

iPSC generation and characterization

WAS fibroblasts obtained from Coriell Repository (Coriell # GM01598) were transduced twice with pMXs retroviruses expressing OCT4, SOX2, KLF4, NANOG, and c-MYC. Reprogrammed iPSC colonies, selected for in hESC media, were subsequently identified based on morphology and live cell staining for Tra-1-81 (Stegment 09-0082), and flow cytometric analysis of Oct4 transcription factor (Oct4-PE BD Biosciences, 560156) and SSEA-4 surface marker expression (SSEA4-APC R&D, FAB1435A). Pluripotency was assayed by teratoma formation and confirmed by quantitative transcriptional profiling (Qiagen RT2 profiler hESC PCR array).

Cell line cultures

hESC WA01 and WA09 and hiPSC WAS and cWAS cell lines were kept in maintenance cultures in an undifferentiated state on inactivated MEFs as described by Thomson et al (Thomson et al., 1998). Before performing differentiation experiments, hESC and hiPSC cell lines were adapted to enzymatic passaging using TrypLE Select (Invitrogen) in the cell expansion phase based on the protocol by Costa et al (Costa et al., 2007). Both maintenance and expansion cultures of hESC and hiPSC displayed stable colony morphology and expression of surface markers such as SSEA-3 and SSEA-4 (Figure S1). OP9 and OP9-DL1 cells (a gift from Juan Carlos Zuniga-Pflücker) were cultured in MEM-a with 20% FBS and supplemented with L-glutamine and the antibiotics streptomycin and penicillin (all from Invitrogen).

ZFN-mediated correction

ZFN plasmids (pVax15755 and pVax15724) were obtained through collaboration with Sangamo Biosciences (Richmond, CA). Zinc finger proteins were cloned in-frame as NH2-terminal fusions to the catalytic domain of FokI, and cloned into pVax (Invitrogen). Coding sequences for ZFNs are as follows:

C2A-3FN-15755-Fok

mrsggqgeqrltcdgveenpgpmdykdhdgykdhdidykdiddkmakppkkrrkvghyerpfpqcjcjmrfdsrnslnsrhirhtgkekpfacdicgrkf
arlndrtahtihhtggsqgpfqrcticrmnfnsfnsflshhirhtgkekpfacdicgrkfarsqhtktehtgsqkpfqrcticrmnfnsfnsflshhirhtgkekpfacdicgrk
rfkadnssrtthhtilrgsqvlkseleekkserlhlklyvpheyielieiarqtdtelemkvmeffmkvygyrgkhhlsrkgpdagiytvspidygvirdtkays
geynlpigademqoryveenqrtnkhpineuwwkvypessvtekfllfsghfksgynkaqltrlnhitncngavlsveelliggemikagtitlevrkkfingeins*

N2A-3FN-15724-Fok

mdykhdgdykdhdidykdiddkmakppkkrrkvghyerpfpqcjcjmrfdsrnslnsrhirhtgkekpfacdicgrkfqaannrthhtkhtgkekpfacdicgrk
nstfylrdrhtdrhtgkekpfacdicgrkfaslrlhthtkjhhqslqvlkseleekkserlhlklyvpheyielieiarqtdtelemkvmeffmkvygyrgkhhlsrkgp
dagiytvspidygvirdtkaysgeynlpigademqoryveenqrtnkhpineuwwkvypessvtekfllfsghfksgynkaqltrlnhitncngavlsveelligg
emikagtitlevrkkfingeinsfrs*

ZFN RNA was generated by first linearizing 1μg of each ZFN plasmid by XbaI digestion (New England Biolabs) as per manufacturer’s protocol. Subsequent to linearization, in vitro generated ZFN RNA was prepared utilizing the following kits and protocols: MessageMAX T7 ARCA-Capped Message Transcription Kit (Cell Script Inc.), A-PlusPolyA Polymerase Tailing Kit (Cell Script Inc.), and MegaClear Kit (Ambion).

iPSCs were dissociated into single-cell suspension and nucleofected with ZFNs together with a 1.6 kb donor construct which included wt WASex2-12 cDNA linked to a GFP reporter via 2A peptide sequences, followed by a loxp-flanked puro-TK selection cassette downstream. Although the 2A linker and GFP reporter were incorporated into our transgene construct for purposes of tracking WASp-expressing cells, these elements would not be utilized for clinical purposes. The donor sequences were flanked by approximately 1.5kb of WAS homology sequences. Puro-resistant colony exhibiting WAS correction were identified by PCR and confirmed via Southern blot analysis. ZFN activity was determined in pooled cells by using the CEL-1 /Surveyor nuclease assay (Transgenomics).

