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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 $CD34^+CD43^+CD45^-$ hematopoietic precursor cells and later in all hematopoietic lineages examined. Whereas differentiation to non-lymphoid lineages was readily obtained from WAS-iPSCs, in vitro T lymphopoiesis from WAS-iPSC was deficient with few $CD4^+CD8^+$ double-positive and mature $CD3^+$ T cells obtained. T cell differentiation was restored for cWAS-iPSCs. Similarly, defects in natural killer cell differentiation and function were restored on targeted correction of the *WAS* locus. These results demonstrate that the defects exhibited by WAS-iPSC-derived lymphoid cells were fully corrected and suggests the potential therapeutic use of gene-corrected WAS-iPSCs.

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 (*WAS*$_{2–12}$) 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 *WAS*$_{2–12}$ cDNA to yield the *WAS-2A-GFP* mRNA; the inclusion of GFP in the *WAS*$_{2–12}$*-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 fialuridine (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, confirmed 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 WAS (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$^-$CD45$^-$ 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

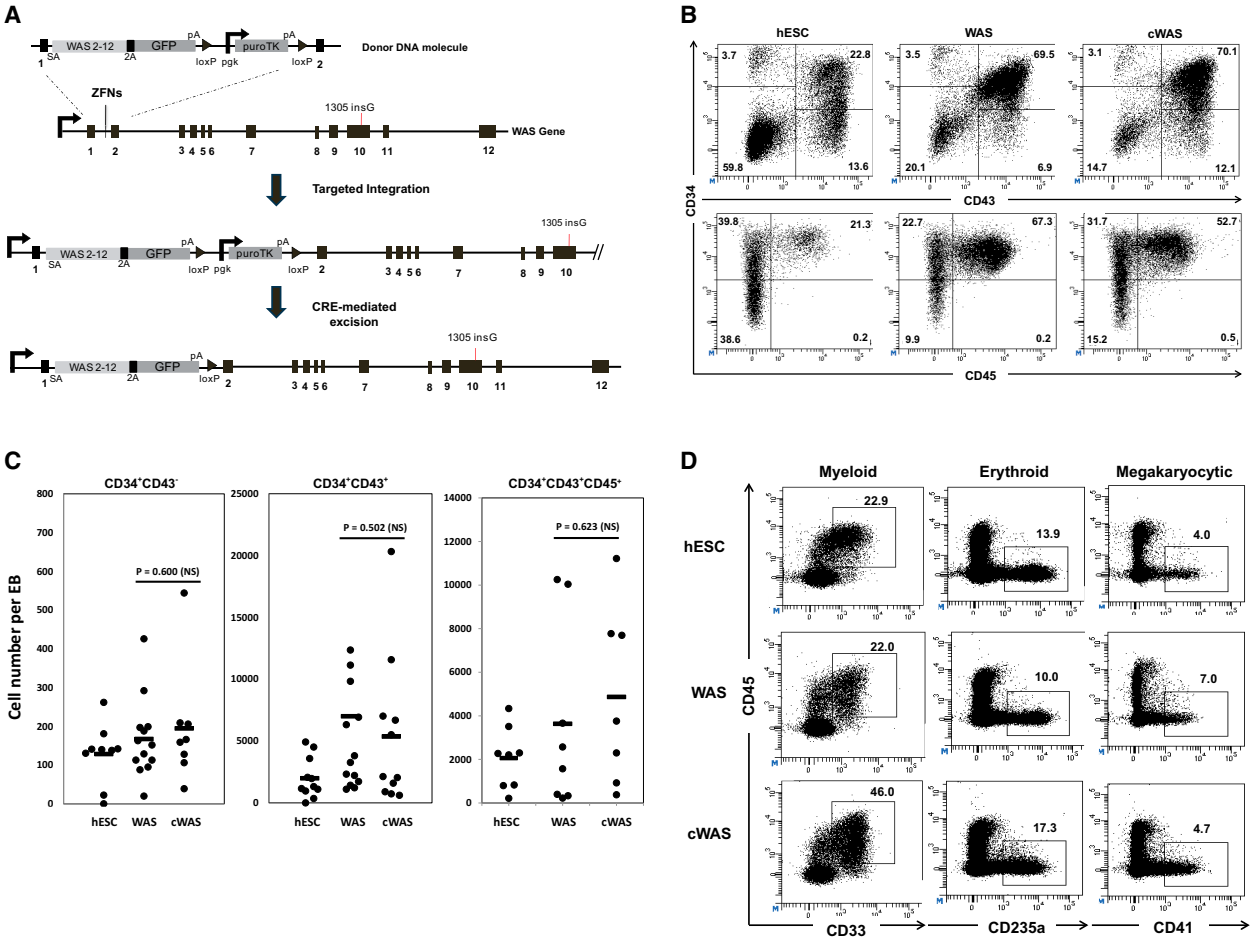

Figure 1. Targeted, ZFN-Mediated Correction of WAS-iPSCs and Derivation of Hematopoietic Progeny

(A) Targeted integration of the *WAS-2A-GFP* transgene into the endogenous mutant *WAS* locus.

