Title

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Journal

Stem Cells, 33(5)

ISSN

1066-5099

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Publication Date

2015-05-01

DOI

10.1002/stem.1957

Peer reviewed

Manuscript Accepted – Please Update SC-14-0646.R2

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Enrichment of Human Hematopoietic Stem/Progenitor Cells Facilitates Transduction for Stem Cell Gene Therapy

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Kismet Baldwin collected and assembled data, performed data analysis and co-wrote the manuscript. Fabrizia Urbinati, Zulema Romero, Beatriz Campo-Fernandez and Michael Kaufman performed portions of the research studies. Aaron Cooper and Roger Hollis provided study materials. Donald Kohn participated in conception and design of the studies, provided financial support through grant funding, co-wrote the manuscript and gave final approval.

Key words: Hematopoietic stem cells, gene therapy, lentiviral vector, stem cell enrichment

This work was supported by The National Institutes of Health [2P01 HL073104 (DBK); T32 HL6699201 {Medical Hematology Training Grant (KMB); and T32 HL08634506 {Developmental Hematology Training Grant} (KMB), the California Institute for Regenerative Medicine (CIRM DR1-01452) along with generous donations from the Hina Patel Foundation.

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Abstract

Autologous hematopoietic stem cell (HSC) gene therapy for sickle cell disease (SCD) has the potential to treat this illness without the major immunological complications associated with allogeneic transplantation. However, transduction efficiency by β-globin lentiviral vectors using CD34-enriched cell populations is sub-optimal and large vector production batches may be needed for clinical trials. Transducing a cell population more enriched for HSC could greatly reduce vector needs and, potentially, increase transduction efficiency. $CD34^+/CD38^-$ cells, comprising ~1-3% of all $CD34^+$ cells, were isolated from healthy cord blood (CB) $CD34^+$ cells by fluorescence activated cell sorting (FACS) and transduced with a lentiviral vector expressing an anti-sickling form of beta-globin (CCL-β^{AS3}-FB). Isolated $CD34^+/CD38^-$ cells were able to generate progeny over an extended period of long term culture (LTC) compared to the $CD34^+$ cells and required up to 40-fold less vector for transduction compared to bulk $CD34^+$ preparations containing an equivalent number of $CD34^+/CD38^-$ cells. Transduction of isolated $CD34^+/CD38^-$ cells was comparable to $CD34^+$ cells measured by quantitative PCR at day 14 with reduced vector needs, and average vector copy/cell remained higher over time for LTC initiated from $CD34^+/38^-$ cells. Following *in vitro* erythroid differentiation, HBBAS3 mRNA expression was similar in cultures derived from $CD34^+/CD38^-$ cells or unfractionated $CD34^+$ cells. *In vivo* studies showed equivalent engraftment of transduced $CD34^+/CD38^-$ cells when transplanted in competition with 100-fold more $CD34^+/CD38^+$ cells. This work provides initial evidence for the beneficial effects from isolating human $CD34^+/CD38^-$ cells to use significantly less vector and potentially improve transduction for HSC gene therapy.

Introduction

Hematopoietic stem cell based therapies can potentially treat a number of inherited and acquired blood cell diseases and exciting clinical progress has been made in recent years (1). Sickle cell disease (SCD) is a multisystem disease associated with severe acute illnesses and progressive organ damage leading to significant morbidity and early mortality (2). It is one of the most common genetic disorders worldwide, affecting approximately 90,000 people in the United States. Current treatments consist mainly of symptomatic therapy of anemia and pain. Hydroxyurea (HU) is another treatment option that induces fetal hemoglobin (HbF) production to inhibit polymerization of sickle hemoglobin (HbS) under low oxygen tension conditions; however HU is not widely used for various reasons (3,4). The only potential cure of SCD is allogeneic hematopoietic stem cell transplant (HSCT). This typically requires a well-matched donor and may be accompanied by the need for long-term immune suppression with the possibility of graft rejection or graft versus host disease, although recent reports of effective reduced intensity condition in adult recipients of matched sibling stem cells holds promise (5).

Gene therapy with autologous hematopoietic stem cells (HSC) is a promising treatment for SCD, potentially without the major immunological complications seen with allogeneic HSCT (6). The anti-sickling β^{AS3}-globin gene when added to mouse and human hematopoietic stem/progenitor cells (HSPC) has been shown to have similar activity as HbF to inhibit red blood cell (RBC) sickling and prevent the manifestations of SCD (7-9). However, lentiviral vectors carrying complex and relatively large human β-globin genomic expression cassettes have low titers; transduction of human $CD34^+$ HSPC is only moderately effective and requires a relatively large amount of vector to be used, while yielding relatively low gene transfer (e.g. average vector copies per cell of 0.5-1.0). Unconcentrated production batches yield titers of $\leq 10^6$ transducing

units (TU)/ml, necessitating large volumes of vector to be produced to perform transductions at clinical scale. Ideally, identifying ways to use less viral vector would avoid high vector production costs and allow treatment of more patients (10).

CD38 is a type II membrane surface glycoprotein expressed on a variety of mature hematopoietic cells. CD38 expression is either low or absent on early HSPC populations (11-13) and most definitions of the primitive, pluripotent human HSC are contained within the $CD34^+/CD38^-$ fraction (14). $CD34^+/CD38^-$ cells comprise only ~1-3% of $CD34^+$ cells and thus are 50-100 times more enriched for HSPC than the unfractionated $CD34^+$ population. $CD34^+/CD38^-$ cells have the capacity for long- term proliferation and blood cell production exceeding that of unfractionated $CD34^+$ cells (11,12). Previously published studies have demonstrated the ability to transduce primary human BM $CD34^+$ cells with the CCL-β^{AS3}-FB LV vector (9) with moderate efficiency using relatively high vector concentrations. We postulated that further purification of HSPC beyond the standard $CD34^+$ cell-enriched fractions by isolating $CD34^+/CD38^-$ cells would reduce the absolute number of target cells to be treated *ex vivo* and significantly less vector would be needed per treated subject.

Here we show that human CB $CD34^+/CD38^-$ cells isolated using FACS could be transduced with up to 40-fold less viral vector and still achieve a vector copy number (VCN) comparable to or higher than that seen in the unfractionated $CD34^+$ cell population. These results demonstrate the potential for using $CD34^+/CD38^-$-enriched HSPC to improve transduction of HSC for increased efficacy in gene therapy of SCD.

Methods and Materials

Vectors

Construction of the CCL-β^{AS3}-FB and CCL-MND-GFP have been described (9). CCL-Ubiq-mCitrine-PRE-FB-2XUSE, CCL-Ubiq-mStrawberry-PRE-FB-2XUSE, and CCL-Ubiq-mCerulean-PRE-FB-2XUSE were constructed using the CCL vector backbone (15), a human ubiquitin promoter (16), the fluorescent report genes purchased from Addgene (Cambridge, MA), an optimized post-transcriptional regulatory element (PRE; 17,18), and two tandem copies of the SV40 polyadenylation enhancer sequences USE (19). Lentiviral vectors were packaged with a VSV-G pseudotype and concentrated and titered as described (20). CCL-β^{AS3}-FB was also packaged with an RD-114 pseudotype using the RD114/TR plasmid (21-23) and titered as described (20). Different preparations of the VSV-G pseudotyped CCL-β^{AS3}-FB had titers of $6x10^8$-$6x10^9$ TU/ml after 300-1,000x concentration, compared to 2.7 $x10^7$ TU/ml for the concentrated RD114/TR pseudotype preparation.

Sample collection

Umbilical CB was obtained after vaginal and caesarean deliveries at UCLA Medical Center (Los Angeles) after clamping and cutting of the cord by drainage of blood from the placenta into sterile collection tubes containing the anticoagulant citrate-phosphate-dextrose. All CB specimens were obtained according to guidelines approved by the University of California, and have been deemed as anonymous medical waste exempt from IRB review. Cells were processed within 48 hours of collection. Mononuclear cells (MNC) were isolated from CB using Ficoll Hypaque (Stem Cell Technologies, Vancouver, BC, Canada) density centrifugation.

Immunomagnetic column separation was then used to enrich for $CD34^+$ cells by incubating the MNCs with anti-CD34 microbeads (Miltenyi Biotec Inc., Bergisch Gladbach, Germany) at 4°C for 30 minutes. The cells were then sent through the immunomagnetic column and $CD34^+$ cells collected. $CD34^+$ cells were placed in cryovials with freezing medium (10% Dimethyl sulfoxide (Sigma Aldrich, St. Louis, MO), 90% FBS) and cryopreserved in liquid nitrogen until needed.

Fluorescent antibody labeling and $CD34^+/CD38^-$ cell sorting

The $CD34^+$ cells were thawed, washed and resuspended in 75µl of phosphate-buffered saline (PBS) for incubation with fluorescent-labeled antibodies. Undiluted phycoerythrin (PE) conjugated anti-CD38 (20µl) and undiluted allophycocyanin (APC) conjugated anti-CD34 (5µl) (all antibodies from BD Sciences, San Jose, CA) were added and the cells were incubated for 30 minutes at 4°C in the dark. After incubation, cells were washed once in PBS. FACSwas performed on a FACS Aria II (BD Biosciences).

The viable mononuclear cell population was gated by forward scatter and 4',6-diamidino-2-phenylindole (DAPI) (Life Technologies, Grand Island, NY) staining. The gated region was used to define the $CD34^+$ cell population (Figure 1a). P3 was used to define the $CD34^+/38^-$ cell population, which was 2.5% of the APC positive cells that were negative for PE. P5 was used to define the $CD34^+/CD38^+$ cells that were positive for APC and positive for PE. These gating strategies were used for all sorting experiments.

