Novel lineage depletion preserves autologous blood stem cells for gene therapy of Fanconi anemia complementation group A

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Haematologica 2018
Volume 103(11):1806-1814

ABSTRACT

A hallmark of Fanconi anemia is accelerated decline in hematopoietic stem and progenitor cells (CD34$^+$) leading to bone marrow failure. Long-term treatment requires hematopoietic cell transplantation from an unaffected donor but is associated with potentially severe side-effects. Gene therapy to correct the genetic defect in the patient's own CD34$^+$ cells has been limited by low CD34$^+$ cell numbers and viability. Here we demonstrate an altered ratio of CD34^{Hi} to CD34^{Lo} cells in Fanconi patients relative to healthy donors, with exclusive *in vitro* repopulating ability in only CD34^{Hi} cells, underscoring a need for novel strategies to preserve limited CD34$^+$ cells. To address this need, we developed a clinical protocol to deplete lineage$^+$ (CD3$^+$, CD14$^+$, CD16$^+$ and CD19$^+$) cells from blood and marrow products. This process depletes >90% of lineage$^+$ cells while retaining ≥60% of the initial CD34$^+$ cell fraction, reduces total nucleated cells by 1-2 logs, and maintains transduction efficiency and cell viability following gene transfer. Importantly, transduced lineage$^-$ cell products engrafted equivalently to that of purified CD34$^+$ cells from the same donor when xenotransplanted at matched CD34$^+$ cell doses. This novel selection strategy has been approved by the regulatory agencies in a gene therapy study for Fanconi anemia patients (*NCI Clinical Trial Reporting Program Registry ID NCI-2011-00202; clinicaltrials.gov identifier: 01331018*).

Introduction

Fanconi anemia (FA) is a rare monogenic disease with a wide array and variable presence of clinical symptoms, the hallmark of which is bone marrow (BM) failure.[1] The genetic basis of FA is a mutation in any one of 21 genes[2] whose protein components make up the FA/breast cancer pathway responsible for DNA repair of interstrand crosslinks through nucleotide excision followed by homologous recombination. Resulting compromises in genetic integrity are associated with a nearly uniform decline in hematopoietic stem and progenitor cells (HSPCs), a 50% incidence of myelodysplastic syndrome or acute myeloid leukemia by adolescence, and a 25% lifetime incidence of head and neck squamous cell carcinoma or gynecological cancer.[3] In some patients, blood cell clones demonstrate spontaneous reversion to wild type (i.e. somatic mosaicism) leading to improved and stable blood cell counts for up to 27 years.[4-6] Thus, correction of the FA hematopoietic defect could significantly alter the disease's clinical course, and this has driven decades of research in HSPC gene therapy for FA.

While FA was recognized as an early candidate disorder for gene therapy, several obstacles have been identified that have delayed clinical success.[3] Initial clinical trials demonstrated a dramatic approximately 50-fold reduction in the number of true HSPCs in FA patients relative to other gene therapy patients, such as those treated for primary immune deficiencies.[7] Moreover, FA HSPCs were exceptionally fragile when manipulated *ex vivo* for gene transfer. No treated patient has demonstrated

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Received: April 6, 2018.

Accepted: July 4, 2018.

Pre-published: July 5, 2018.

doi:10.3324/haematol.2018.194571

Check the online version for the most updated information on this article, online supplements, and information on authorship & disclosures: www.haematologica.org/content/103/11/1806

Table 1. Clinical characteristics of 3 patients with Fanconi Anemia A genetic defect enrolled in clinical trial NCT01331018.

Patient	Age (years)	Weight (kg)	Sex	Baseline average ANC/platelet count (thousand/μL)	Marrow cellularity	FANCA defect
1	22	70.7	Male	1.3/65	10-30%	Exon22 splice variant (c. 1827-1 G>A)
2	10	19.6	Male	1.0/62	~20%	Exons6-31
3	5	14.7	Male	1.7/32	~30%	Not determined

One adult and 2 pediatric patients were treated with lentivirus gene therapy for Fanconi Anemia-A (FA-A) defect. Three patients demonstrated steadily declining: absolute neutrophil count (ANC) and platelet counts in the peripheral blood prior to treatment and less than 30% marrow cellularity. Molecular characterization of the FANCA gene defect performed by gene sequencing demonstrated that Patient 1 was homozygous in the FANCA gene for the splicing variant. For Patient 2, Multiplex Ligation-dependent Probe Amplification (MLBA) on the FANCA gene identified a homozygous gross deletion of exons 6-31. No sequence analysis was performed for Patient 3, but complementation testing confirmed FANCA defect.

stable improvements in blood cell counts with long-term persistence of gene-corrected blood cells. These studies highlighted two needs for innovation in *FA* gene therapy: 1) to increase the number of available HSPCs for gene transfer and infusion; and 2) to increase the engraftment potential of these cells after gene transfer and infusion. Following the recommendations of the International FA Gene Therapy Working Group,[8] we launched a phase I clinical trial of gene therapy for FA complementation group A (FA-A) patients in 2011 (*clinicaltrials.gov identifier: 01331018*). This trial design incorporates several features aiming to improve HSPC numbers and fitness. These include: i) a self-inactivating (SIN) lentiviral vector (LV) for transfer of the *FANCA* cDNA regulated by a human phosphoglycerate kinase (hPGK) promoter; ii) a short, overnight transduction to minimize *ex vivo* manipulation, as well as addition of the antioxidant N-acetylcysteine (NAC) throughout manipulation; and iii) culture under reduced oxygen (5%) to limit oxidative DNA damage.[9]

The target HSPC population for gene transfer expresses the CD34 cell surface protein ($CD34^+$). When stained with fluorophore-conjugated antibody against CD34 and analyzed by flow cytometry, a small proportion of BM cells are $CD34^+$, representing both primitive stem cells and more committed progenitors.[10] The standard clinical procedure for isolating these cells first involves either BM collection or mobilization of the cells into circulation through cytokine stimulation with granulocyte colony stimulating factor (G-CSF) or, in certain clinical scenarios, a combination of G-CSF and the chemokine receptor CXCR4 antagonist plerixafor, followed by peripheral blood leukapheresis (mAPH). Initial isolation technologies relied on CD34 antigen expression on the cell surface and utilized biotin-avidin affinity, panning, or immununomagnetic bead-based approaches. Expected yields were 50% of available $CD34^+$ cells with highly variable purities, ranging from 20-90% across techniques.[11] Of these, immunomagnetic bead-based positive selection is the most widely-applied today, with the first US Food and Drug Administration (FDA) approval of a clinical device for human use in 2014. Advances in this technology to include automation have improved reliability in recovery to a mean yield of 70% with purities regularly over 90%.[12,13] However, these values are based on BM and mAPH products wherein 1-3% of total cells express CD34 antigen, and the majority of these cells display high levels of CD34. For FA patients, the frequency of $CD34^+$ cells is much lower: 0.1-1.5% in BM.[14,15] This implies that non-standard processes may be required to preserve the limited numbers of HSPCs for gene transfer in FA.