Generation and delivery of corrective donor template

A 1.6kb-long region of WAS sequences encompassing exons 1 and 2, and spanning the ZFN-cleavage site in intron 1 was amplified from wild-type K562 cells and subsequently cloned into the pSC-B–amp/kan vector (Agilent Technologies) to generate the WAS homology arms. Utilizing site-directed mutagenesis (QuickChange Lightening Site-Directed Mutagenesis kit, Agilent Technologies), an Nsil endonuclease site was introduced in intron 1, near the
ZFN cleavage site, leaving 751 bp of WAS-homology sequences to the right of the enzyme site, and 815 bp to the left. A vector encoding the WAS cDNA sequences was commercially obtained and by site-directed mutagenesis, we introduced a base-pair change (C→A) at position 995 in exon 10 (QuickChange Lightening Site-Directed Mutagenesis kit, Agilent Technologies). WAS exons 2-12 cDNA sequences were linked to an eGFP reporter followed by bovine growth hormone polyadenylation sequences and cloned into a homologous WAS donor plasmid. A loxP-flanked PGK-PuroTK selection cassette was subsequently cloned downstream of the reporter.

For targeted integration experiments, 2 μg of each ZFN RNA and 4 μg of donor template were co-delivered by Amaxa Nucleofection (Lonza) to 2x10^6 iPSC in single-cell suspension. Transfected cells were plated onto irradiated puromycin-resistant MEFs, and at day 4 post-transfection, Puromycin selection was initiated. Approximately 2-5 days later, puromycin-resistant colonies were picked and transferred to new 6-well plates for expansion.

### Generation of hematopoietic progenitor cells

Spin embryoid bodies were generated from hESC and hiPSC in the expansion phase. The protocol described by Ng et al was slightly adapted (Ng et al., 2005). In brief, hESC and hiPSC were dissociated using TrypLE Select (Gibco) and resuspended in APEL medium at a density of 50,000 cells per ml. Medium was supplemented with bone-morphogenetic protein (BMP-4; 2 ng/ml: R&D), vascular endothelial growth factor (VEGF: 20 ng/ml; Peprotech), stem cell factor (SCF: 40 ng/ml; Amgen) and 10μM Y-27632 (Selleck). 5000-7000 cells were transferred into one well of an ultra low-attachment 96-well plate (Costar) and centrifuged at 480g for 7 minutes. Following 3-4 days of differentiation, individual EBs were transferred onto OP9 stromal cells. Medium was refreshed by half-medium changes on day 7, absent of Y-27632. EBs were harvested for analysis and further experiments at different timepoints after complete dissociation either by incubating in TrypLE for 3 minutes at 37°C and subsequent mechanically disrupting EBs using a 21-gauge needle, or using collagenase IV for 25 minutes at 37°C followed by 0.1% trypsin (Invitrogen) for 15 minutes at 37°C followed by vigorous pipetting.

### Immunostaining and flow cytometric analysis for hematopoietic markers

For cell analysis and cell sorting, spin EBs were enzymatically dissociated as described above, while OP9-DL1 cultures were mechanically dissociated without use of enzymes. Cells were stained, following manufacturer’s protocol, for known hematopoietic surface markers: Allophycocyanin (APC)-conjugated CD34 (BD Biosciences, 340862); Brilliant Violet 421 (BV421)-conjugated CD43 (BD Horizon, 562916); Phycoerythrin (PE)-conjugated CD45 (BD Pharmingen, 555483); APC-conjugated: CD4 (Miltenyi, 130-091-232), CD33 (Miltenyi, 130-091-731), CD34 (Miltenyi, 130-098-139), TCRβ (Miltenyi, 130-091-237); PE-conjugated: CD7 (Beckman Coulter, IM1429U), CD8 (BD Biosciences, 345573), CD8β (Beckman Coulter, IM2217U), CD3 (BD Pharmingen, 555450), CD41α (BD Pharmingen, 555467), CD43 (BD Biosciences, 561999), CD235a (Beckman Coulter, A07792), TCRαβ (Miltenyi, 130-091-236); PECy7-conjugated: CD3 (25-0038-42), CD4 (25-0049-42), CD5 (25-0059-42), CD8αβ (25-5273-42) (all from eBioscience), TCRγδ (BD Biosciences, 655410); PerCP-Cy5.5 conjugated: CD34 (BD Biosciences, 347222), CD45 (eBioscience, 45-0459-42); APC-Vio770 conjugated CD45R (Miltenyi, 130-47-0038-42096-609); APC-eFluor-780 conjugated CD5 (eBioscience, 47-0038-42); eFluor-450 conjugated CD14 (eBioscience, 48-0149-42), V450 conjugated CD7 (BD Horizon, 642916), APC-Cy7 conjugated CD8 (BD Pharmingen, 348813). Respective isotype controls were always generated for each fluorophore used in the panel. Samples were incubated on ice for 30 minutes, and subsequently washed twice with 1mL FACS buffer per 1x10^6 cells. Flow cytometric analysis was performed using either a BDFACS Aria II or LSR II Cytometer (BD Biosciences) and cell sorting was performed with the FACSAria II cell sorter. Viable cells were gated based on forward and side scatter and on lack of propidium iodide (Invitrogen) uptake and were further analyzed for surface-marker expression and eGFP expression.