Lentiviral vector transduction

After cell sorting, $CD34^+$ and $CD34^+/CD38^-$ cells were placed in individual wells of a non-tissue culture treated plate coated with retronectin (20μg/ml retronectin, Takara Shuzo Co., Japan) at a cell density of $6.3x10^4$-$7.5x10^5$ cells/ml. Pre-stimulation was performed for 18-24 hours at 37˚C, 5% CO_2 in Transduction Medium [serum free X-vivo 15 medium (Lonza, Basel Switzerland) containing 1x L-glutamine/penicillin/streptomycin (L-Glut/Pen/Strep) (Gemini BioProducts, West Sacramento, CA), 50 ng/ml human stem cell factor (hSCF) (StemGent, Cambridge, MA), 20 ng/ml human interleukin-3 (hIL-3) (R&D Systems, Minneapolis, MN), 50 ng/ml human thrombopoietin (hTPO) (R&D Systems), 50 ng/ml human Flt-3 ligand (Flt-3) (PeproTech, Rocky Hill, NJ)]. After pre-stimulation, the desired viral vector (CCL-β^{AS3}-FB, CCL-MND-GFP, mStrawberry, mCerulean, mCitrine or β^{AS3}-FB-RD114) was added to each well at the specified vector concentration (typically $2x10^7$ TU/ml unless otherwise specified) and again incubated at 37°C, 5% CO_2 for 24 hours. The cells were then washed and transferred to a tissue cultured treated plate for myeloid differentiation in Basal Bone Marrow Medium (BBMM) [Iscove's Modified Dulbecco's Medium (IMDM) (Life Technologies, Grand Island, NY), 1x L-Glut/Pen/Strep, 20% FBS, 0.52% BSA] with 5 ng/ml IL-3, 10 ng/ml IL-6, 25 ng/ml hSCF at 37°C, 5% CO_2. Fresh medium was added as needed over a 14 day period. After 14 days in culture, VCN was determined by qPCR or digital droplet PCR (ddPCR) (29) and fluorescent reporter gene expression was analyzed using flow cytometry.

Long-term stromal cultures and methylcellulose cultures

Two days prior to planned $CD34^+$ cell sorting, MS5 murine stromal cells (24) were thawed, irradiated at 10,000 cGy, and then plated ($3x10^4$ cells/well) in 96 well plates in stromal medium [IMDM (Life Technologies Grand Island, NY), FBS 10%, 2-mercaptoethanol] to form pre-established stromal layers for the long-term cultures. Sorted $CD34^+$ and $CD34^+/CD38^-$ cells were co-cultured on the irradiated stroma in long-term culture medium [IMDM, 30% FBS, 10% bovine serum albumin (BSA), 2-mercaptoethanol, 10^6 mol/L hydrocortisone, 1X L-Glut/Pen/Strep, along with 10 ng/ml interleukin-3 (IL-3), 50U/ml IL-6, 50 ng/ml human stem cell factor (hSCF)] (12,25-27). At 2-4 week intervals, samples of non-adherent cells were removed from cultures. Their numbers were determined by viable cell counting with a hemocytometer (Thermo Fisher Scientific, Pittsburgh, PA) and genomic DNA was extracted using Purelink Genomic DNA Mini kit (Invitrogen, Carlsbad, CA) for qPCR, as described below.

Differentiation and mRNA expression analysis

The *in vitro* erythroid differentiation assay is based on a protocol adapted from Douay et al (28) as modified by Romero, Urbinati et al (9). After pre-stimulating and transducing the FACS isolated $CD34^+/CD38^-$ cells and unfractionated $CD34^+$ cells, the cells were transferred to erythroid culture. The VCN was analyzed using qPCR of the HIV-1 packaging signal sequence Psi in the LV provirus and normalized to the human cellular autosomal gene syndecan 4 (SDC4) to calculate the VCN as described by Cooper et al (20). HBBAS3 mRNA expression was determined as previously described by Romero et al. (9).

Transplantation of transduced human CB CD34$^+$/CD38$^-$ cells in immune deficient mice

Unfractionated CD34$^+$, CD34$^+$/CD38$^+$ and CD34$^+$/CD38$^-$ cells from healthy donor CB were transduced separately with lentiviral vectors carrying the different fluorescent marker genes all at 2x10^7 TU/ml (MOI 5-140). Mock transduced (5x10^5) and control unfractionated transduced CD34$^+$ (5x10^5) cells were individually transplanted by tail vein injection into 6-10 week old, immune-deficient NOD.Cg-*Prkd*^{scid}Il2rg^{tm1Wjil}/SzJ (NSG) mice (Jackson Laboratory, Sacramento, CA) after 250 cGy total body irradiation. CD34$^+$/CD38$^-$ and CD34$^+$/CD38$^+$ cells were mixed at a 1:99 ratio so that 2x10^3 CD34$^+$/CD38$^-$ cells and 2x10^5 CD34$^+$/CD38$^+$ cells were co-transplanted by tail vein injection into 6-10 week old NSG mice after irradiation.

After 8-12 weeks, the mice were euthanized and the BM was analyzed for engraftment of human cells by flow cytometry using APC-conjugated anti-human CD45 vs. Horizon V450-conjugated anti-murine CD45 (BD Biosciences). After antibody incubation, RBCs were lysed using BD FACS-Lysing Solution (BD Biosciences, San Jose, CA). The percentage of engrafted human cells was defined as the %huCD45$^+$/(%huCD45$^+$ + %muCD45$^+$ cells). From among the huCD45$^+$ cells, expression of mCitrine, mStrawberry and mCerulean was analyzed using flow cytometry.

The average VC/human cell was measured in the murine BM samples with positive engraftment of human cells using ddPCR (Table 1). Reaction mixtures were prepared consisting of 22 µl volumes containing 1× ddPCR Master Mix (Bio-Rad, Hercules, CA), primers and probe specific to either the HIV-1 Psi region, to detect all vectors, or to each of the fluorescent reporter genes (400nM and 100nM for primers and probe, respectively), DraI (40U; New England Biolabs, Ipswich, MA), and 1.1 µl (4µl for cfu) of the genomic DNA sample. Droplet generation

was performed as described in Hindson *et al*. (29). Thermal cycling conditions consisted of 95°C 10min, 94°C 30s and 60°C 1min (55 cycles), 98°C 10min (1 cycle), and 12°C hold (Supplemental Table 1). The specific amplified portions of the gene were normalized for the percentage of human cells present in the marrow collected from the NSG mice using primers to the autosomal human gene SDC4 gene to adjust for the presence of murine cells in the samples.

All animals involved in experiments were cared for and handled in accordance with protocols approved by the UCLA Animal Research Committee under the Division of Laboratory Medicine.

LDL receptor expression analysis

Cells were collected and sorted as previously described in the fluorescent antibody staining and cell sorting section. $CD34^+$ and $CD34^+/CD38^-$ cells were placed into individual wells of a non-tissue culture treated plate coated with retronectin (20µg/ml retronectin, Takara Shuzo Co., Japan) in Transduction Medium for at 37°C, 5% CO_2. At 24 hours and 48 hours in culture, the cells were harvested for analysis of LDL receptor expression, compared to the cells prior to culture (0 hours) using flow cytometry. The cells were washed and resuspended in 90µl of PBS for incubation with fluorescent-labeled antibody, 10µl undiluted APC conjugated anti- human LDL receptor (R&D Systems, Minneapolis, MN.) The cells were incubated for 30 minutes at 4°C in the dark. After incubation, cells were washed once in PBS and analyzed with the LSR Fortessa for analysis. APC positive cells were considered to be positive for expression of the LDL receptor.

Statistical Analyses

Continuous outcome variables such as means and standard errors by experimental conditions are presented in figures. Pairwise comparison was performed by either unpaired t-test within the framework of one-way or two-way ANOVA. Two group comparisons by Wilcoxon rank sum test was performed when the assumption of normality was not met. Mixed linear model was used to compare two groups over time. A p-value of 0.05 was used as the significance threshold.

Results

Isolation of CB $CD34^+/CD38^-$ cells using fluorescence activated cell sorting (FACS)

Healthy donor CB was enriched for $CD34^+$ cells using immunomagnetic columns. Portions of the unfractionated $CD34^+$ cells were sorted by flow cytometry to isolate $CD34^+/CD38^-$ cells, operationally defined as cells with the lowest 2.5% for CD38 expression (Fig. 1A). Starting with 2.2-6.8 x 10^6 $CD34^+$ cells isolated from CB units, $6x10^3$-$6x10^4$ (mean= $2.5x10^4$) $CD34^+/CD38^-$ cells were isolated, representing a range of 36-99% of the theoretical yield (n= 11).

When put into long- term culture, the unfractionated $CD34^+$ cells expanded ~10-fold over the first month, and then declined in numbers (Fig. 1B). Long-term cultures initiated with $CD34^+/CD38^-$ cells expanded to a greater extent (~100-fold) and maintained stable cell numbers over three months (Fig.1B), demonstrating the greater generative capacity of the more primitive $CD34^+/CD38^-$ populations, compared to the bulk $CD34^+$ cells.

Assessment of transduction of CB $CD34^+$ vs. $CD34^+/CD38^-$ cells

Transduction of $CD34^+$ and $CD34^+/CD38^-$ cells from CB of healthy donors (n=11) with the CCL-β^{AS3}-FB lentiviral vector was compared. Cell density and vector concentration, and hence multiplicity of infection (MOI), were kept constant for the two cell types within an experiment, using either equal numbers of $CD34^+$ and $CD34^+/CD38^-$ cells in identical volumes or adjusting the total volume of the culture when different cells numbers were transduced. Transduced cells were either cultured for two weeks under short-term *in vitro* myeloid differentiation conditions, grown in methylcellulose colony-forming unit (CFU) assay (14 days), or grown in long-term myeloid cultures (90 days) to compare colony-forming capabilities and VCN.

Genomic DNA isolated from cells was analyzed by quantitative (qPCR) for the HIV-1 psi region of the vector at day 14 to determine average vector copy number/cell (VCN). In each sample, transduction of the $CD34^+/CD38^-$ cells was equal to or greater than transduction of $CD34^+$ cells (Supplemental Table 2). The cells produced from the transduced $CD34^+/CD38^-$ cells had a significantly higher VCN of 2.43 ± 0.41 compared to 1.25±0.28 from the transduced $CD34^+$ cultures (n=11, p=0.02) (Fig.2A).

