RUNX3 levels in human hematopoietic progenitors are regulated by aging and dictate erythroid-myeloid balance

Peter Balogh,[1] Emmalee R. Adelman,[2] John V. Pluvinage,[3] Brian J. Capaldo,[4] Katie C. Freeman,[1] Sandeep Singh,[1] Kamaleldin E. Elagib,[1] Yukio Nakamura,[5] Ryo Kurita,[6] Goro Sashida,[7] Eli R. Zunder,[8] Hui Li,[1] Alejandro A. Gru,[1] Elizabeth A. Price,[9] Stanley L. Schrier,[9] Irving L. Weissman,[10] Maria E. Figueroa,[2] Wendy W. Pang,[11]* and Adam N. Goldfarb[1]*

[1]Department of Pathology, University of Virginia School of Medicine, Charlottesville, USA; [2]Sylvester Comprehensive Cancer Center, University of Miami Health System, Miami, Florida, USA; [3]Department of Medicine, Stanford University, Stanford, California, USA; [4]Flow Cytometry Core Facility, University of Virginia School of Medicine, Charlottesville, Virginia, USA; [5]Cell Engineering Division, RIKEN BioResource Center, Tsukuba, Ibaraki, Japan; [6]Research and Development Department, Central Blood Institute, Blood Service Headquarters, Japanese Red Cross Society, Tatsumi, Koto-ku, Tokyo, Japan; [7]Laboratory of Transcriptional Regulation in Leukemogenesis IRCMS, Kumamoto University, Chuo-ku, Kumamoto, Japan; [8]Department of Biomedical Engineering, University of Virginia, Charlottesville, Virginia, USA; [9]Department of Medicine, Division of Hematology, Stanford University, Stanford, California, USA; [10]Institute for Stem Cell Biology and Regenerative Medicine, Stanford University, Stanford, California, USA and [11]Department of Medicine, Division of Blood and Bone Marrow Transplantation, Stanford University, Stanford, California, USA

**WWP and ANG contributed equally to this work*

©2020 Ferrata Storti Foundation. This is an open-access paper. doi:10.3324/haematol.2018.208918

Received: October 19, 2018.

Accepted: June 5, 2019.

Pre-published: June 6, 2019.

Correspondence: *ADAM N. GOLDFARB* -ang3x@virginia.edu

CRITICAL REAGENTS AND RESOURCES

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
RUNX1	Cell Signaling Technology	4334S
RUNX2	Cell Signaling Technology	12556S
RUNX3	Cell Signaling Technology	9647S
Tubulin	Sigma Aldrich	T9026-.2ML
RUNX3	R&D Systems	MAD3765
Goat anti-mouse secondary	Thermo Fisher Scientific	A-11003
CD235a (FITC)	Invitrogen	11-9987-82
CD235a (PE)	BD Pharmingen	555570
CD41a (APC)	BD Pharmingen	559777
CD15 (FITC)	BioLegend	301903
CD36 (APC)	BD Biosciences	561822
CD71 (APC)	BD Pharmingen	551374
CD41a (89 Yb)	Fluidigm	3089004B
CD117 (143 Nd)	Fluidigm	3143001B
CD123 (151 Eu)	Fluidigm	3151001B
CD45RA (153 Eu)	Fluidigm	3153001B
CD36 (155 Gd)	Fluidigm	3155012B
Ki-67 (162 Dy)	Fluidigm	3162012B
CD235a (163 Dy)	Fluidigm	3163008B
CD34 (166 Er)	Fluidigm	3166012B
CD38 (172 Yb)	Fluidigm	3172007B
CD71 (175 Lu)	Fluidigm	3175011B
CD11b (209 Bi)	Fluidigm	3209003B
RUNX3 (148 Nd)	This study	R&D Systems RUNX3 antibody (MAB3765); Fluidigm Maxpar X8 antibody labeling kit (201148)
Cleaved Caspase-3 (173 Yb)	This study	BD Biosciences cleaved Caspase-3 antibody (559565); Fluidigm Maxpar X8 antibody labeling kit (201173)
Biological Samples		
Adult PBSC CD34 (1 million)	Fred Hutchinson Cancer Research Center	N/A
Adult PBSC CD34 (5 million)	Fred Hutchinson Cancer Research Center	N/A
Chemicals, Peptides, and Recombinant Proteins		
TPO	PeproTech	300-18-50UG
SCF	PeproTech	300-07-50UG
Flt3-ligand	PeproTech	300-19-50UG
IL-3	PeproTech	200-03-10UG
G-CSF	PeproTech	300-23-10UG
SDF1a	PeproTech	300-28A-50UG
IL-6	PeproTech	200-06-5UG
EPO	Procrit, Janssen	609-10-98-5