### Immunostaining and flow cytometric analysis for WASp

For WASp analysis, sorted CD34^-CD43^+ hematopoietic progenitor cells were cultured for 24-36 hours and subsequently harvested for analysis. Cells were then re-suspended in 100μL of fixation medium (Reagent A, Invitrogen Fix & Perm kit) and incubated at room temperature for 15 minutes. Following incubation, cells were washed with 2mL of PBS supplemented with 5%FBS, and subsequently centrifuged at 300g for 5 minutes. Again cell pellets were dislodged by vortexing, and resuspended in 100μL (final volume) of permeabilization medium (Reagent B, Invitrogen Fix & Perm kit) containing either 20μL Alexa 647-conjugated anti-WASp B-9 IgG2a, mouse monoclonal antibody (Santa Cruz Biotechnology, sc-13139AF647), or the respective isotype control. Following 20-minute incubation at room temperature, cells were washed with PBS/5%FBS and re-suspended in 300μL of FACS buffer. Cells were analyzed on BDFACS Aria II instrument (BD Biosciences). For WASp analysis of hESC/iPSC-derived NK cells, cells recovered from OP9-DL1 NK differentiation cultures were first stained for surface expression of CD56 with BV421-conjugated anti-CD56 (BD Biosciences, 562752), followed by intracellular staining for WASp...
with Alexa 647-conjugated anti-WASp B-9 IgG2a mouse monoclonal antibody (Santa Cruz Biotechnology).

**RT-PCR analysis**

RNA was extracted from approximately 2x10^5 sorted CD34^-CD43^- hematopoietic progenitor cells harvested at day 14 of differentiation, using RNease Mini Kit (Qiagen). cDNA was synthesized by reverse transcription using Improm-II Reverse Transcription System (Promega). Approximately 150ng of cDNA was added into a PCR reaction for specific amplification of WAS mRNA using primers binding to exon 9 and the exon junctions between exons 10 and 11, yielding a 445bp-long amplicon (WASex9_F 5’- GTGCGGCAGGAGATGAGGCG-3’, WASex10-11_R 5’-GGGCCCAAGGTTTGTTCCTGA-3’). RT-PCR reactions were performed using Platinum Taq High Fidelity Polymerase (Invitrogen) utilizing 1X Enhancer (Invitrogen) and, in both cases, cDNA was amplified for 30 cycles, and then subsequently resolved by agarose gel electrophoresis. Subsequent to PCR-amplification, material generated for analysis of WASp mRNA was purified by a spin-column system (NucleoSpin® Gel and PCR Clean-Up, Clontech) and submitted for Sanger sequencing analysis to LoneStar Sequencing (Houston, TX). We utilized the same forward and reverse PCR primers for bi-directional sequencing of purified material. We analyzed sequencing results using Lasergene SeqMan analysis software.

For Real-time RT-qPCR, cDNA from day 14 hematopoietic progenitors was Synthesized using SuperScript II Reverse Transcriptase (Invitrogen) and analyzed using LightCycler 480 SYBR Green I Master Kit (Roche) on a Lightcycler 480 II (Roche), according to the manufacturer’s protocol. The following primers were used: WAS exon11-3’UTR forward (fw) 5’-GATGCACGTGATGCAGAAGAGAA-3’ and WAS exon11-3’UTR reverse (rev) 5’-AGCAGGGCCAGCAAGTAACTCA-3’; WAS exon10-eGFP fw 5’-GAGCGCTTTTGGATCAAATC-3’ and rev 5’-TCCCCGATGTTAGAAGACT-3’; eGFP fw 5’-TGTTCTGCTGTAGTGTTGCG-3’ and rev 5’-TATATCATGCGCAGCAAGCA-3’. Results were analyzed using the delta Ct and the delta-delta Ct method. Negative control cells included undifferentiated hESC and hiPSC, and as a positive control for WAS expression, CD14^+ monocytes isolated from peripheral blood were used.