Here we report HSPC collection results for the first 3 patients treated on our study. Initially, this protocol proposed direct isolation of $CD34^+$ cells from BM without prior attempts at mobilization. The addition of a mobilization regimen with subsequent leukapheresis collections has permitted the evaluation of CD34 expression patterns in both product types and provided evidence for the need for alternative HSPC isolation strategies.

Methods

Patient selection

This study was approved by an Institutional Review Board at Fred Hutchinson Cancer Research Center (Fred Hutch) in accordance with the Declaration of Helsinki and the FDA, and conformed to the National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules. Informed consent was obtained from all patients or guardians. FA patients aged 4 years or over were diagnosed by a positive test for increased sensitivity to chromosomal breakage with mitomycin C (MMC) or diepoxybutane. Correction of melphalan hypersensitivity following retroviral transduction of the FANCA cDNA identified Patient 3 as belonging to the FA-A complementation group. (*Online Supplementary Table S1*). FA-A patients who demonstrated normal karyotype in BM analyses as defined in the trial were considered eligible for the study. Characteristics of enrolled patients are available in Table 1.

Lentiviral vectors

All SIN lentiviral (LV) vectors were produced with a third-generation split packaging system and pseudotyped with vesicular stomatitis virus glycoprotein. LV used to transduce healthy donor cells encoded either an enhanced green fluorescent protein (eGFP) transgene (pRSC-PGK.eGFP-sW) or the full-length *FANCA* cDNA (pRSC-PGK.FANCA-sW), both regulated by an hPGK promoter. Research-grade vectors were produced by the Fred Hutch Vector Production Core (Principal Investigator: HPK). Clinical-grade LV (pRSC-PGK. *FANCA*-sW), was produced by the Indiana University Vector Production Facility (IUVPF, IN, USA) using a large-scale, validated process following Good Manufacturing Practices standards under an approved Drug Master File held by IUVPF. Infectious titer was determined by serial transduction of HT1080 human fibrosarcoma-derived cells and evaluated either by flow cytometry for eGFP expression or by quantitative polymerase chain reaction (qPCR).

Table 2. Isolation and lentiviral vector transduction of autologous Fanconi Anemia A genetic defect HSPC.

Patient	Product collected	Product volume processed	Total CD34+ cells collected	CD34+ cell purification method	CD34+ cells transduced	CD34+ cell dose/kg	CD34Hi cell dose/kg	CD34Lo cell dose/kg
1	BM	1278 mL	1.69E+08	Direct enrichment	5.34E+06	3.57E+04	1.77E+04	1.65E+04
2	BM	534 mL	1.59E+07	None	8.15E+06	3.57E+05	4.62E+04	3.15E+05
3	mAPH	513 mL	9.5E+07	Lineage depletion	5.28E+07	2.44E+06	3.69E+05	2.06E+06

$CD34^{Hi}$: high CD34 expression; $CD34^{Lo}$: low CD34 expression; BM: bone marrow product; mAPH: mobilized apheresis product.

Table 3. Transduction efficiency.

Patient	MOI (IU/cell)	Viability of infusion product (%)	VCN of infusion product	Plating efficiency in CFCs, 0 nM MMC (%)	Plating efficiency in CFCs, 10 nM MMC (%)	% Gene transfer in CFCs, 0 nM MMC (%)	% Gene transfer in CFCs, 10 nM MMC (%
1	10 IU/ cell	79	0.33	4.14	0.22	17.7	80
2	10 IU/ cell	99.5	1.83	3.8	0.03	42.73	100
3	5 IU/ cell	99.3	0.67	2.75	0.04	26.23	100

Viability of the infusion product was determined by trypan blue dye exclusion. The vector copy number (VCN) in the bulk transduced population was determined by quantitative polymerase chain reaction (qPCR) method against a reference standard curve. Plating efficiency of the infusion product was determined as the percentage of $CD34^+$ cells plated with colony-forming capacity. Functional correction of the FANCA gene defect was determined by calculating the plating efficiency under stress of various concentrations of MMC. Gene transfer in colony-forming cells (CFCs) was determined as the percentage of colonies analyzed positive for the presence of lentivirus backbone by PCR analysis on DNA extracted from individual colonies. MOI: multiplicity of infection.

Study design and HSPC isolation

Patients underwent either BM harvest with a target collection goal of 15 cc/kg body weight or were administered daily G-CSF (filgrastim; 16 μg/kg BID; days 1-6) and plerixafor (240 μg/kg/day; days 4-6) subcutaneously to mobilize $CD34^+$ cells. Mobilized patients were subjected to large volume leukapheresis when circulating $CD34^+$ blood cell counts were ≥5 cells/μL. Healthy donor blood products were purchased from a commercial source (BM products; StemExpress, Folsom, CA, USA) or institutional shared resources (mAPH products). Immunomagnetic beads were from Miltenyi Biotech, GmbH (Auburn, CA, USA). For BM products, RBC were debulked by hetastarch sedimentation prior to labeling on a CliniMACS Prodigy™ device (Miltenyi Biotec GmbH, Germany). For mAPH products, an initial platelet wash was performed prior to labeling. Custom programming for lineage depletion was designed and executed on the CliniMACS Prodigy™ device (Miltenyi Biotec, GmbH). Complete processing methods are included in the *Online Supplementary Materials and Methods*.

Transduction

CD34-enriched cells were cultured on RetroNectin™ (Takara Bio, Mountain View, CA, USA)-coated culture flasks at a density of $1x10^6$ cells/mL and $2.9x10^5$ cells/cm^2 in StemSpan™ ACF media (StemCell Technologies, Vancouver, BC, Canada), supplemented with 4 μg/mL of protamine sulfate (American Pharmaceutical Partners; APP, East Shaumburg, IL, USA), 100 ng/mL each of recombinant human stem cell factor (rhSCF), thrombopoietin (rhTPO) and Flt-3 ligand (rhFLT3L) (all from CellGenix GmbH, Freiburg, Germany), and 1 mM NAC (Cumberland Pharmaceuticals, Nashville, TN, USA). Cells were immediately transduced at a multiplicity of infection (MOI) of 5-10 infectious units (IU)/cell. Following 12-24 hours of incubation at 37°C, 5% CO_2 and 5% O_2 , cells were harvested for infusion and/or analyses.