The types of colonies formed by $CD34^+$ cells and $CD34^+/CD38^-$ cells were not different (Fig. 2B). Colonies were formed by 25.7% of the non-transduced $CD34^+$ (NT-$CD34^+$), 24.3% of transduced $CD34^+$ and 22.3% of transduced $CD34^+/38^-$ cells plated in methylcellulose (Fig. 2C). qPCR of individual CFU to detect and quantify the CCL-β^{AS3}-FB vector sequences demonstrated that the percentage of transduced colony-forming progenitors from $CD34^+/CD38^-$ cells (73.8% with a mean VCN=2.12) was higher than from CB $CD34^+$ cells (56.2% with a mean VCN=1.75) (n=80 colonies, each) (Fig. 2D) (p=0.52). CFU formed from $CD34^+/38^-$ cells showed a larger

percentage of colonies with 1-2 VC/cell (47.5%) compared to those formed from unfractionated $CD34^+$ cells (36.2%) (Fig. 2D).

Vector dose-response experiments were performed to examine the relative ability of the CCL-β^{AS3}-FB vector to transduce human CB $CD34^+$ and $CD34^+/CD38^-$ cells, using a range of vector concentrations during transduction from $2x10^6$ to $2x10^7$ TU/ml. A dose-related increase in gene transfer (VCN measured by qPCR) at day 14 was seen with increasing vector concentrations in both cell populations (Fig. 2E). However, at every vector concentration tested, the resultant VCN was higher for the $CD34^+/CD38^-$ cells ($p=0.05$ at $6.6x10^6$ TU/ml, $p=0.002$ at $2x10^7$ TU/ml) than for the $CD34^+$ cells; thus considerably lower concentrations of viral vector ($2x10^6$ TU/ml) could be used to transduce the $CD34^+/CD38^-$ cells and still match the level of transduction achieved with $CD34^+$ cells at higher vector concentration ($2x10^7$ TU/ml) (Fig.2E).

Transduced CB $CD34^+$ and $CD34^+/CD38^-$ cells (n=3) were grown for 90 days in long-term culture on MS5 stromal cells, and cell samples were analyzed at several time-points for VCN (Fig. 2F). At each time point, there was a higher VCN in the cultures from $CD34^+/CD38^-$ cells (1.6-2.3) compared to cultures from the unfractionated $CD34^+$ population (0.3-0.6) ($p=0.0004$), with statistically significant time trend difference ($p=0.03$).

Long-term cultures initiated from $CD34^+/CD38^-$ cells had increasingly higher frequencies of colony-forming cells compared to cultures initiated form $CD34^+$ cells. At day 30 of the LTC, 0.05% of the cells from cultures of non-transduced $CD34^+$ cells, 0.04% of transduced $CD34^+$ and 0.13% of transduced $CD34^+/38^-$ cells ($p<0.0001$) plated in methylcellulose produced colonies (Supplemental Fig. 1). At day 60 of the LTC, colonies were produced by 0.0017% of the cells

derived from non-transduced $CD34^+$ cells, 0.0025% from transduced $CD34^+$ and 0.0067% from transduced $CD34^+/CD38^-$ cells plated in methylcellulose (Supplemental Fig. 2).

Higher percentages of transduced colony-forming progenitors were also present in the cultures initiated from $CD34^+/CD38^-$ cells than from those initiated with $CD34^+$ cells. When analyzed at day 30 of LTC by ddPCR, 83.7% (average VCN=2.3) formed from $CD34^+/CD38^-$ cells were positive for the CCL-βAS3-FB vector compared to 77.3% (average VCN=2.2) of individual CFU derived from cultures of unfractionated $CD34^+$ (Supplement Fig. 1). At 60 days, the unfractionated $CD34^+$ cells produced only three colonies, one of which had a VCN of 0.5 while the other two had a VCN of 0. Twenty-two CFU were formed from $CD34^+/CD38^-$ cells and 16 (88.8%) were positive for the CCL-β^{AS3}-FB vector (average VCN=1.52) (Supplemental Fig. 2).

To determine if the higher transduction of the $CD34^+/CD38^-$ cells would occur with vectors other than the CCL-β^{AS3}-FB vector, the transduction efficiency of $CD34^+$ and $CD34^+/CD38^-$ cells by a high titer GFP-expressing LV vector (CCL-MND-GFP) was assessed in dose response experiments. Both cell populations were transduced at equal cell densities with the CCL-MND-GFP LV vector at the concentration of $2x10^6$, $6.6x10^6$ and $2x10^7$ TU/ml (MOI = 4, 40, and 400, respectively). Again a dose-related increase in gene transfer (VCN measured by ddPCR) at day 14 was seen with increasing vector concentrations in both cell populations (Fig. 3A). At vector concentrations of $6.6x10^6$ and $2x10^7$ TU/ml , the resultant VCN was higher for the $CD34^+/CD38^-$ cells ($CD34^+$ mean VCN= 2.25±0.15 vs. 1.36±0.05 at $6.6x10^6$ TU/ml and 4.32±0.6 vs. 3.37± 0.07, p=0.02 at $2x10^7$ TU/ml) than for the $CD34^+$ cells. On day 14, the percentages of GFP expressing cells were determined using flow cytometry (Fig. 3B) and ranged from 45-81% in

$CD34^+$ cells compared to 45-97% in $CD34^+/CD38^-$ cells (Fig. 3C), which was not significantly different, p=0.29.

In vitro erythroid differentiation of CB $CD34^+/CD38^-$ cells

To compare β^{AS3}-globin expression by the CCL-β^{AS3}-FB vector after transduction of $CD34^+$ and $CD34^+/CD38^-$ cells, transduced cells were put into an *in vitro* erythroid differentiation model (28) to produce mature RBCs that support expression by the β-globin gene cassette. Vector expression was measured using quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) to specifically quantify both the HBBAS3 transcript from the vector and the total β-globin-like transcripts (endogenous HBB and HBBAS3). $CD34^+/CD38^-$ and $CD34^+$ cells from healthy CB donors were transduced with the CCL-β^{AS3}-FB LV vector and control samples were mock-transduced. After 24 hours, the cells were differentiated into erythroid cells for 27 days. Enucleated RBCs were identified at the end of the differentiation (day 27) by double staining with an antibody to the erythroid membrane glycoprotein GpA and the DNA labeling fluorescent dye, DRAQ5. Enucleated RBCs were defined as being $GpA^+/DRAQ5^-$ (Supplemental Fig. 3). Final cell numbers, differentiation markers and percentage enucleation were similar between the two cell populations, ranging from 52.6% ± 1.06 enucleated erythrocytes from the unfractionated $CD34^+$ cells and 52.7% ± 1.08 from the $CD34^+/CD38^-$ cells (p=0.87).

Higher VCN were present in the erythroid progeny of the $CD34^+/CD38^-$ cells, compared to the progeny of the $CD34^+$ cells. At 2 weeks of culture, the cultures from the $CD34^+/CD38^-$ cells had an average VCN of 3.08±0.71 compared to an average VCN of 1.84±0.44 from the $CD34^+$ cells (n=3, p=0.26) (Fig.4A).

Cells transduced with the CCL-β^{AS3}-FB LV and collected at day 14 of the erythroid differentiation culture assay were assessed for their HBBAS3 mRNA expression by qRT-PCR and compared to expression from the endogenous adult HBB mRNA. The level of expression was similar in all of the cultures; HBBAS3 mRNA levels made up 55.2 ± 18.1% of the total HBB-like mRNA in erythroid cells from cultures of CB $CD34^+/38^-$ cells, compared to 45.4% ± 16.7% from cultures of CB $CD34^+$ cells (p=0.59) (Fig. 4B), which was similar to amounts reported in previous studies using the CCL-β^{AS3}-FB LV in healthy and SCD bone marrow $CD34^+$ cells (9).

Assessment of LDL receptor expression after pre-stimulation and transduction

We evaluated whether differences in the recently identified cellular receptor for VSV-G-pseudotyped vectors (30), the human low density lipoprotein (LDL) receptor, were responsible for the more effective transduction of $CD34^+/CD38^-$ cells compared to $CD34^+$ cells. Flow cytometry was used to analyze LDL receptor expression in fresh CB $CD34^+$ and $CD34^+/CD38^-$ cells stained with APC anti-human LDL receptor antibodies and then again after 24 and 48 hours in culture in Transduction Medium with multiple cytokines (time points correlating to completion of pre-stimulation and transduction, respectively).

7.9% of fresh $CD34^+$ cells expressed the LDL receptor compared to 4.8% of fresh $CD34^+/CD38^-$ cells (Fig. 5A, 5D). At 24 hours, ~91% of $CD34^+$ cells were expressing the LDL receptor compared to 74.6% of $CD34^+/CD38^-$ cells (Fig. 5B, 5E). At 48 hours, LDL receptor was expressed on 99% of the $CD34^+$ cells and on 90% of the $CD34^+/CD38^-$ cells (Fig. 5C, 5F). The geometric mean fluorescence intensity of the LDL receptor was similar on the $CD34^+$ and

$CD34^+/CD38^-$ cells ($1.6x10^3$-$3x10^3$ and $1.5x10^3$-$3.5x10^3$, respectively). Fresh $CD34^+$ and $CD34^+/CD38^-$ cells had similarly low percentages expressing the LDL receptor, and it was induced equally on these cells by culture under conditions used for pre-stimulation and transduction. Thus, a difference in LDL receptor expression was not the basis for the better transduction of $CD34^+/CD38^-$ cells.

RD114 retroviral envelope pseudotyped LV

To determine whether the improved transduction of the $CD34^+/CD38^-$ cells is specifically related to the VSV-G envelope, we produced a batch of the CCL-β^{AS3}-FB LV with an alternative pseudotype, using the RD114 retroviral envelope (21,22). $CD34^+/CD38^-$ cells transduced with the RD114- pseudotyped CCL-β^{AS3}-FB LV vector and then cultured for 14 days had a higher average VCN (0.86 ± 0.46) compared to $CD34^+$ cells transduced and analyzed under the same conditions (0.006 ± 0.05, n=3) (Fig. 5G). Therefore, the higher transduction of $CD34^+/CD38^-$ cells is not specific to the VSV-G pseudotype.