**Generation of NK cells**

Sorted CD34^-CD43^- progenitors derived from each cell line – WAS, cWAS, and WA09 were seeded at 3x10^4 to 5x10^4 cells per well onto 24-well plates pre-coated with 5x10^4 live OP9-DL1 murine stromal cells that express the Notch ligand Delta–like 1. Cells were maintained in NK cell differentiation medium containing: 56.6% DMEM-high glucose, 28.3% HAMS/F12 (Invitrogen), 15% heat-inactivated human AB serum (Valley Biomedical), 2 mM L-glutamine (Invitrogen), 1 uM β-mercaptoethanol (Sigma), 5 ng/mL sodium selenite (Sigma), 50 uM ethanolamine (MP Biomedicals), 20 mg/L ascorbic acid (Sigma), 1% P/S (Invitrogen), 20 ng/mL SCF, 20 ng/mL IL-7 (PeproTech), 10 ng/mL IL-15 (PeproTech), 10ng/mL Flt3 ligand (Flt3L) (PeproTech), and 5 ng/mL IL-3 (first week only) (PeproTech) [76]. Medium was prepared fresh on the day of experiment. Cells were cultured in this system for 28-32 days, with half-medium changes occurring every 3-4 days. Umbilical cord blood (UCB)-derived CD34^- cells were plated under the same conditions and used as NK differentiation control.

**NK stimulation and FACS analysis**

Sorted NK cells were stimulated with K562 cells at a 1:1 effector-target ratio stimulation for 1 hour. Subsequently, brefeldine was added and cells were incubated for 1 hour. Cells were harvested, fixed, and permeabilized (BD Cytofix/Cytoperm with addition of 2mM EDTA), and subsequently stained for FACS analysis or known NK cell surface markers (CD56-APC (BD Biosciences, 341026), CD45-PerCP-Cy5.5 (eBioscience, 45-0459-42), and for intracellular cytotoxic cytokines (IFNγ-PE (BD Biosciences, 404524) and TNFα-PECy7 (BD Pharmingen, 557647).

**Western Blotting**

Protein was harvested from approximately 1.5x10^6 to 3x10^6 sorted CD34^- progenitor cells or CD56^- NK cells from each line in RIPA buffer containing 1 protease inhibitor cocktail tablet (Complete Protease Inhibitor Cocktail tablet, Roche Applied Science). 10ug of total protein were run on a 7% Tris-Acetate gel (Life Technologies). Transferred protein was incubated overnight in mouse anti-human WASp antibody solution (1:1000 dilution, BD Biosciences), and subsequently with anti-mouse IgG HRP-conjugated secondary antibody (1:5000 dilution, Cell Signaling Technology).
**T-cell differentiation**

The T-cell protocol was based on the protocol described by Timmermans et al (Timmermans et al., 2009) with minor modifications. Briefly, day 12 spin EBs were dissociated as described above and cells were resuspended in MEM-α medium supplemented with 20% FBS, SCF (10 ng/ml), fms-like tyrosine kinases 3 receptor (Flt3-L: 5 ng/ml; Peprotech) and rhIL-7 (5 ng/ml; R&D Systems). Cells were transferred onto subconfluent OP9-DL1 feeder layers. Every 3-4 days, half of the medium was changed, and every 5-7 days, cells were transferred on a fresh OP9-DL1 monolayer, this for up to 6 weeks.

**Southern blot analysis**

A radio-labeled DNA probe was generated by digesting the donor plasmid with PvuII and Scal and resolved on a 0.8% agarose gel. The 2.3kb long probe with [alpha-32P]dCTP (Prime-It II Random Primer Labeling kit (Agilent Technologies)) encompassing the pgk-Puro-TK selection cassette was used to detect integration sites in puromycin-resistant clones. Subsequent to transfer onto membrane, material was exposed and an image was generated using a phosphorimager system (Molecular Dynamics).

**Comparative Genomic Hybridization Array**

We isolated DNA from WAS iPSC and cWAS iPSC, and 8ug of each purified material was sent to WiCell Research Institute for comparative genomic hybridization and Single-nucleotide polymorphism (SNP) analyses. Samples were analyzed using the Agilent SurePrint G3 Human Genome CGH+SNP Microarray. This array analysis is capable of detecting copy number gains and losses, copy neutral aberrations, and mosaicism. Data analysis was also conducted by WiCell Research Institute.