Transplantation in NSG mice

All animal work was performed under protocol 1864 approved by the Fred Hutch Institutional Animal Care and Use Committee. NOD.Cg-PrkdcscidIL2rγtmlWj/Szj (NOD/SCID/IL2rγ^{null}, NSG) mice were housed at Fred Hutch in pathogen-free conditions approved by the American Association for Accreditation of Laboratory Animal Care. 8-12-week old mice received 275 cGy total body irradiation (TBI) from a Cesium source. Four hours after TBI, $1x10^6$ gene-modified total nucleated cells (TNCs) re-suspended in 200 μL phosphate buffered saline (D-PBS, Life Technologies Corporation, Grand Island, NY, USA) containing 1% heparin (APP) were infused *via* tail vein. Blood samples were collected into ethylenediaminetetraacetic acid (EDTA) Microtainers (BD Bioscience, San Jose, CA, USA) by retro-orbital puncture and diluted 1:1 with PBS prior to analysis. At necropsy, spleen and BM were collected. Tissues were filtered through 70 μm mesh (BD Bioscience) and washed with Dulbecco's PBS (D-PBS).

Colony-forming cell assays

Transduced cell products were seeded in standard CFC assays in methylcellulose media (H4230, Stem Cell Technologies) as previously described[16] with the following exceptions: to assess FANCA gene function, MMC (Sigma Aldrich, St. Louis, MO, USA) was added at concentrations of 0 nM, 5 nM, 10 nM, or 20 nM. Complete colony DNA extraction and PCR methods are included in the *Online Supplementary Materials and Methods*.

Quantitative real-time PCR-based measurement of vector copy number

Vector copy number (VCN) per genome equivalent was assessed by TaqMan 5' nuclease quantitative real-time PCR assay in duplicate reactions with an LV-specific primer/probe combination [forward, 5'-TGAAAGCGAAAGGGAAACCA;

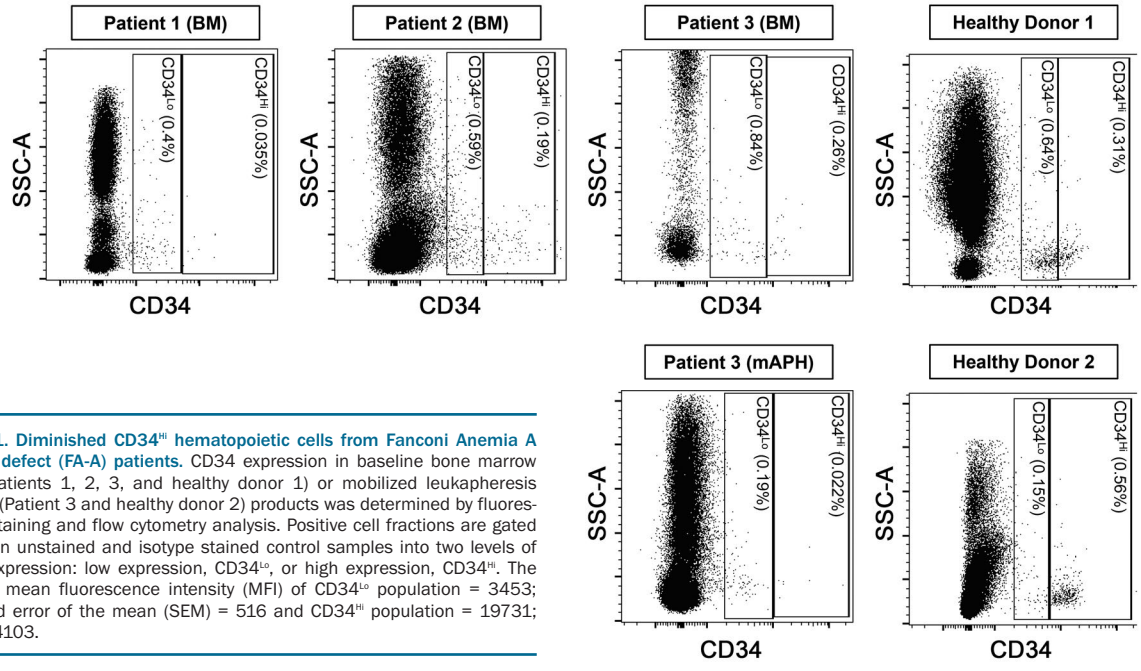

Figure 1. Diminished CD34^{Hi} hematopoietic cells from Fanconi Anemia A genetic defect (FA-A) patients. CD34 expression in baseline bone marrow (BM) (Patients 1, 2, 3, and healthy donor 1) or mobilized leukapheresis (mAPH) (Patient 3 and healthy donor 2) products was determined by fluorescence staining and flow cytometry analysis. Positive cell fractions are gated based on unstained and isotype stained control samples into two levels of CD34 expression: low expression, CD34^{Lo}, or high expression, CD34^{Hi}. The average mean fluorescence intensity (MFI) of CD34^{Lo} population = 3453; standard error of the mean (SEM) = 516 and CD34^{Hi} population = 19731; SEM = 4103.

reverse, 5'-CCGTGCGCGCTTCAG; probe, 5'-AGCTCTCTC-GACGCAGGACTCGGC (Integrated DNA Technologies; IDT, Coralville, IA, USA)] and in a separate reaction with a β-globin-specific primer/probe combination [forward, 5'-CCTATCA-GAAAGTGGTGGCTGG; reverse, 5'-TTGGACAGCAA-GAAAGTGAGCTT; probe, 5'-TGGCTAATGCCCTGGCCCA-CAAGTA (IDT)]. Two standard curves were established by serial dilution of gDNA isolated from a human cell line (HT1080) confirmed to contain a single integrant of the same LV backbone and from peripheral leukocytes collected from a healthy donor using both primer-probe sets independently.

Individual colony gDNA samples were subjected to multiplex real-time TaqMan qPCR to amplify the LV-specific product and an endogenous control (TaqMan Copy Number Reference assay RNaseP, Thermo Fisher Scientific, Pittsburgh, PA, USA). Samples with an average VCN ≥0.5 were considered transduced.