Assessment of engraftment potential of $CD34^+/CD38^-$ cells *in vivo*

The prior studies compared transduction of $CD34^+/CD38^-$ cells isolated by FACS to unfractionated $CD34^+$ cells. For subsequent studies, we compared the transduction and engraftment potential of $CD34^+/CD38^-$ cells and $CD34^+/CD38^+$ cells, both isolated by FACS from the same populations of unfractionated $CD34^+$. Each population was transduced with a lentiviral vector carrying different fluorescent reporter genes (CCLc-UBC-mCitrine-PRE-FB-

2XUSE LV, CCLc-UBC-mCerulean-PRE-FB-2XUSE LV and CCLc-UBC-mStrawberry-PRE-FB-2XUSE LV vectors). To ensure that the results were not influenced by the vector, the vector used to transduce each cell fraction was alternated for each study (n=3). Transduced $CD34^+$, $CD34^+/CD38^+$ and $CD34^+/CD38^-$ cells were xeno-transplanted into NSG mice at their appropriate physiologic proportions (99% $CD34^+/CD38^+$ cells + 1% $CD34^+/CD38^-$ cells; or 100% $CD34^+$ cells). Transduction conditions were the same as used for the *in vitro* analyses and the cells were transplanted immediately after 24 hour transduction. Each mouse received a total cell dose of $5x10^5$ cells consisting of (a) non-transduced $CD34^+$ cells (Mock), (b) transduced $CD34^+$ cells (Control), or (c) a combination of transduced $CD34^+/CD38^+$ and transduced $CD34^+/CD38^-$ cells (Test), each mixed with irradiated (10,000cGy) $CD34^-$ cells as "fillers" (Supplemental Table 3). The mice were euthanized 80-90 days after transplantation and their bone marrow was harvested to analyze engraftment of the human cells by flow cytometry and to measure VCN of engrafted cells. The percent engraftment was defined as the percentage of human $CD45^+$ cells of the total $CD45^+$ population (murine $CD45^+$ plus human $CD45^+$). BM from human engrafted mice was then further analyzed by flow cytometry for the percentage of the different transduced cell fractions present in the human engrafted cells, based on the fluorescent markers used and by ddPCR.

Three *in vivo* mouse transplants were conducted, each consisting of six mice, for a total of 18 mice transplanted [5 mock (non-transduced $CD34^+$ cells), 4 controls (transduced $CD34^+$ cells), 9 test mice (mixture of transduced $CD34^+/38^+$ and transduced $CD34^+/38^-$ cells)] (Supplemental Table 3). A portion of the transduced cells were grown in vitro for two weeks and assayed for VCN (Table 1). ddPCR primers and probes were designed to specifically detect each of the fluorescent marker genes (Supplemental Table 1). Samples of transduced cells analyzed after 2

week *in vitro* cultures using the HIV-1 Psi region primers to measure all vectors showed that the $CD34^+/38^-$ cells consistently had a higher VCN (6.89 ± 0.75) than the unfractionated $CD34^+$ cells (VCN=2.19 ± 0.47) or the $CD34^+/CD38^+$ cells (VCN=1.66 ± 0.36) irrespective of the vector used for transduction (mCitrine, mStrawberry, mCerulean) (Table 1).

Of the 18 total mice, 14 had successful engraftment of human $CD45^+$ cells at the time of BM harvest (Supplemental Figure 4). Among the engrafted mice, mice #'s 1, 6 and 7 received only mock-transduced human $CD34^+$ cells. Control mice #'s 8, 9, 12 received $CD34^+$ cells transduced by a single vector. Mice #'s 2, 3, 4, 5, 10, 11, 13 and 14 received test mixtures of $CD34^+/CD38^-$ and $CD34^+/CD38^+$ cells (at 1:99 cell ratios) transduced with different vectors. Overall, there was a trend toward better engraftment with $CD34^+/CD38^-$ cells compared to unfractionated $CD34^+$ cells (p=0.06).

In the 8 engrafted test mice, there was higher engraftment by the vector-labelled $CD34^+/CD38^-$ cells in 6 mice (#'s 2, 3, 4, 5, 11 and 14), equivalent engraftment of $CD34^+/CD38^+$ and $CD34^+/CD38^-$ cells in 1 mouse (#10), and higher $CD34^+/CD38^+$ engraftment in one mouse (#13) (Fig. 6A, Supplemental Fig. 4). Overall the gene marking levels by transplanted bulk $CD34^+$ cells, or the fractionated $CD34^+/CD38^-$ and the $CD34^+/CD38^-$ cells were not different (Supplemental Table 4) and thus, transduced $CD34^+/CD38^-$ cells were approximately 100-times more potent for engraftment than the unfractionated $CD34^+$ or $CD34^+/CD38^+$ cells.

The two mice transplanted with bulk $CD34^+$ cells transduced with a single vector had VCN of 12 and 6, with similar values measured using the HIV-1 Psi region primers or with the fluorescent marker-specific primers. The mice transplanted with a mixture of $CD34^+/CD38^+$ and $CD34^+/CD38^-$ cells showed similar levels of gene marking with the two vectors (3.60 ± 0.26 and

2.17 ± 0.15 respectively), and in each mouse, the sum of the VCN for the two individual vectors was similar to the total VCN measured using the Psi region primers (Fig. 6B).

Discussion

Stem cell gene therapy is advancing toward the clinic for multiple diseases including SCD. For it to be efficacious for SCD, transduction must be efficient with an adequate number of HSC transduced to express enough β^{AS3}-globin to change the pathophysiology of the disease. Clinical scale HSC transduction can be a challenging process made more difficult with large, complex gene cassettes being delivered and inserted, such as the β^{AS3}-globin gene. Although transduction of human $CD34^+$ hematopoietic stem/progenitor cells with the CCL-β^{AS3}-FB LV vector has been demonstrated (9), due to the suboptimal unconcentrated titers of the viral vector, high volumes of viral vector are required to attain the level of gene transfer to engrafting HSC to correct RBC disease manifestation of SCD. For these reasons, an alternate cell population that can be transduced using less viral vector to achieve comparable gene transduction efficiency with effective engraftment capabilities is appealing. To date, several studies have shown that $CD34^+/CD38^-$ cells can be transduced with LV vectors since these vectors are able to transduce cells that are not actively dividing, unlike γ- retroviruses (31-33). However, the capacity of $CD34^+/CD38^-$ cells to be transduced and engrafted for gene therapy has not been explored due to the absence of clinical-grade reagents and the challenges of large-scale GMP cell sorting.

We performed studies using human CB $CD34^+/CD38^-$ cells to assess the potential suitability of these cells for gene therapy of SCD while using less viral vector for transduction. Our studies have shown $CD34^+/CD38^-$ cells isolated from cord blood to be susceptible to transduction with lentiviral vectors, requiring markedly lower amounts of viral vector to achieve comparable or

higher gene transfer compared to $CD34^+$ cells. Importantly, clonal analysis of colony-forming progenitors indicated that a higher percentage were transduced when targeting the $CD34^+/CD38^-$ populations, compared to bulk $CD34^+$ targets, rather than transducing a constant fraction but with higher vector copies. Ideally, clinical applications would lead to a similar higher percentage of transduced HSC for better efficacy, and limit the VCN per transduced cell for better safety.

Interestingly, *in vivo* transplant studies in NSG mice demonstrated that $CD34^+/CD38^-$ cells were approximately 100-fold more potent for engraftment than the counterpart $CD34^+/CD38^+$ cells, with essentially equivalent engraftment contributions. We were not able to obtain sufficient human cells from the bone marrow of the engrafted NSG mice to sort out the cells derived from the CD34+/CD38+ and CD34+/CD38- cells based on their fluorescent marker, so that absolute vector copy numbers per cell could not be directly measured to determine if there was higher vector copies in the engrafted descendants of the CD34+/CD38- cells, as was seen *in vitro*. Rather, ddPCR was performed with total marrow cells from engrafted NSG mice to quantify the specific fluorescent reporter genes used to mark the CD34+/CD38- cells or the CD34+/CD38+ cells, normalized for the human cell content of the marrow and indicated similar contribution to hematopoiesis by the 1% CD34+/CD38- cells as by the 99% CD34+/CD38+ that were transplanted. However, it is possible that the lack of higher average VCN of the vectors used to mark the CD34+/CD38- cells in the marrow may indicate that high VCN led to cytotoxicity to transduced HSC and decreased contribution to engraftment.

Overall, these findings may have applications to any approach to gene therapy using HSC, in addition to the specific benefits for SCD gene therapy shown here. The use of $CD34^+/CD38^-$ cells in gene therapy would allow the use of lesser amounts of vector to transduce the target cells, but may still result in adequate engraftment, based on our observations in the xeno-

transplant studies. The present findings are consistent with other studies demonstrating the good engraftment capability of $CD34^+/CD38^-$ cells (31-34).

Recent publications have described the LDL receptor as the major receptor for vesicular stomatitis virus (VSV) (30) that is most commonly used to pseudotype lentiviral vectors. If LDL receptor expression was higher in the $CD34^+/CD38^-$ cells, there is the possibility of more vector binding to the cell, being taken into the cell and eventually integrating into the genome, leading to the higher VCN seen. When expression of the LDL receptor was analyzed, in unfractionated $CD34^+$ and $CD34^+/CD38^-$ cell populations, there were few cells expressing the LDL receptor at rest and relatively equivalent induction of expression 48 hours after stimulation with cytokines, corresponding to the time for transduction. It is therefore unlikely, that higher expression of the receptor for the VSV-G pseudotyped vector is the mechanism of increased transduction of $CD34^+/CD38^-$ cells.