(B) Flow cytometric analysis of in vitro hematopoietic differentiation assays showing efficient generation of hematopoietic progenitors ($CD34^+CD43^+$ and $CD34^+CD45^+$); data shown are of day 12 cultures from a representative experiment initiated with spin EBs from WA01 hESCs, WAS-iPSCs, and cWAS-iPSCs. Expression of CD34/CD45 is shown in the lower panels after gating on the $CD43^+$ cells. Numbers shown are the percentage of analyzed cells in each region. Regions were set based on control staining with isotype control antibodies.

(C) Quantitative analysis of the number of $CD34^+CD43^-$ endothelial cells, $CD34^+CD43^+$ hematopoietic progenitor cells (HPCs), and $CD34^+CD45^+$ HPCs generated per EB. Each data point represents a separate experiment. N (number of differentiated experimental samples; biological replicates) for $CD34^+CD43^-$ equals 9, 13, and 8 for hESC, WAS, and WAS, respectively. Similarly, for $CD34^+CD43^+$ N equals 11, 13, and 11, respectively, and for $CD34^+CD43^+CD45^+$ N equals 8, 8, and 7, respectively. Statistical significance was assessed via the Mann-Whitney U test.

(D) Flow cytometric analysis of in vitro derivation of myeloid ($CD45^+CD33^+$), erythroid ($CD45^-CD235a^+$), and megakaryocytic/platelet ($CD45^-CD41^+$) progeny. Data are of day 12 cultures from a representative experiment.

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 $WAS_{2–12}$ 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,