Flow cytometry analysis of hematopoietic subsets

Stained cells were acquired on a FACSCanto™ II, FACSAria™ II or FACS LSR II (all from BD Bioscience) and analyzed using FlowJo software v.10.0.8 (Tree Star Inc., Ashland, OR, USA). Analysis was performed on up to 20,000 cells. Gates were established using Full Minus One stained controls.

Antibodies included anti-human CD34 (clone 563), CD16 (clone 3G8), CD3 (clone UCHT1), CD4 (clone L200), CD8 (clone RPA-T8), all from BD Biosciences; CD14 (clone 61D3, Thermo Fisher Scientific, Pittsburgh, PA, USA); CD19 (clone 4G7, BD Pharmingen, San Diego, CA, USA); CD90 (clone 5E10), CD20 (clone 2H7), CD15 (clone W6D3), all from Biolegend (San Diego, CA, USA); CD133 (clone 293C3, Miltenyi Biotec, GmbH); CD45 (clone D058-1283) and CD45RA (clone 5H9), both from BD Horizon (San Jose, CA, USA).

For mouse samples, antibodies were anti-mouse CD45-V500 (561487, clone 30-F11), anti-human CD45-PerCP (347464, clone 2D1), CD3-FITC (555332, clone UCHT1), CD4-V450 (560345, clone RPA-T4), CD8-APCCy7 (557834, clone SK1), CD20-PE (555623, clone 2H7), and CD14-APC (555824, clone 581), all from BD Biosciences.

Results

Diminished CD34^{Hi} expressing cells in FA-A BM and mAPH

Two enrolled patients underwent BM harvest to collect available CD34^{+} HSPCs (Patients 1 and 2). The third patient underwent mobilization with filgrastim and plerixafor followed by peripheral blood leukapheresis (Patient 3). All 3 patients demonstrated reduced CD34 expression and estimated numbers of CD34^{+} cells in screening BM aspirate samples prior to collection and treatment, relative to healthy donor BM products, as well as in cell products collected for CD34^{+} cell isolation and gene transfer (Figure 1). Two levels of CD34 expression were observed, CD34^{Lo} [mean fluorescence intensity (MFI)=3453±516], and CD34^{Hi} (MFI=19731±4103). Notably, the proportion of CD34^{Hi} cells were markedly reduced in FA-A patients relative to those observed in healthy donors (Figure 1).

FA-A CD34^{Hi} cells, but not CD34^{Lo} cells, demonstrate *in vitro* repopulating capacity

To determine which CD34^{+} cells demonstrated repopulation potential, we used colony-forming cell (CFC) potential as a surrogate. This required sufficient blood product to flow-sort CD34^{Lo} and CD34^{Hi} cells for *in vitro* assays. Only the mAPH product collected from Patient 3 was sufficient for this study. For direct comparison, we sort-purified CD34^{Lo} and CD34^{Hi} cells from a healthy donor mAPH product. Only CD34^{Hi} cells from the FA-A patient demonstrated colony-forming potential (Figure 2A). In the healthy donor, CD34^{Hi} cells also demonstrated the majority of CFC capacity in comparison with CD34^{Lo} cells, and at much higher levels as compared to the FA-A patient (Figure 2B). These data suggest repopulating capacity is restricted to CD34^{Hi} cell fractions, underscoring the need to preserve as many of these cells as possible for gene transfer processes.

Extensive loss of FA-A CD34^{Hi} cells with direct clinical purification protocols

The current clinical standard for CD34$^+$ cell enrichment is optimized for collection of CD34^{Hi} cells. However, in Patient 1, direct enrichment of CD34$^+$ cells using this protocol was inefficient, resulting in an approximately 3% yield and only 5.34x10^6 total CD34$^+$ cells available for gene transfer (Table 2). Moreover, the purity of the enriched cell product was only 58.9%, and approximately 47% loss in viable cells was observed during culture and gene transfer. Resulting gene-modified cells retained colony-forming capacity and demonstrated acquired resistance to the potent DNA crosslinking agent MMC following LV-mediated FANCA gene transfer (Table 3).

In Patient 2, estimated losses during direct CD34 enrichment and gene transfer were expected to reduce the cell product available for transduction to a level lower than observed for Patient 1. Thus, an urgent amendment was filed with the FDA to permit elimination of the direct CD34 enrichment steps and allow transduction of the entire red blood cell (RBC)-depleted BM product. This processing change preserved more CD34$^+$ cells (Table 2), with improved transduction and viability (Table 3). Together, these data suggested that minimal manipulation of target CD34$^+$ cells from FA-A patients could improve yield, gene transfer efficiency, and function *in vivo*.

Development of a novel strategy to deplete lineage$^+$ cells

We hypothesized that depleting non-target mature B cells, T cells, monocytes, and granulocytes would retain precious CD34$^+$ cells with minimal manipulation, since CD34-expressing cells would not be directly labeled, selected, or washed (Figure 3). Building on our previous work automating cell selection and gene transfer using the CliniMACS Prodigy™ device,[17] we designed a customized, automated RBC debulking and immunomagnetic bead-based lineage specific depletion strategy (*Online Supplementary Materials and Methods*). Four different bead-conjugated antibody reagents were used in this approach: anti-CD3 (T-cell removal), anti-CD14 (monocyte removal), anti-CD16 (granulocyte and NK-cell removal), and anti-CD19 (B-cell removal). This protocol was designed for both BM and mAPH products.