When both cell populations were transduced with the CCL-β^{AS3}-FB LV vector pseudotyped with the RD-114 retroviral envelope protein, there was again higher transduction of the $CD34^+/CD38^-$ cells compared to $CD34^+$ cells. These results reinforce the observation of increased susceptibility to transduction of $CD34^+/CD38^-$ cells despite use of a different envelope protein (21-23).

Potential limitations to applying this extended HSC enrichment methods for clinical purposes would be the ability to isolate $CD34^+/CD38^-$ cells with sufficient efficiency to maintain engraftment capacity. Since these preliminary data are based on small scale experiments using CB, large scale experiments will also need to be performed using BM samples, as that is the intended clinical HSC source for gene therapy of SCD due to the contra-indication to use of G-

CSF to mobilize peripheral blood stem cells in patients with SCD. The efficiency of $CD34^+/CD38^-$ cell enrichment and transduction can then be assessed to help determine the feasibility of using these cells for transplantation into patients. Further studies to determine the mechanism of increased VCN of $CD34^+/CD38^-$ cells also need to be performed, to better delineate the cell differences affecting transduction using LV vectors. Additionally, there is not currently a GMP approved anti-CD38 antibody available to use for immunomagnetic column isolation of these cells, although a monoclonal antibody to CD38 is under study for systemic administration for the treatment of chronic lymphocytic leukemia (35,36); this type of reagent would need to be developed if these cells are to be used in clinical practice. In all, our results in human CB $CD34^+/CD38^-$ cell may improve not only the efficacy of transduction with the CCL-β^{AS3}-FB-LV vector, but also improve the logistics of transducing large numbers of stem cells for the treatment of older pediatric and adult patients with sickle cell and other blood cell disease.

Acknowledgements

This work was supported by The National Institutes of Health [2P01 HL073104 (DBK); T32 HL6699201 {Medical Hematology Training Grant (KMB); and T32 HL08634506 {Developmental Hematology Training Grant} (KMB)], the California Institute for Regenerative Medicine (CIRM DR1-01452) along with generous donations from the Hina Patel Foundation. The UCLA Broad Stem Cell Research Center Flow Cytometry Core supported FACS analyses and sorting. We thank the cord blood donors, the nurses who collected the samples, Rebecca Chan who processed the cord blood samples to $CD34^+$ cells, as well as Michelle Ho who prepared and provided the lentiviral vectors used in the experiments and Xiaoyan Wang and Chi-Hong Wang provided the statistical analyses.

The authors have no financial conflicts of interest related to this work.

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Table 1. *In vitro* vector copy number of cells transplanted into NSG mice

Transplant #	1	2	3
Experimental Arm	***In Vitro* Vector Copy Number**		
Non-Transduced CD34+	0	0	0
Transduced CD34+	-	1.58	2.81
Transduced CD34+/CD38+	1.48	0.40	2.66
Transduced CD34+/CD38-	1.94	7.59	11.50

Figure Legends

Figure 1. Isolation and growth properties of human $CD34^+$ and $CD34^+/CD38^-$ cells. (A) Flow cytometry of CD34-enriched cells showing gating strategy used to define $CD34^+/CD38^+$ cells (Region P5) and $CD34^+/CD38^-$ cells (Region P3). (B) Cell expansion from $CD34^+$ and $CD34^+/CD38^-$ cells from CB. Cells were co-cultured with irradiated MS5 stromal cells in long term culture medium. The mean fold increase over cell number plated on day 0 is shown at each time point of long-term culture. Data represent cell expansion ± SEM over time (n=3, $p<0.0001$).

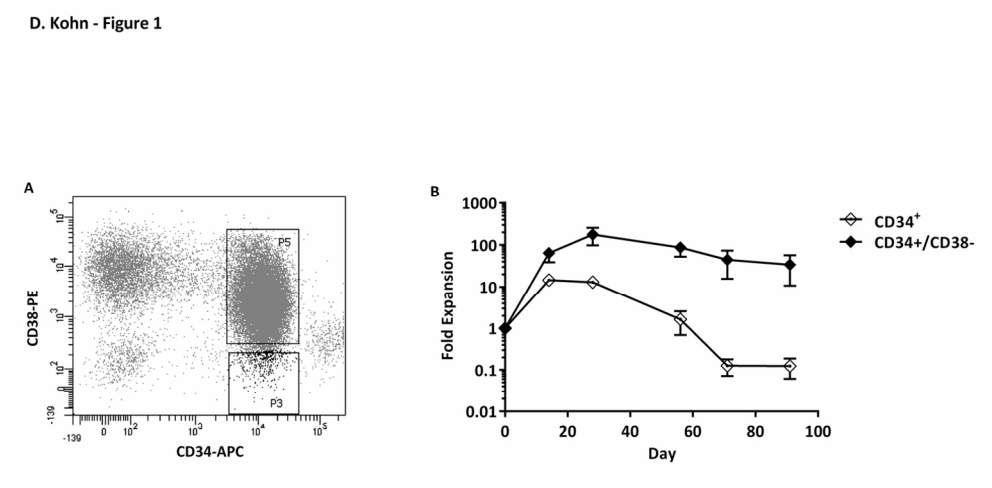
Figure 2. Analysis of transduction of $CD34^+$ and $CD34^+/CD38^-$ cells with the CCL-β^{AS3}-FB LV vector. (A) Vector copy number (VCN) ± SEM in transduced $CD34^+$ and $CD34^+/CD38^-$ cells (n=9, p=0.02). **(B)** Distribution of hematopoietic colony types (n=80 colonies) formed by non-transduced CB $CD34^+$ (NT-$CD34^+$), transduced $CD34^+$ ($CD34^+$) and $CD34^+/CD38^-$ cells. **(C)** Percentage of plated NT-$CD34^+$, $CD34^+$ and $CD34^+/CD38^-$ cells that grew into hematopoietic colonies *in vitro*. Values represent the mean ± SD. **(D)** Single CFU grown from transduced $CD34^+$ (left) and $CD34^+/CD38^-$ (right) CB cells were analyzed for VCN by ddPCR (n=80 colonies). Graph indicates percentages of the CFU that were negative for vector by digital PCR (0 VC/cell) or that had VC/cell of 1-2, 3-4, 5-6 or >6. **(E)** Vector transduction dose-response for $CD34^+$ and $CD34^+/CD38^-$ cells (n=3, p=0.05 at $6.6x10^6$ TU/ml, p=0.002 at $2x10^7$ TU/ml). (F) VCN over time in long-term culture [± SEM (n=3)] (time trend difference p=0.03, VCN difference p=0.004, linear mixed model). Asterisk indicates significance, *$p\leq 0.05$, **$p\leq 0.01$.

Figure 3. Analysis of transduction of $CD34^+$ and $CD34^+/CD38^-$ cells by the CCL-MND-GFP LV vector. (A) Comparison of mean VCN± SEM after transduction with a dose range of CCL-MND-GFP LV analyzed by qPCR at day 14 of culture. **(B)** Representative histogram showing relative GFP expression of transduced $CD34^+$ and $CD34^+/CD38^-$ cells. (C) Percentages of GFP+ cells determined by flow cytometry in CCL-MND-GFP-transduced $CD34^+$ and $CD34^+/CD38^-$ cells (n=6, p=0.02).

Figure 4. **Erythroid differentiation of $CD34^+$ and $CD34^+/CD38^-$ cells transduced by the CCL-β^{AS3}-FB LV vector. (A)** Comparison of VCN ± SEM during differentiation, at day 14 after transduction (n=3). **(B)** Percentage of HBBAS3 mRNA expression of all β-globin transcripts per vector copy (VCN) (%AS3/VCN) in erythroid cells differentiated from transduced $CD34^+$ and $CD34^+/CD38^-$ cells analyzed by qRT-PCR (n=3).

Figure 5. **Role of vector envelope and receptor on transduction by the CCL-β^{AS3}-FB LV vector.** Low-density lipoprotein receptor (LDL-R) expression by $CD34^+$ and $CD34^+/CD38^-$ cells on: **(A)** Freshly isolated $CD34^+$ cells, **(B)** $CD34^+$ cells at 48 hours of culture in cytokines, **(C)** Freshly isolated $CD34^+/CD38^-$ cells, and **(D)** $CD34^+/CD38^-$ cells at 48 hours of culture in cytokines. **(E)** Transduction of $CD34^+$ and $CD34^+/CD38^-$ cells with the RD114 pseudotyped CCL-β^{AS3}-FB LV vector. The graph represents the mean VCN of $CD34^+$ and $CD34^+/CD38^-$ cells ± SEM analyzed by qPCR at day 14 of culture (n=3).

Figure 6. **Comparison of engraftment of NSG mice. (A)** Contribution to human $CD45^+$ cell engraftment in NSG mice by transduced, transplanted cell populations. Mock mice were transplanted with non-transduced human CB $CD34^+$ cells; control mice were transplanted with transduced $CD34^+$ cells; all other mice were transplanted with a combination of $CD34^+/CD38^-$ (1%) and $CD34^+/CD38^+$ cells (99%). Vectors used for transduction (CCLc-UBC-mStrawberry-FB, CCLc-UBC-mCitrine-FB and CCLc-UBC-mCerulean-FB LV) were alternated among the cell populations for each transplant. BM harvested from NSG mice with human cell engraftment (%huCD45$^+$/%huCD45$^+$+muCD45$^+$ cells) was further analyzed for percent vector expression using flow cytometry. **(B)** VCN of cells analyzed *in vivo* mouse transplantation. *In vivo* VCN was analyzed from BM harvested 80-90 days after transplantation into NSG mice using ddPCR with primers and probes specific to each fluorescent reporter. The *in vivo* VCN/mouse for each population of cells is displayed separately.