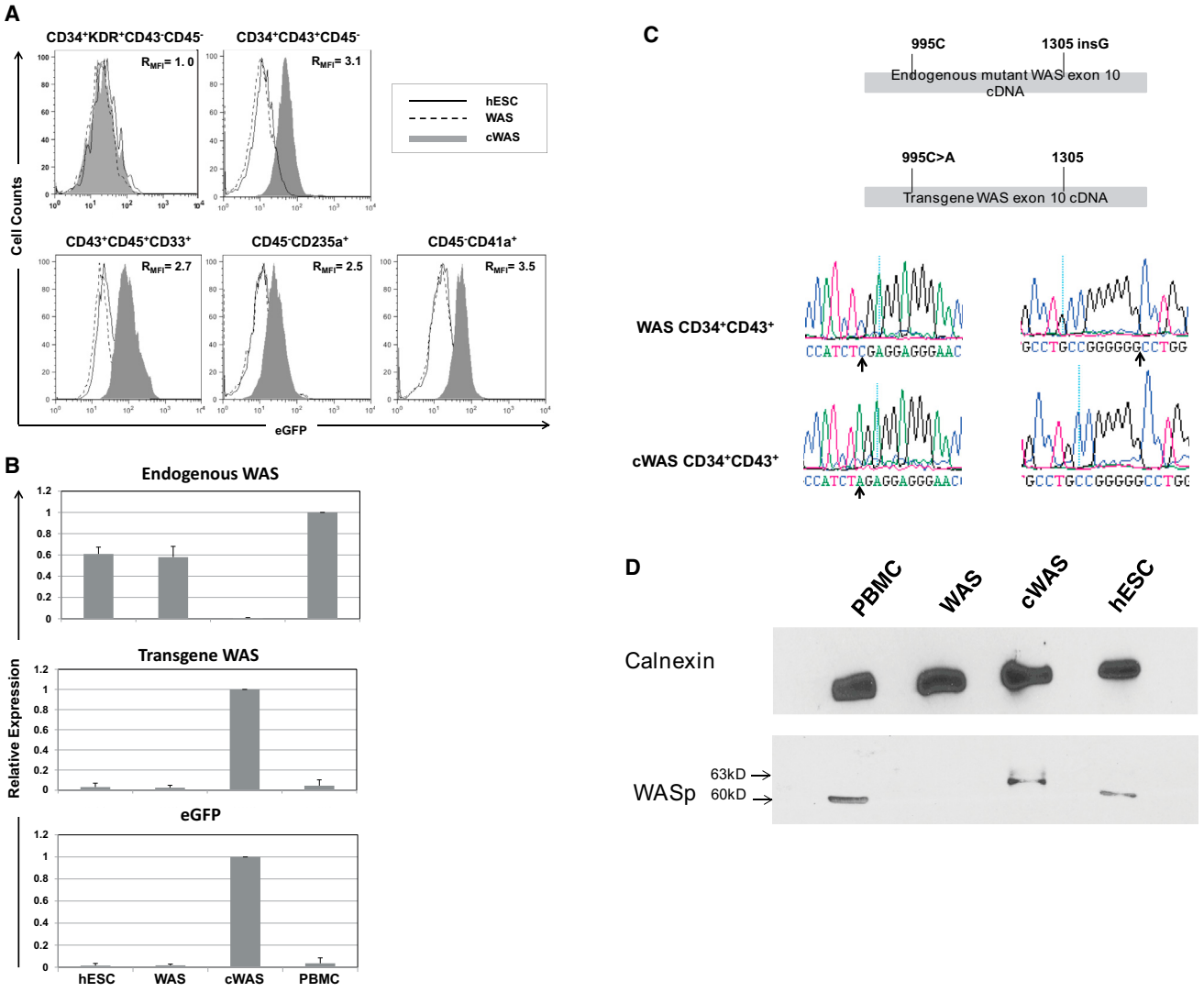

Figure 2. Expression of the *WAS-2A-GFP* Transgene in cWAS-iPSC-Derived Hematopoietic Cells

(A) Assessment of GFP expression in various cWAS-iPSC-derived cell populations. WA01- and WAS-iPSC-derived cells are shown as controls. R_{MFI} (the ratio of the mean fluorescence intensity [MFI] for cWAS-iPSC-derived cells to the MFI for WAS-iPSC-derived cells) is shown for each cell type.

(B) Quantitative RT-PCR analysis, expressed as ΔΔ Ct of endogenous *WAS* and transgene (*WAS*, *GFP*) expression in $CD34^+CD43^+$ HPCs. Shown are the mean and SD of the mean for a total of two experimental cultures, each analyzed in duplicate by quantitative RT-PCR. Endogenous *WAS* expression was normalized to PBMCs. Transgene *WAS* and *EGFP* expression was normalized to cWAS HPCs.

(C) Sequence analysis of *WAS* cDNA in $CD34^+CD43^+$ HPCs. RT-PCR primers were designed to amplify *WAS* cDNA, irrespective of whether it was endogenous or transgenic.

(D) Western blot analysis of WASp expression in $CD34^+CD43^+$ HPCs; human PBMCs served as positive control. Immunostaining for calnexin demonstrated relatively equal loading of protein.

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.