Lineage depletion preserves available CD34$^+$ cells for gene transfer

A total of nine BM and ten mAPH products were processed to establish process validity. An average 60% of BM CD45$^+$ cells and 50% of mAPH CD45$^+$ cells expressed one of the four target markers (CD3, CD14, CD16, or CD19) (*Online Supplementary Figure S1A* and *B*, respectively). CD34$^+$ cell content in these products ranged from 0.35-1.4% in BM and 0.06-0.9% in mAPH products. The average process run time for BM products was ten hours, whereas mAPH products were processed over 13 hours. Observed total nucleated cell (TNC) reduction was approximately 1 log for both BM and mAPH products following lineage depletion (Figure 4A). All target lineage$^+$ cells were depleted to less than 10% of initial numbers, and CD34$^+$ cells were retained at 94.62±4.61% for BM products and 70.69±11.4% for mAPH products (Figure 4B). Retention of available CD34^{Hi} and CD34^{Lo} cells was observed and comparable or superior to that observed for the same products by direct CD34-enrichment (*Online Supplementary Figure S2*). Approximately 24% of BM CD34$^+$ cells were colony-forming in a standard methylcellulose assay, while 51% of mAPH CD34$^+$ cells formed colonies (Figure 4C and *Online Supplementary Figure S3*). However, following LV transduction of these cells using the same protocol proposed for FA-A patient cells, we observed consistent 50% rates of gene transfer into CFCs from both cell product types (Figure 4D). Analysis of single colonies demonstrated an average VCN per CFC of 0.7 for BM CD34$^+$ cells and 1.6 for mAPH CD34$^+$ cells. VCN was also assessed in bulk transduced cells cultured for ten days *in vitro*, demonstrating an average value of 5 for both BM and mAPH products (Figure 4E). Final cell products tested for mycoplasma and sterility were negative, and endotoxin testing demonstrated values within criteria for patient infusion. Lineage-depleted and transduced cells from six mAPH and BM products each were infused into immunodeficient (NSG) mice at a target cell dose of 1x10^6 TNC per mouse. On average, the CD34$^+$ cell dose per mouse for BM products was 2.86x10^4 CD34$^+$ cells [standard error of the mean (SEM)=6.67x10^3] and for mAPH products was 1.08x10^5 CD34$^+$ cells (SEM=1.45×10^4). Flow cytometry analysis on peripheral blood was used to evaluate engraftment (human CD45$^+$) and lineage development into T cells (human CD3$^+$), B cells (human CD20$^+$),

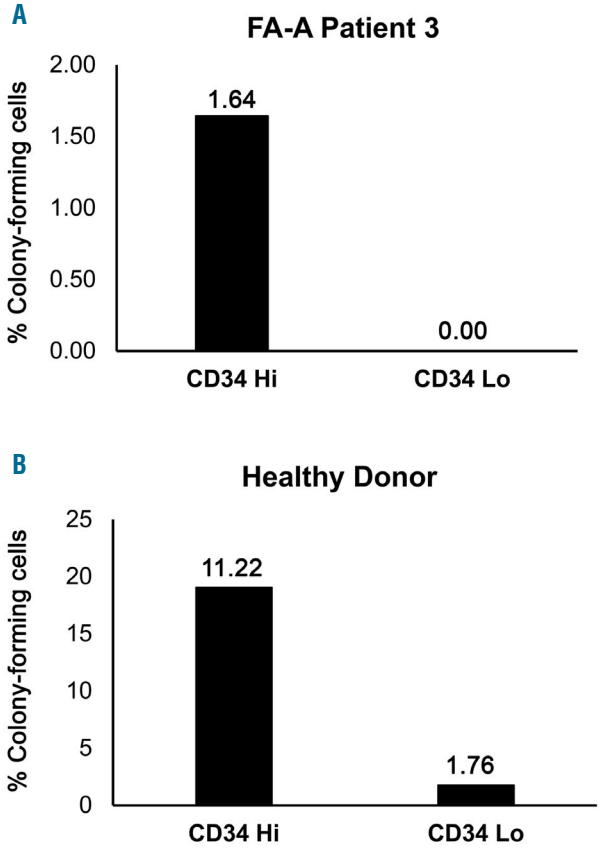

Figure 2. *In vitro* repopulation potential restricted to CD34^{Hi} hematopoietic cells. Mobilized leukapheresis from FA-A Patient 3 (Panel A) and a healthy donor (Panel B) were in parallel fluorescence stained with anti-CD34 antibody and sort-purified for CD34^{Hi} and CD34^{Lo} cells. Total nucleated cells (TNC) equivalent to 1500 CD34-expressing cells were seeded in CFC assays. Percentage of CD34$^+$ cells seeded in the assay that gave rise to colonies is represented as the % of colony-forming cells.

and monocytes (human CD14$^+$) over time (Figure 4F). Both mAPH and BM products demonstrated long-term engraftment over 20 weeks of monitoring. Engraftment levels were comparable to results reported by Wiekmeijer *et al*. with CD34$^+$ cells purified from BM and infused at similar cell doses.[18]

Lineage-depleted cell products xenoengraft equivalently to CD34-enriched products

In this experiment, healthy donor BM products were divided into two aliquots. One was lineage-depleted and the other CD34-enriched. Resulting cell populations were transduced with the same LV vector under identical conditions and infused into NSG mice at matched CD34$^+$ cell doses. We observed higher CD34$^+$ cell retention with lineage depletion compared to CD34 selection, with no differences in transduction efficiency or colony-forming potential (Figure 5A and B). We observed slightly higher, but not significantly different, levels of human CD45$^+$ blood cell engraftment in mice receiving transduced, lineage-depleted cells relative to mice receiving CD34-selected cells. We also observed more stability of T- and B-cell engraftment in mice receiving lineage-depleted cell products relative to mice receiving CD34-selected cell products (Figure 5C).

Lineage depletion protocol preserves limited FA CD34^{Hi} cells

These data collectively suggest that lineage-specific depletion preserved available CD34$^+$ cells without compromising transduction efficiency or cell fitness. Under FDA approval, the clinical protocol was modified to include both BM and/or mAPH products, with lineage depletion as the method of CD34$^+$ cell enrichment. Patient 3 (the first treated under the modified protocol) was a 5-year old male with FA-A confirmed by complementation studies. Baseline neutrophils averaged 1.7x10^9/L and baseline platelets averaged 32x10^9/L in the six months prior to treatment, with declining neutrophils and platelets over the prior 2-year interval (*Online Supplementary Figure S4*). Mobilization of ≥10 CD34$^+$ cells/μL peripheral blood was achieved (*Online Supplementary Figure S5A*), and two successive apheresis collections resulted in 8.5x10^{10} TNC containing a total 1.6x10^8 CD34$^+$ cells (Table 2). The patient required a total of two platelet transfusions and two packed red blood cell transfusions during mobilization and leukapheresis (*Online Supplementary Figure S5B*). Due to column limitations, 5x10^{10} TNC (equivalent to 9.5x10^7 total CD34$^+$ cells) were subjected to lineage depletion, and the remainder were cryopreserved. Lineage depletion resulted in a 94% reduction in TNC and a 56% retention of available CD34$^+$ cells. CD34 purity was 1.6%, representing a 1-2 log-fold increase in the total number of CD34$^+$ cells per kg available for transduction and infusion relative to Patients 1 and 2 (Table 2). A total of 52.8x10^6 CD34$^+$ cells were transduced at an MOI of 5 IU/cell, resulting in a final cell dose of 2.4x10^6 total CD34$^+$ cells per kg with 99.3% viability based on trypan blue dye exclusion. Approximately 26% of CFCs in this cell product were transduced, displaying a mean VCN of approximately 1 (0.9) (Table 3). Thus, limited numbers of available CD34$^+$ cells were indirectly enriched using lineage deple-