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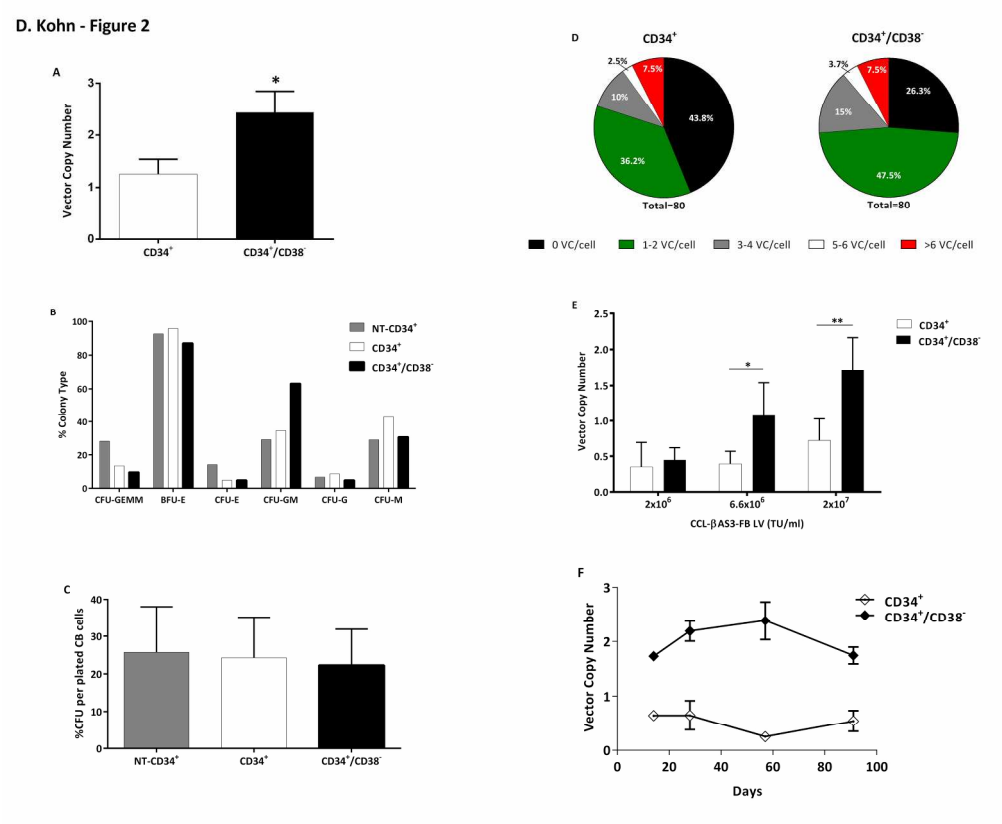

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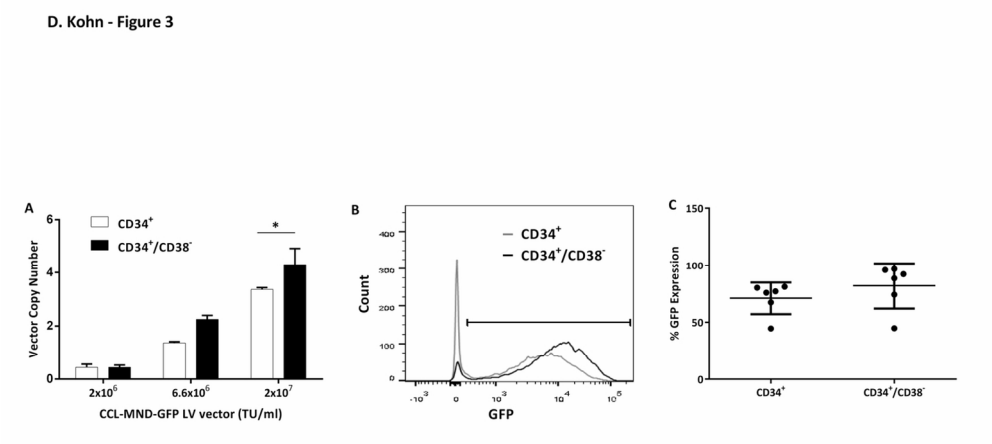

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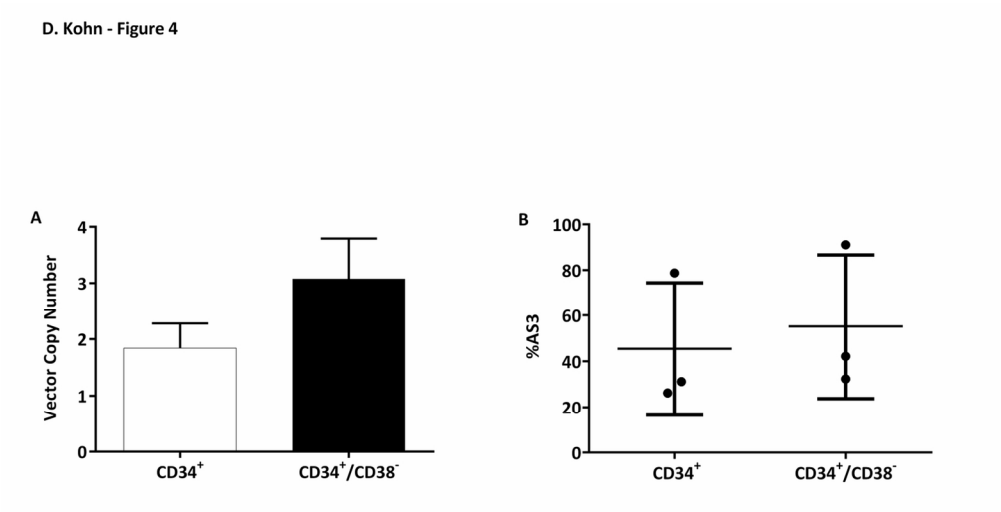

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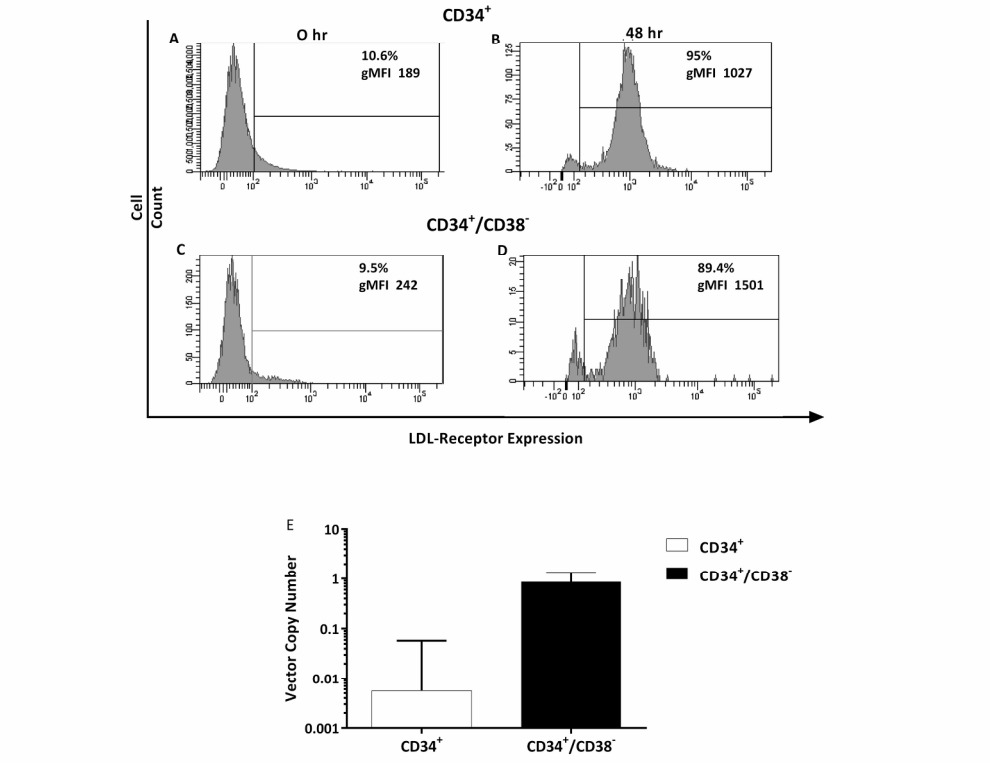
D. Kohn - Figure 5



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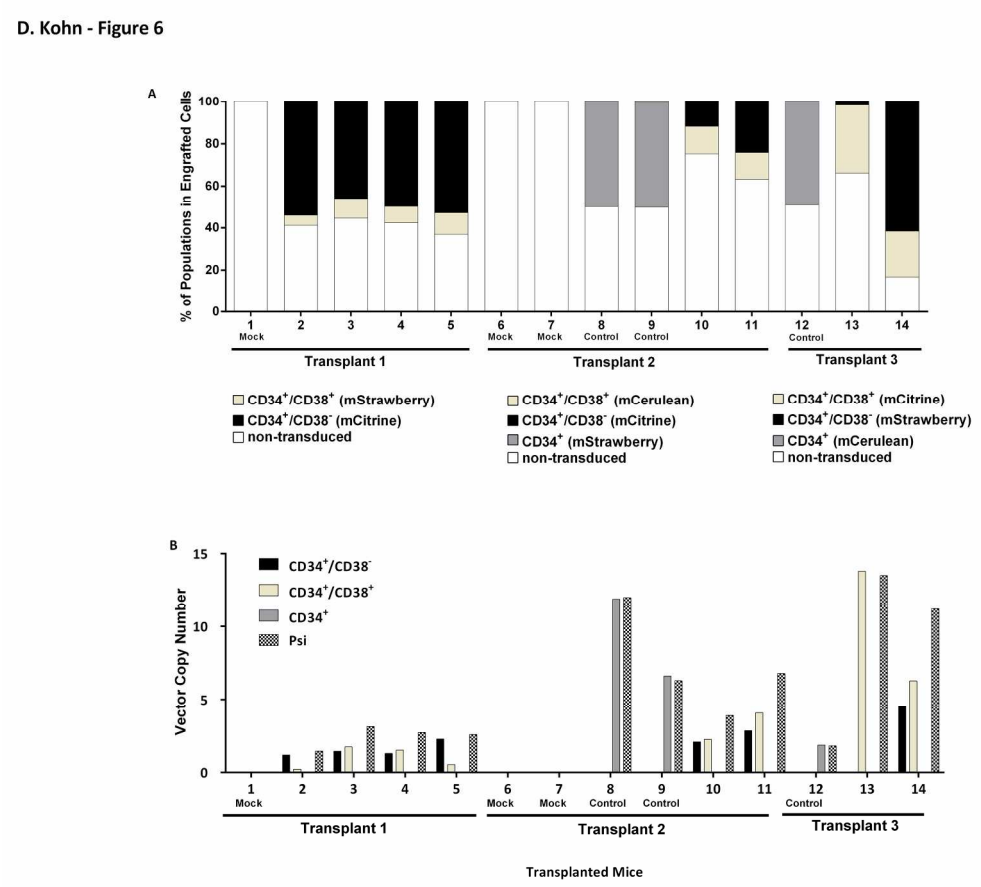
D. Kohn - Figure 6