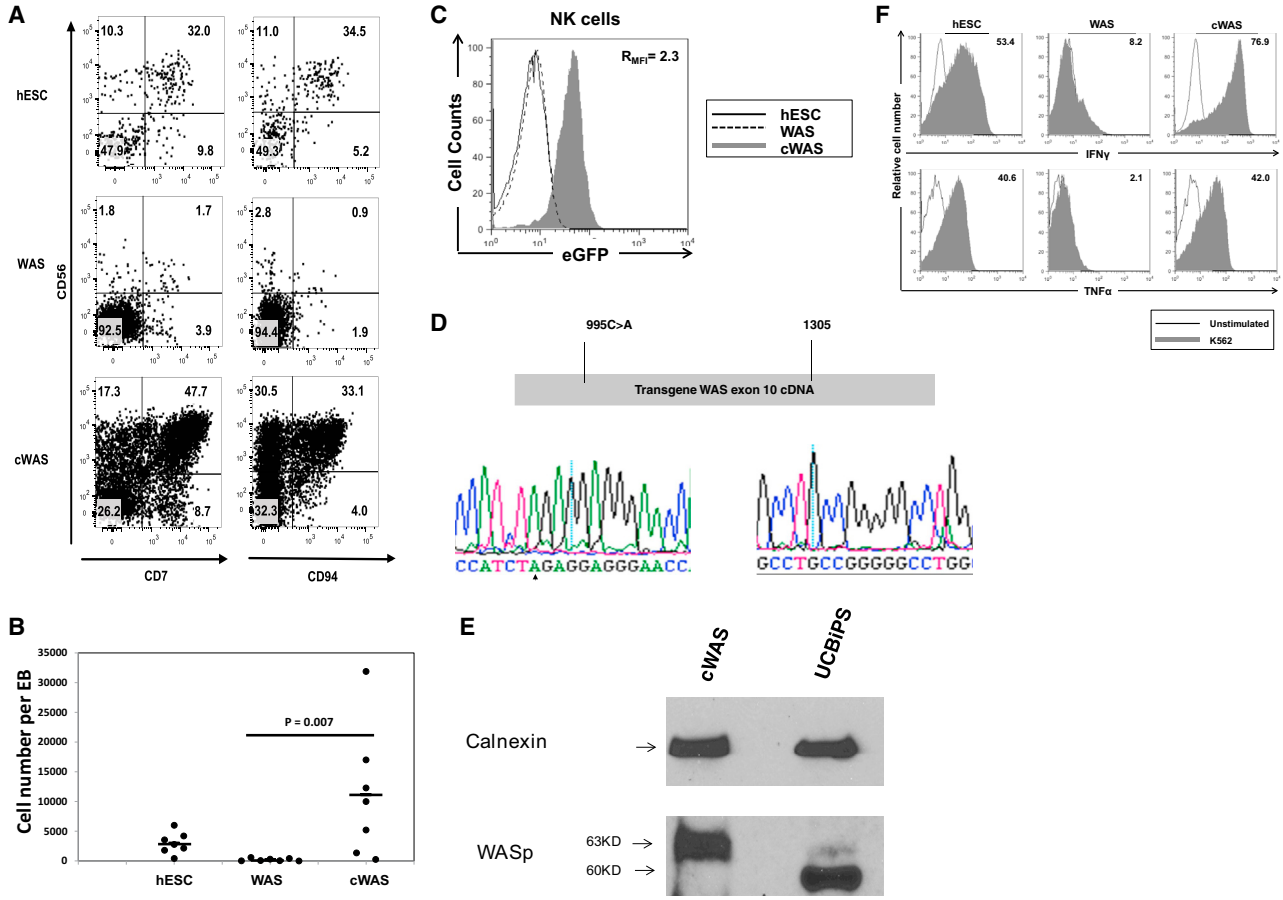

Figure 3. In Vitro Derivation and Functional Assessment of CD56$^+$ NK Cells from WAS and cWAS-iPSCs

(A) Flow cytometric analysis of CD56$^+$ NK cells obtained from EB-derived CD34$^+$CD43$^+$ HPCs plated onto OP9-DL1 feeder layers. These data are of day 13 cultures from a representative experiment. The HPC-derived CD56$^+$ NK cells also express early lymphoid marker CD7 and NK marker CD94.

(B) Quantitative analysis of the number of CD56$^+$ NK cells generated per EB in experiments performed with either OP9-DL1 or OP9-DL4 feeders (N = 7; five independent differentiation experiments for hESC, WAS, and cWAS in which three experiments were sampled at a single time point and two experiments were sampled at two time points).