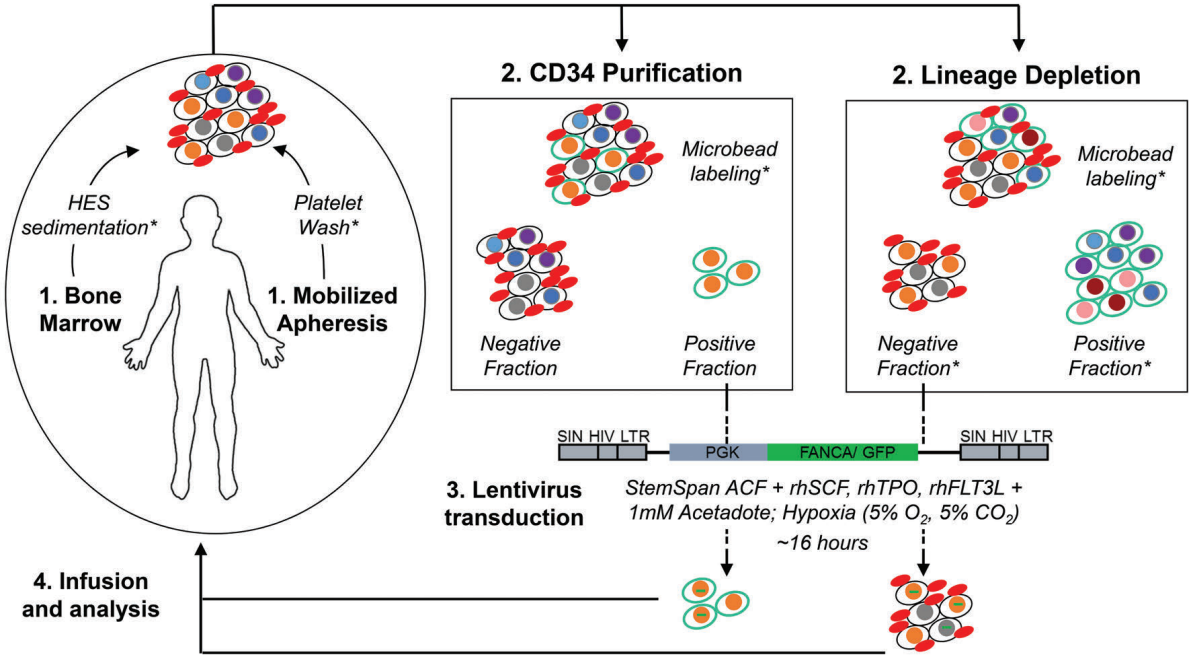

Figure 3. Direct CD34 enrichment *versus* depletion of lineage positive (+) cells. Products can include bone marrow (BM) or mobilized apheresis product (mAPH) (1). BM products were first processed through hetastarch sedimentation to deplete red blood cells (RBCs). Leukapheresis products were first subjected to several washes to deplete platelets. For direct CD34$^+$ cell selection, anti-CD34 antibody-bound immunomagnetic beads (microbeads) are used, whereas for lineage depletion anti-CD3$^+$, CD14$^+$, CD16$^+$, and CD19$^+$, microbeads are used (2). In both cases, microbead-bound cells are retained on the column and subjected to wash steps. When lineage depletion is used, CD34-expressing cells undergo minimal manipulation during purification. Following purification, cells are cultured and transduced with a VSV-G pseudotyped lentiviral vector at a multiplicity of infection (MOI) of 5–10 IU/ cell (3). Following ~16 hours of incubation cells are harvested (4). *These processes were performed on the CliniMACS Prodigy™ device from Miltenyi Biotec GmbH.