The great majority of CGH-identified copy number variations (CNV) (amplifications or loss of heterozygosity) were common to both WAS and cWAS iPSCs; only 4 CNV were present in cWAS but not WAS, and 3 CNVs were unique to WAS (Table S1). Although we have not determined the origination of CNVs unique to either cWAS or WAS, similar gain or loss of sequences has been observed upon culturing of other hESC/iPSC lines.

**Whole exome analysis**

Exom Capture and Sequencing.

Human iPSCs were first depleted of MEFs by passage on matrigel in MEF-conditioned media. Genomic DNA was extracted with the QIAPrep Spin Miniprep Kit. Two ug of genomic DNA was submitted to Axeq Technologies for human exome capture sequencing using TrueSeq 62 Mb target enrichment (http://www.illumina.com/documents/products/datasheets/datasheet_truseq_exome_enrichment_kit.pdf). Axeq Technologies performed sample validation, library preparation, exon enrichment, clustering and sequencing using illumina HiSeq 2000 Sequencer. We received, from Axeq, two fastq files (one file per orientation) per sample with approximately 38,000,000 reads of an average size of 101 bp. Next-generation sequencing data can be downloaded from http://www.ncbi.nlm.nih.gov/Traces/sra/ using the study accession number SRP074068 for Illumina exome sequencing data (in .bam file format).

Read Mapping.

Each pair of fastq files were aligned to human genome (hg19) using Novoalign (http://www.novocraft.com). All parameters were kept at the default settings, as recommended by Novocraft. SAMtools (http://samtools.sourceforge.net/) (Li et al., 2009) was used to sort the SAM files, create BAM files and generate their index files. Picard (http://picard.sourceforge.net/) was used to remove all the PCR duplicates from the BAM files. The Genome Analysis Toolkit (GATK)(McKenna et al., 2010) was used for local realignments, base quality recalibration, and variant calling. Parameters were set as described in GATK’s Best Practices v3. GATK’s unified genotyper generated standard variant call format files (VCF http://www.1000genomes.org/wiki/Analysis/Variant%20Call%20Format/vcf-variant-call-format-version-41). The VCF files were annotated using snpEff (snpeff.sourceforge.net)(Cingolani et al., 2012). De novo variant were identified using two tools: a)DeNovoGear under the paired sample mode (Ramu et al., 2013) and b) VarScan2(Koboldt et al., 2012). Putative de novo variants identified by both packages were ranked at the top of our list. All the putative de novo variants were visually inspected using the Integrative Genomics Viewer (IGV)(Thorvaldsdottir et al., 2013). Annotations for the de novo variants were retrieved from our original vcf file that was previously annotated by snpEff and indexed with tabix(Li, 2011). De novo Non-synonymous coding variants (NSCV) were found by using the tags generated by snpEff (SNPEFF_FUNCTIONAL and SNPEFF_EFFECT).
Whole exome sequencing identified a total of 4 non-synonymous coding variants (NSCVs) in cWAS iPSCs not present in WAS iPSCs; these changes consisted entirely of single bp substitutions (Table S2). The DNA sequences flanking these variants were examined to determine whether the DNA variations in the ZFN-corrected iPSCs possibly resulted from non-homologous end joining (NHEJ) at sites of off-target cutting by the ZFNs. Importantly, the NSCV found in the cWAS iPSC line did not share significant homology to any permutation of the ZFN target site. Furthermore, all DNA variations identified were base pair substitutions rather than indel mutations, the latter being the hallmark of nuclease-driven error-prone repair. Base pair substitutions were similarly identified in Yusa et al. (Yusa et al., 2011) and attributed to culturing of cells.

SELEX analyses were performed essentially as described (Miller et al., 2011) to identify consensus binding preferences for both right and left zinc-finger proteins (15724 and 15755) comprising the WAS-ZFNs. The resulting position weight matrices were then used to identify and rank the 100 potential off-target sites in the human genome, taking into account either homodimer or heterodimer binding (Perez et al., 2008). Whole exome sequences for corrected WAS iPSC were interrogated at these potential off-target sites for any evidence of mutation. No evidence of NHEJ-induced mutation was identified at any of the 5 predicted sites falling within exons (Table S3).

**Teratoma formation assay**

For analysis of pluripotency, approximately 3 million cells from each uncorrected (WAS) iPSC as well as corrected (cWAS) iPSC cultured in 30% matrigel were injected into either the kidney capsule or the testis of six-week old Fox Chase SCID beige mice (Charles River, 3 mice per cell line). Animals were monitored weekly for tumor growth. At six to eight weeks post injection, tumors were removed and subsequently paraffin-embedded. Histological examination and analysis of tumor samples was then performed (Applied Stem Cell Inc.)
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