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Supplemental Table 1. PCR primers

SDC4	
forward primer	5' CAGGGTCTGGGAGCCAAGT 3'
reverse primer	5' GCACAGTGCTGGACATTGACA 3'
Probe	5' HEX-CCCACCGAA-ZEN-CCCAAGAAACTAGAGGAGAAT-IBFQ* 3'
Psi U5	
forward primer	5' AAGTAGTGTGTGCCCGTCTG 3'
reverse primer	5' CCTCTGGTTTCCCTTTCGCT 3'
Probe	5' FAM-CCCTCAGAC-ZEN-CCTTTTAGTCAGTGTGGAAAATCTCTAG-IBFQ 3'
mCerulean	
forward primer	5' GACCACCCTGACCTGG3'
reverse primer	5' CGCTCCTGGACGTAGCCTT3'
Probe	5' FAM- AGCACGACT-ZEN-TCTTCAAGTCCGCCAT-IBFQ 3'
mStrawberry	
forward primer	5' TCAAGACCACCTACAAGGCCAAGA 3'
reverse primer	5' ACAGTTCCACGATGGTGTAGTCCT 3'
Probe	5' FAM- ATCGTCGGC-ZEN-ATCAAGTTGGACATCACCT-IBFQ 3'
mCitrine	
forward primer	5' TTCGGCTACGGCCTGATG T 3'
reverse primer	5' CGCTCCTGGACGTAGCCTT 3'
Probe	5' FAM- AGCACGACT-ZEN-TCTTCAAGTCCGCCAT-IBFQ 3'

*IBFQ=Iowa Black® FQ, ZEN=internal modification from IDT-Integrated DNA Technologies

Supplemental Table 2. Vector copy number of transduced cells after 14 day culture.

		Vector Copy Number in Transduced Cell Populations	
	Experiment Number	**$CD34^+$**	**$CD34^+/CD38^-$**
VSV-G Pseudotype	1	3.22	3.52
	2	0.98	2.50
	3	0.96	2.07
	4	0.63	1.81
	5	0.66	1.64
	6	1.54	3.40
	7	1.40	1.02
	8	0.36	1.16
	9	1.03	1.75
	10	1.612	4.110
	11	1.56	2.03
RD114 Pseudotype	1	0.002	1.5
	2	0.015	0.87
	3	0.0008	0.22

Supplemental Table 3. Experimental design of xenograft transplantation studies – cell doses and fluorescent markers.

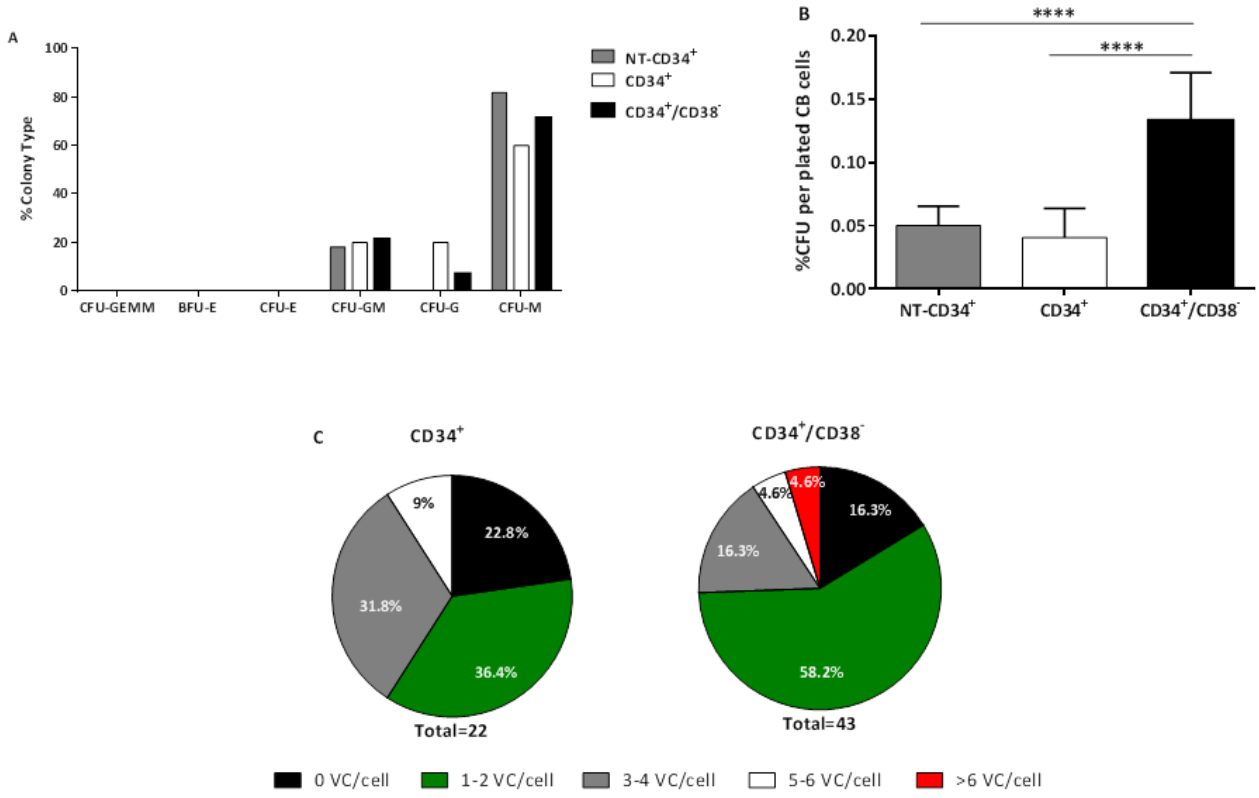
	Transplant 1		**Transplant 2**			**Transplant 3**	
Experimental Arm	**Mock**	**Test**	**Mock**	**Control**	**Test**	**Control**	**Test**
Mouse #'s	1	2,3,4,5	6,7	8,9	10,11	12	13,14
NT* $CD34^+$	2x10^5		2x10^5				
TD** $CD34^+$				2x10^5 (mStrawberry)		2x10^5 (mCerulean)	
TD $CD34^+/CD38^+$		2x10^5 (mStrawberry)			2X10^5 (mCerulean)		2x10^5 (mCitrine)
TD $CD34^+/CD38^-$		2x10^3 (mCitrine)			2x10^3 (mCitrine)		2x10^3 (mStrawberry)
NT $CD34^-$ (irradiated)	3x10^5	3x10^5	3x10^5	3x10^5	3x10^5	3x10^5	3x10^5

*NT = not transduced; **TD=transduced

Supplemental Table 4. Digital droplet PCR (ddPCR) of bone marrow from NSG mice for each specific fluorescent marker gene used to mark individual transplanted cell populations and for total vector proviruses (Psi), normalized to human genomes in each marrow samples.

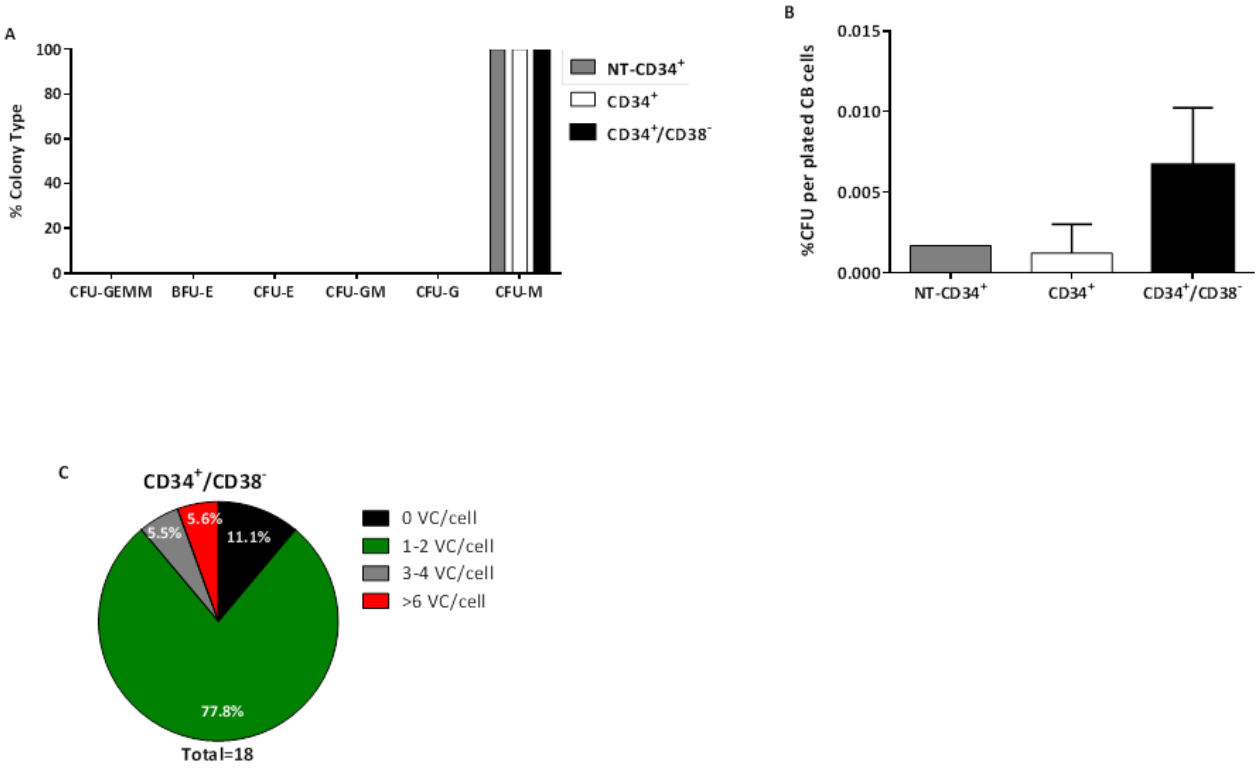
	Transplant Arm	Fluorescent Marker(s)	Fluorescent Gene Specific ddPCR				Total Vector ddPCR	p value*
Mouse #			**34+**	**34+/38-**	**34+/38+**	**Sum (38- + 38+)**	**(Psi)**	
8	Bulk CD34+	mStrawberry	11.9				12	
9	Bulk CD34+	mStrawberry	6.61				6.29	
12	Bulk CD34+	mCerulean	1.86				1.81	
Mean +/- S.D.			6.79 +/- 5.02				6.70 +/- 5.11	0.98
2	34+/38- vs. 34+/38+	mCitrine vs. mStrawberry		1.19	0.215	1.405	1.45	
3	34+/38- vs. 34+/38+	mCitrine vs. mStrawberry		1.44	1.74	3.18	3.12	
4	34+/38- vs. 34+/38+	mCitrine vs. mStrawberry		1.29	1.52	2.81	2.72	
5	34+/38- vs. 34+/38+	mCitrine vs. mStrawberry		2.27	0.54	2.81	2.58	
10	34+/38- vs. 34+/38+	mCitrine vs. mCerulean		2.08	2.25	4.33	3.88	
11	34+/38- vs. 34+/38+	mCitrine vs. mCerulean		2.84	4.06	6.9	6.79	
13	34+/38- vs. 34+/38+	mStrawberry vs. mCitrine		0	13.8	13.8	13.5	
14	34+/38- vs. 34+/38+	mStrawberry vs. mCitrine		4.49	6.28	10.77	11.2	
Mean +/- S.D.						5.76 +/- 4.41	5.66 +/- 4.45	0.97
Mean +/- S.D.				1.95 +/- 1.33	3.8 +/- 4.5			0.3