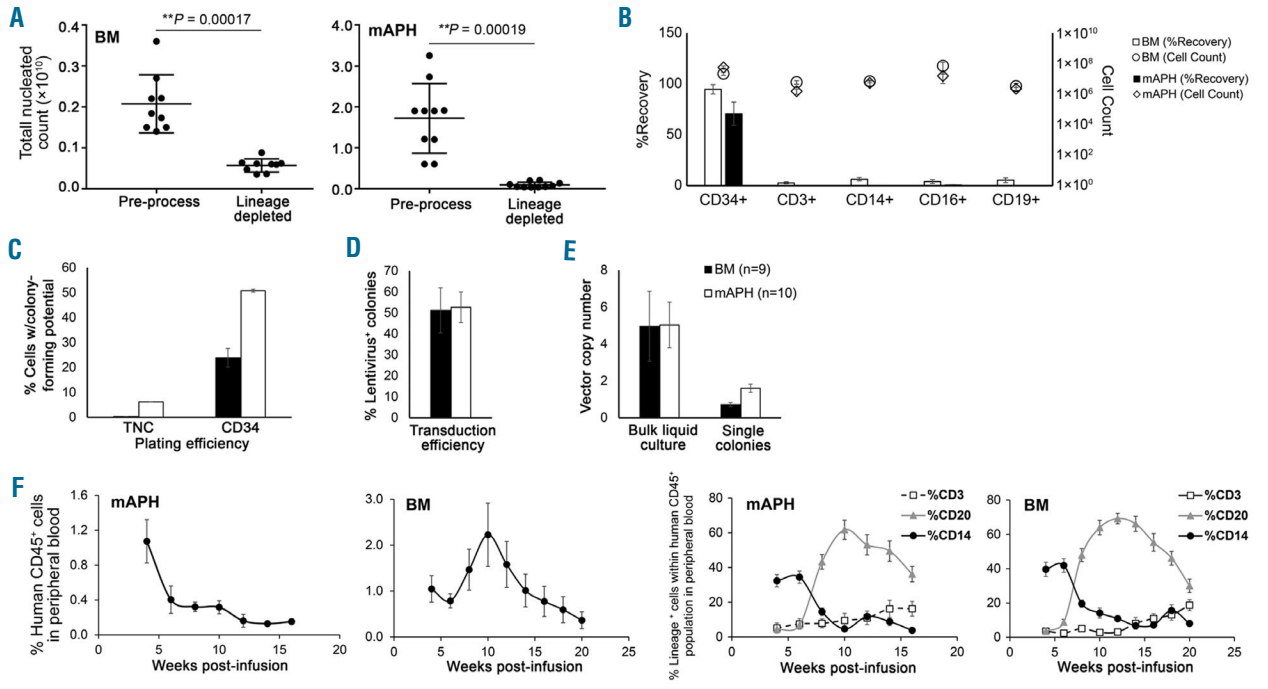

Figure 4. Multi-lineage engraftment of lineage depleted and transduced bone marrow (BM) and mobilized apheresis products (mAPH) in NSG mice. Recovery of total nucleated cells (TNC), CD34$^+$ cells and lineage positive (+) cells (A and B). (C-E) Gene transfer efficiency. The colony-forming potential of transduced cells in standard CFC assays is defined as the plating efficiency (TNC). The colony-forming potential normalized to the number of CD34$^+$ cells seeded is depicted as plating efficiency (CD34$^+$). The percentage of colonies analyzed positive for the presence of lentivirus (LV) backbone by PCR analysis on DNA extracted from individual colonies is depicted as transduction efficiency. The vector copy number per cell in the bulk transduced population is depicted as VCN. The average VCN per cell in the individual CFC is depicted as single colony VCN. Data are representative of the average of 9 healthy BM products and 10 healthy mAPH products. Error bars represent the standard error of the mean. (F) Engraftment of human CD45$^+$ cells and lineage development into T cells (CD3$^+$), monocytes (CD14$^+$) and B cells (CD20$^+$) was determined by flow cytometry over 20 weeks following infusion of lineage-depleted cell products. Data are representative of 36 mice from 6 mAPH donors and 42 mice from 6 BM donors, respectively. Error bars represent the Standard Error of the Mean.

tion on a blood product from an FA-A patient without compromising transduction efficiency.

Discussion

Here we confirm prior reports of inefficient CD34$^+$ cell enrichment from FA patient blood products by direct, immunomagnetic bead-based separation, which is the current standard protocol for isolating HSPCs.[15,19-21] We also demonstrate substantially reduced levels of CD34^{Hi} cells in FA patients relative to healthy donors, which likely contributes to poor positive selection results in blood products from FA patients. Colony seeding assays demonstrate that only CD34^{Hi} cells contribute to *in vitro* colony-forming potential in both FA and healthy donor blood products, underscoring the need to preserve as many available CD34$^+$ cells as possible during *ex vivo* manipulation for gene transfer. We demonstrate a clinically viable procedure for depleting lineage positive cells to indirectly enrich for CD34$^+$ cells that preserves the limited numbers of these cells in FA-A patients without compromising viability, gene transfer, or engraftment potential.

Importantly, the phenotype of limiting CD34$^+$ cell numbers is not restricted to FA alone. Sickle cell disease (SCD) patients treated with hydroxyurea also display reduced CD34$^+$ cell frequencies in BM, and there is a contraindication to mobilization of available CD34$^+$ cells owing to an increased risk of vaso-occlusive crisis.[22] Other inherited BM failure syndromes such as dyskeratosis congenita also are associated with abnormal CD34$^+$ cell frequencies and behavior.[23] As a larger number of disease targets become relevant for gene therapy, additional patient populations will likely display variable CD34$^+$ cell frequency and antigen expression. These disease targets could also benefit from clinically viable alternative selection procedures such as we have developed here.

Our observation of CFC potential in only the CD34^{Hi} fraction in both FA and healthy samples suggests that CD34^{Lo} cells may not be contributing to hematopoietic reconstitution. Notably, our data are from mAPH samples not BM, and we will need more patients for confirmation. Additionally, the standard colony-forming assay best defines progenitor cells, more so than true long-term repopulating hematopoietic stem cells.[24] Alternatively, xenotransplant of purified cells into immunodeficient mice could provide the most robust evidence for CD34$^+$ cell function *in vivo*, but the very small numbers of these cells may prove problematic to achieving relevant cell doses needed for these experiments. Another *in vitro* assay, such as the long-term culture-initiating cell assay,[25] may provide additional insight into the desired target CD34$^+$ subpopulations for gene therapy if they are present in either the CD34^{Hi} or CD34^{Lo} populations in FA patients. In this regard, we recently demonstrated that the CD34^{Hi}CD45RA$^-$CD90$^+$ phenotype is responsible for hematopoietic repopulation in non-human primates in the autologous, myeloablative setting,[16] and evaluation of this phenotype in the enrolled FA patients is ongoing. Critically, our strategy of depleting cells expressing mature