(C) Assessment of GFP expression in cWAS-iPSC-derived NK cells. WA09 hESC- and WAS-derived cells are shown as controls. R_{MFI} is defined as in Figure 2A.

(D) Sequence analysis of *WAS* cDNA in cWAS CD56$^+$ NK cells. The sequence confirms expression of the transgene (995C>A [marked with arrowhead] and absent 1305 insG).

(E) Western blot analysis of WASp expression in cWAS-derived CD56$^+$ NK cells; NK cells generated from healthy donor umbilical cord blood-derived iPSCs served as control. Calnexin served as control for protein loading.

(F) Functional assessment of IFN-γ and TNF-α expression in NK cells after activation. 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 to K562 cells. 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.

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 $WAS_{2–12}$ 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

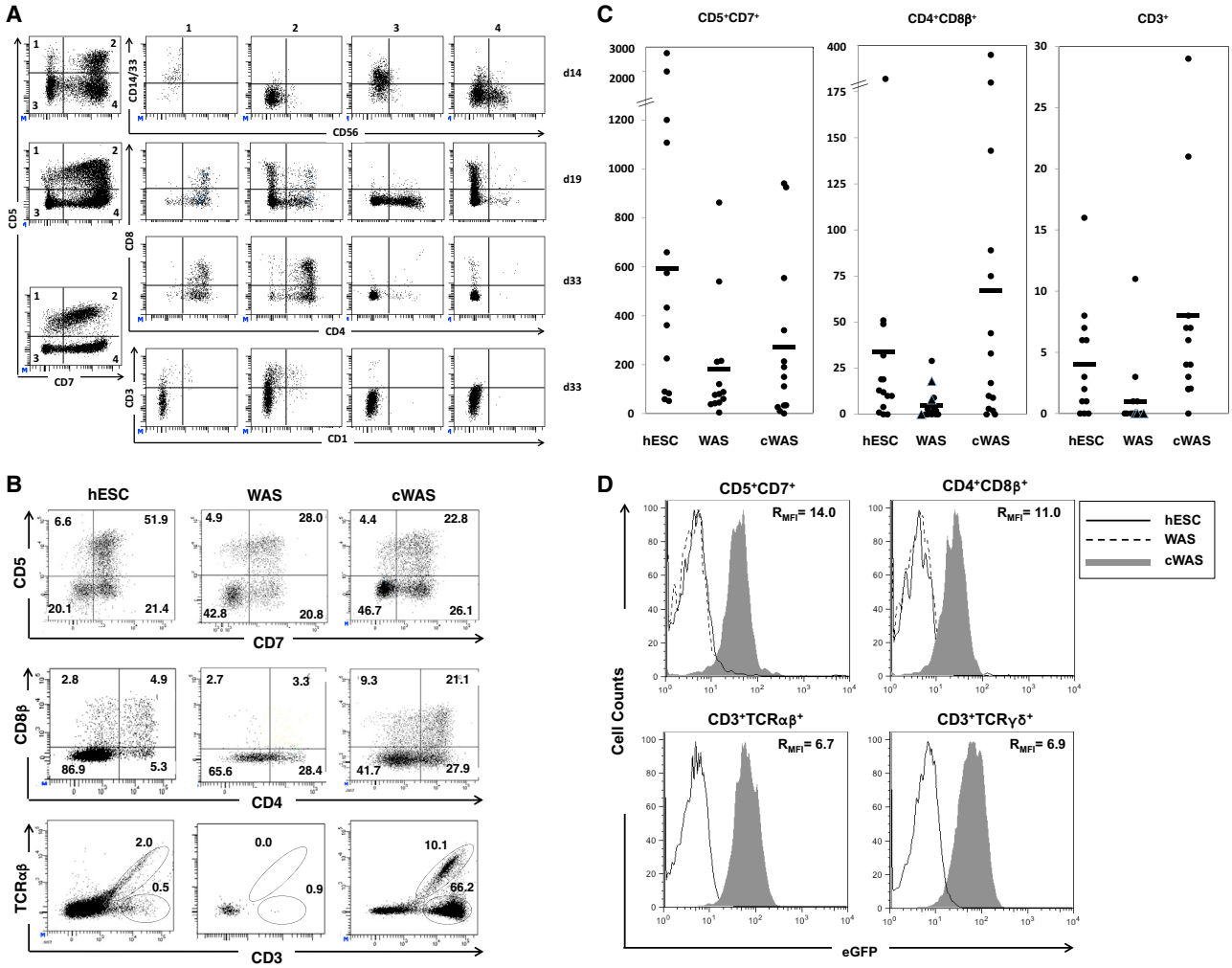

Figure 4. In Vitro Derivation of Precursor and Mature T Cells from WAS and cWAS-iPSCs

(A) Flow cytometric analysis of a cWAS OP9-DL1 culture. On the left, CD7/CD5 plots are shown at days 14, 19, and 33 of culture. On the right, the phenotype of the cells in each of the four quadrants (1–4) is shown. Note the presence of $CD7^+CD56^+$ NK cells in early cultures (quadrant 4). Note that only $CD5^+$ cells generate $CD4^+CD8\beta^+$ and $CD3^+$ cells (quadrant 1 and 2).

(B) Representative experiment of OP9-DL1 cultures: dot plots of WA01 (hESC), WAS, and cWAS cultures. EB-derived $CD34^+$ cells were plated into OP9-DL1 feeder layers and sampled for emergence of $CD7^+CD5^+$ (day 14, top panels), $CD4^+CD8\beta^+$ (day 28, middle panels), and $CD3^+TCR\alpha\beta^+$ and $CD3^+TCR\gamma\delta^+$ (day 33, bottom panels) T cells. For CD7/CD5 and CD4/CD8β dot plots, the quadrants were determined based on staining with isotype-matched antibody controls. The percentage of cells in each quadrant is shown. For the bottom panels, the circled populations represent $CD3^+$ cells that are also either $TCR\alpha\beta^+$ (e.g., 2% for hESC) or $TCR\alpha\beta^-$ (e.g., 0.5% for hESC). Cells with phenotype $CD3^+TCR\alpha\beta^-$ are assumed to be $CD3^+TCR\gamma\delta^+$.