*By unpaired T test.



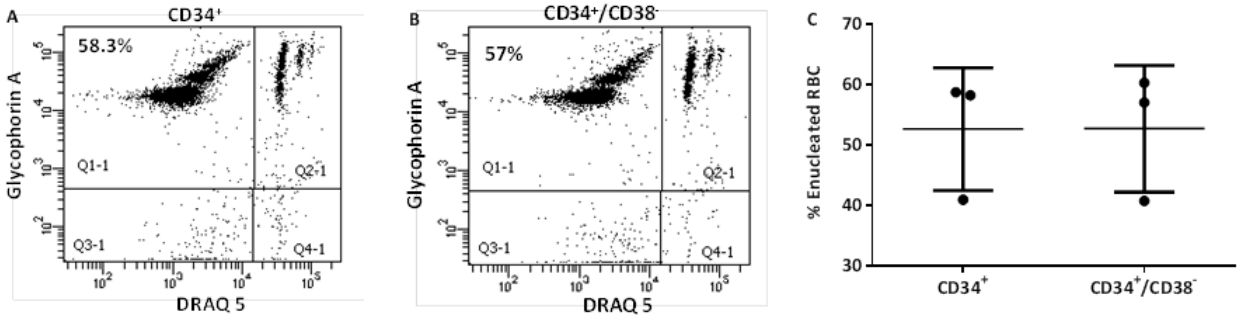
Supplemental Figure 1. Analysis of CD34$^+$ and CD34$^+$/CD38$^-$ cells transduction with the CCL-β^{AS3}-FB LV vector and hematopoietic potential at day 30 of long-term culture.

(A) Distribution of hematopoietic colony types formed by non-transduced (NT)-CD34$^+$ cells (n=37 colonies), transduced CD34$^+$ cells (n=29 colonies) and CD34$^+$/CD38$^-$ cells (n=81 colonies). **(B)** Percentage of plated NT-CD34$^+$, transduced CD34$^+$ and CD34$^+$/CD38$^-$ cell that grew into hematopoietic colonies *in vitro*. Values represent the mean ± SD; asterisk indicates significance, ****p≤ 0.0001. **(C)** VCN distribution of *in vitro* single CFU grown from transduced CD34$^+$ analyzed by ddPCR (n=22 colonies). Graph indicates percentages of the CFU that were negative for vector (0 VC/cell) or that had VC/cell of 1-2, 3-4, 5-6 or > 6. VCN distribution for in vitro CFU grown from transduced CD34$^+$/CD38$^-$ cells (n=43 colonies).

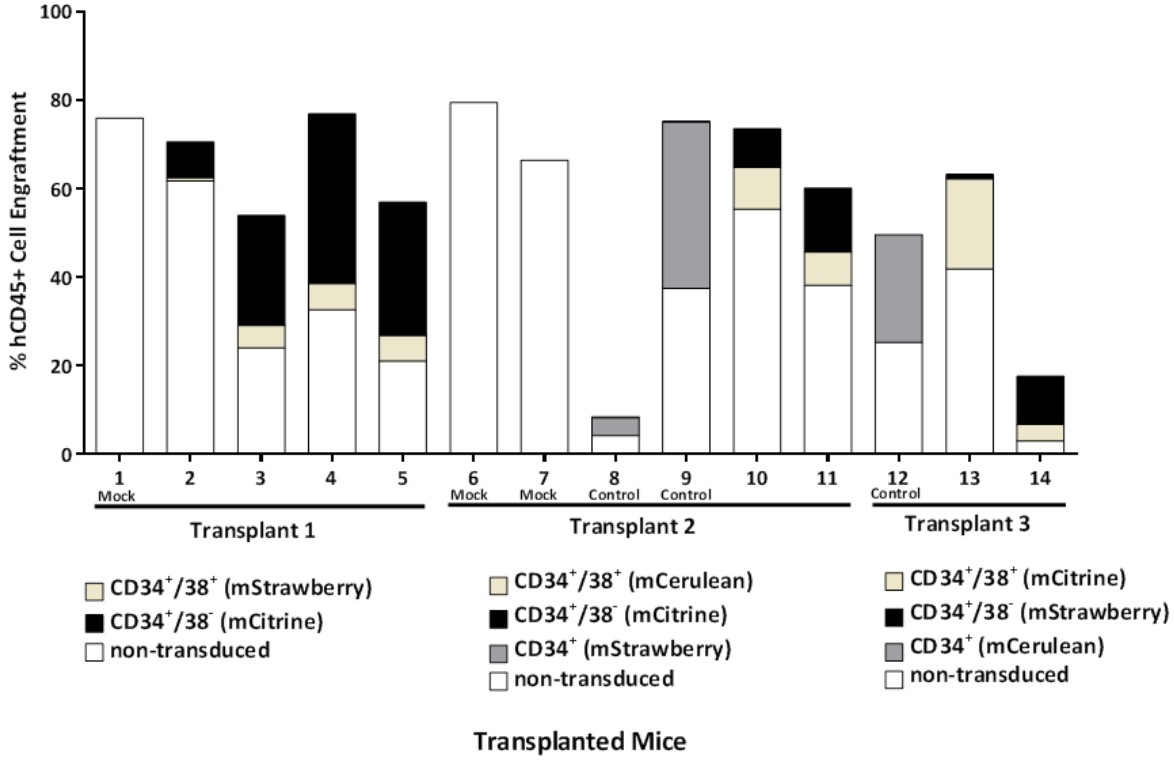


Supplemental Figure 2. Analysis of CD34$^+$ and CD34$^+$/CD38$^-$ cells transduction with the CCL-β^{AS3}-FB LV vector and hematopoietic potential at day 60 of long-term culture.

(A) Distribution of hematopoietic colony types formed by non-transduced (NT)-CD34$^+$ cells (n=5 colonies), transduced CD34$^+$ cells (n=3 colonies) and CD34$^+$/CD38$^-$ cells (n=22 colonies). **(B)** Percentage of plated NT-CD34$^+$, transduced CD34$^+$ and CD34$^+$/CD38$^-$ cell that grew into hematopoietic colonies *in vitro*. **(C)** VCN distribution of *in vitro* single CFU grown from transduced CD34$^+$/CD38$^-$ cells analyzed by ddPCR (n=18 colonies). Graph indicates percentages of the CFU that were negative for vector (0 VC/cell) or that had VC/cell of 1-2, 3-4, 5-6 or > 6.



Supplemental Figure 3. Erythroid differentiation of $CD34^+$ and $CD34^+/CD38^-$ cells transduced by the CCL-β^{AS3}-FB LV vector. Flow cytometry analysis of cells from erythroid cultures from (**A**) unfractionated CD34+ cell and **(B)** $CD34^+/CD38^-$ cells. Enucleated erythrocytes are present in the left upper quadrant as DRAQ5 negative, glycophorin A (GpA) positive cells. **(C)** Percentage of enucleated RBC at the end of erythroid differentiation.



Supplemental Figure 4. Contribution to total human engraftment in NSG mice by transduced, transplanted cell populations. Mock mice were transplanted with non-transduced human CB $CD34^+$ cells; control mice were transplanted with transduced $CD34^+$ cells; all other mice were transplanted with a combination of $CD34^+/CD38^-$ (1%) and $CD34^+/CD38^+$ cells (99%). Vectors used for transduction (CCLc-UBC-mStrawberry-FB, CCLc-UBC-mCitrine-FB and CCLc-UBC-mCerulean-FB LV) were alternated among the cell populations for each transplant. BM harvested from NSG mice with human cell engraftment ($\%huCD45^+/\%huCD45^+ + muCD45^+$ cells) was further analyzed for percent vector expression using flow cytometry.