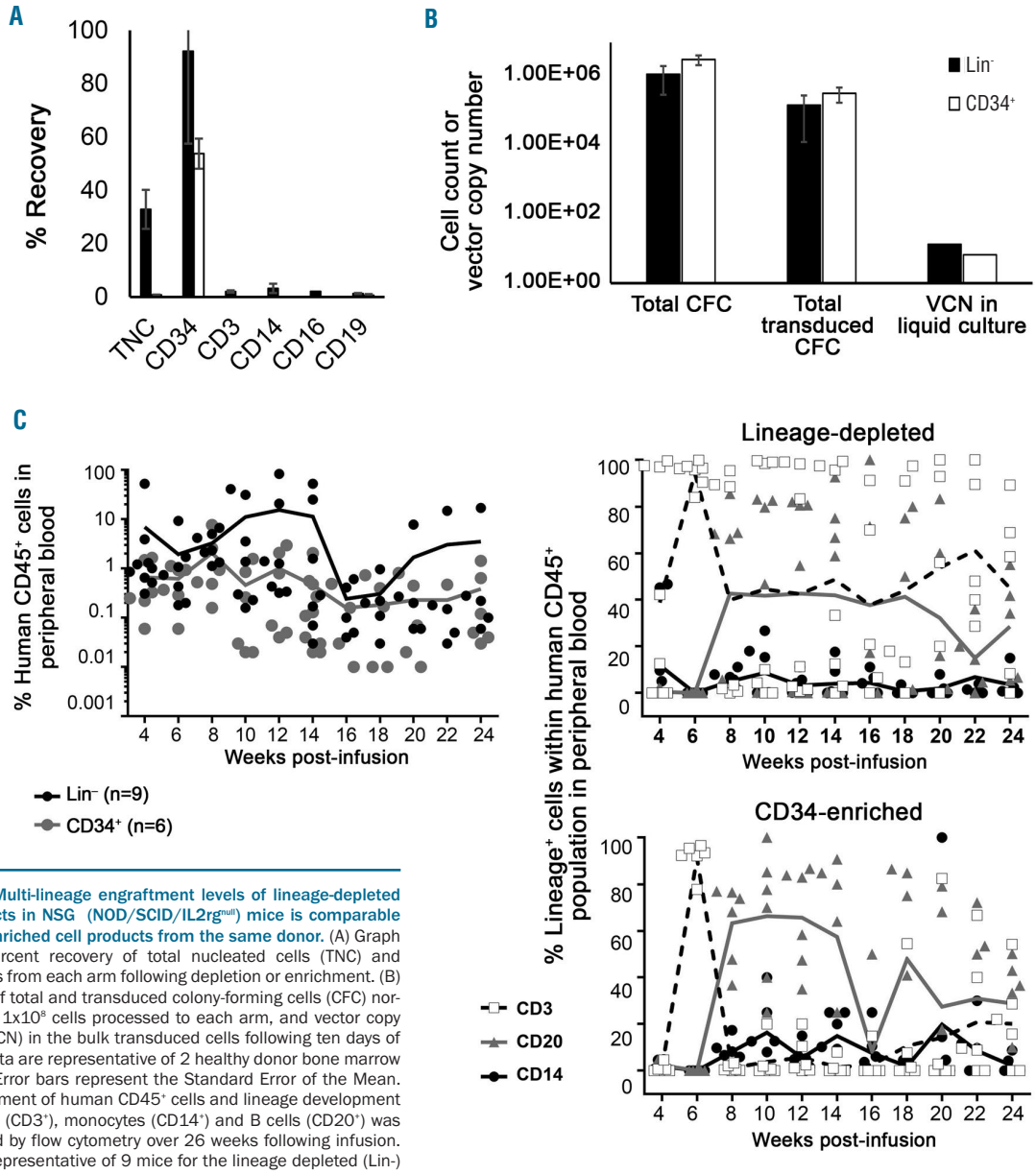

Figure 5. Multi-lineage engraftment levels of lineage-depleted cell products in NSG (NOD/SCID/IL2rg^{null}) mice is comparable to CD34-enriched cell products from the same donor. (A) Graph depicts percent recovery of total nucleated cells (TNC) and CD34$^+$ cells from each arm following depletion or enrichment. (B) Numbers of total and transduced colony-forming cells (CFC) normalized to 1x10^8 cells processed to each arm, and vector copy number (VCN) in the bulk transduced cells following ten days of culture. Data are representative of 2 healthy donor bone marrow products. Error bars represent the Standard Error of the Mean. (C) Engraftment of human CD45$^+$ cells and lineage development into T cells (CD3$^+$), monocytes (CD14$^+$) and B cells (CD20$^+$) was determined by flow cytometry over 26 weeks following infusion. Data are representative of 9 mice for the lineage depleted (Lin-) arm and 6 mice for the CD34 enriched (CD34) arm, respectively.

blood cell lineage markers preserves all CD34$^+$ cell phenotypes for gene transfer and infusion, as demonstrated by Patient 3, whose infused CD34$^+$ cell dose was the largest received to date.

One characteristic of lineage-depleted cell products requiring additional study is the presence and impact of other supporting cells on engraftment. Especially for BM-derived products, our procedure does not include a marker to deplete mesenchymal stem cells (MSC). While the engraftment potential of MSC manipulated *ex vivo* in CD34$^+$ cell supportive media is unexplored, two recent reports suggest that these cells are integral to BM function in FA, and can be LV-transduced and functionally corrected to facilitate hematopoietic recovery and function in a mouse model of FA.[26,27] For mAPH-derived products, such as that infused into Patient 3, additional follow up will be required to determine if a selective advantage is observed *in vivo*. The improved transduction efficiency of lineage-depleted cell products could reflect non-repopulating CD34$^-$ cell uptake of LV. However, we still observed a benefit in transduction of hematopoietic CFC, even at the lower MOI of 5 IU/cell. One other possible explanation is the age and clinical condition of Patient 3. To address this concern we compared our results in Patient 3 to the 4 FA patients enrolled in the FANCOSTEM clinical trial in Spain (*clinicaltrials.gov identifier: 02931071*).[28] These 4 patients were aged 3-7 years and demonstrated higher baseline blood cell counts at the time of collection. All 4 patients received the same mobilization regimen as Patient 3 reported here, but resulting mAPH products were subjected to direct CD34 enrichment prior to transduction at an MOI of 100 IU/cell. The reported mean VCN was 0.4±0.1 and ranged from 0.1 to 0.4 copies in individual CFC. Our data with a higher VCN at lower

MOI suggest that the mixed cell culture supports transduction of hematopoietic progenitor cells, at least.

In conclusion, we describe an alternative strategy to a direct, immunomagnetic bead-based selection of CD34-expressing cells that overcomes current barriers in isolation of blood stem and progenitor cells especially for diseases like FA. Our novel approach to preserve available CD34$^+$ cells during initial blood product processing has the potential to improve gene therapy and gene editing in settings of limited CD34$^+$ cell availability, including FA and other diseases in which direct CD34 enrichment has proven inefficient, such as SCD.

Acknowledgments

We thank our patients and their families. We thank the Fanconi Anemia Research Fund for sponsoring patient travel and housing arrangements associated with this study. We especially thank the Fred Hutch Cell Processing Facility and Seattle Cancer Care Alliance for their support in enrollment and treatment of clinical trial patients. We thank H. Crawford and K. Gonzalez for help in preparing the manuscript. We also thank J. Chen and C. Ironside for excellent support in our mouse studies. This work was primarily funded by a Sponsored Research Agreement between Fred Hutch and Rocket Pharmaceuticals.

Funding

This work was also supported in part by grants and contracts to H-PK from the NIH HHSN2680004 and HL122173 and by funds to JEA from the Fred Hutch. This research was also funded in part through the NIH/NCI Cancer Center Support Grant P30 CA015704. H-PK is a Markey Molecular Medicine Investigator and received support as the inaugural recipient of the José Carreras/E. Donnall Thomas Endowed Chair for Cancer Research and the Fred Hutch Endowed Chair for Cell and Gene Therapy. JEA and H-PK are co-inventors on U.S. Provisional Patent Applications #62/491,116 and #62/503,801 "Novel Manufacturing of Gene Corrected Autologous Blood Cells for Gene Therapy." JEA has previously served as a consultant for Rocket Pharmaceuticals. H-PK is an active consultant for Rocket Pharmaceuticals. The other authors declare that they have no competing interests.

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