(C) Quantitative analysis of the number of $CD5^+CD7^+$ (day 14), $CD4^+CD8\beta^+$ (day 28), and $CD3^+$ cells (day 33) generated per EB. A total of 13 independent differentiated experimental samples are included for $CD5^+CD7^+$ (p values: WAS-cWAS p = 0.834 [NS], WAS-hESC p = 0.016), a total of 14 for $CD4^+CD8^+$ (p values: WAS-cWAS p = 0.008, WAS-hESC p = 0.013), and a total of 12 for $CD3^+$ cells (p values: WAS-cWAS p = 0.002, WAS-hESC p = 0.05). Included in the WAS data are three experiments performed with a different WAS clone than the cWAS parent line (triangles).

(D) Assessment of GFP expression in T cell populations. WA01 hESC- and WAS-derived cells are shown as controls.

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 $CD4^{-}CD8^{-}$ double-negative (DN) to the $CD4^{+}CD8^{+}$ 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 $WAS^{+/-}$ 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 $CD4^{-}CD8^{-}$ 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_{2-12} 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 ($CD34^{+}CD43^{+}$ or $CD34^{+}CD43^{+}CD45^{+}$), or $CD5^{+}CD7^{+}$ 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 3×10^4 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 subconfluent 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.

ACCESSION NUMBERS

The accession number for the exome sequencing data reported in this paper is SRP074068 (http://www.ncbi.nlm.nih.gov/Traces/sra/).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2016.06.003.

AUTHOR CONTRIBUTIONS

B.V., D.K., and B.R.D. designed and oversaw the experiments. T.J.L., Y.V.C., R.P., C.M., Z.N., Z.G., A.M.C., X.S.L., and W.L. performed the targeted correction, in vitro differentiation, and functional analysis with valuable input from J.C.S. and M.C.H. D.E.P., E.J.R., and M.C.H. designed and generated the ZFNs. M.L.G.G., T.J.L., and M.C.H. performed the assessment of genome integrity.

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