Doxycycline	Sigma Aldrich	D3447-500MG
Dexamethasone	Sigma Aldrich	D2915
Fibronectin	Corning	354008
Retronectin	Takara	T100B
Puromycin	Sigma Aldrich	P8833-10MG
Saponin	Sigma Aldrich	S7900
Sodium Azide	Sigma Aldrich	S2002
IC (Intracellular) Fixation Buffer	eBioscience	00-8222-49
Paraformaldehyde, 16% Solution, EM grade	Electron Microscopy Sciences	15710
Cisplatin	Sigma Aldrich	P4394
Cell-ID Intercalator-Ir—125 µM	Fluidigm	201192A
Commercial Assays		
RNeasy Plus Mini Kit	QIAGEN	74134
PKH26 Red Fluorescent Cell Linker Kit	Sigma Aldrich	MINI26-1KT
CalPhos Mammalian Transfection Kit	Clontech	631312
Zombie Violet Fixable Viability Kit	BD Biolegend	423113
Deposited Data		
RNA-seq	This study	GSE119264, GSE104406
Microarray	This study	GSE123991
Experimental Models: Cell Lines		
HEK293T	ATCC	CRL-3216
HUDEP-2	Kurita, et al. 2013	N/A
Recombinant DNA		
TRC RUNX3 shRNA (TRCN0000013668, TRCN0000013670, TRCN0000013671, TRCN0000013672)	GE Dharmacon	RHS4533-EG864
pLKO.1 plasmid	GE Dharmacon	RHS4080
pCMV-dR8.2 dvpr	Addgene	8455
pMD2.G	Addgene	12259
Software and Algorithms		
Trimmomatic	Bolger et al., 2014.	usegalaxy.org
HISAT2	Kim et al., 2015.	usegalaxy.org
RmDup	Li et al., 2009; Li et al., 2011; Li et al., 2011; Danecek et al.; Durbin et al.; Li et al.	usegalaxy.org
featureCounts	Liao et al., 2013.	usegalaxy.org
DESeq2	Love et al., 2014.	usegalaxy.org
The Synergizer	Roth laboratory	http://llama.mshri.on.ca/synergizer/translate/
Cufflinks	Cole et al., 2010.	usegalaxy.org
FlowSOM	Van Gassen et al. 2017.	bioconductor.org
flowType	Aghaeepour et al. 2014.	bioconductor.org

cydar	Lun 2017.	bioconductor.org
edgeR	Robinson et al. 2010; McCarthy et al. 2012.	bioconductor.org
Enrichment Analysis	Ashburner et al. Gene ontology: tool for the unification of biology (2000) Nat Genet 25(1):25-9. GO Consortium, Nucleic Acids Res., 2017	geneontology.org
Single cell RNA-seq Gene Visualizer	Tusi et al. 2018; Klein laboratory	https://kleintools.hms.harvard.edu/paper_websites/tusi_et_al/

CONTACT FOR REAGENT AND RESOURCE SHARING

Please contact Dr. Adam Goldfarb at ang3x@virginia.edu for all reagent and resource inquiries.

METHODS

Cell Culture

Cryopreserved primary human adult G-CSF-mobilized CD34+ cells were purchased from Fred Hutchinson Cancer Research Center. Cells were thawed and expanded 2-3 days in medium composed of IMDM (GIBCO BRL) supplemented with BIT 9500 (Stem Cell Technologies), and the following cytokines: 100 ng/ml each of rhTPO, rhSCF, and rhFlt3-l, plus 10 ng/ml rhIL-3 (all from PeproTech). After expansion, cells were moved into unilineage media, and cultured from 1-8 days. Erythroid medium is composed of IMDM supplemented with BIT 9500, and the following cytokines: 4.5 U/ml rhEPO (Procrit, Janssen) and 25 ng/ml rhSCF (PeproTech). Megakaryocyte medium is composed of IMDM supplemented with BIT 9500, and the following cytokines: 40 ng/ml rhTPO, 25 ng/ml rhSCF, and 20 ng/ml rhSDF1-alpha (PeproTech). Megakaryocyte cultures utilized fibronectin-coated plates, which were prepared by incubating plates at room temperature for one hour with IMDM containing 20 µg/ml fibronectin (Becton Dickinson). Granulocyte medium is composed of IMDM supplemented with BIT 9500, and the following cytokines: 25 ng/ml rhSCF, 10 ng/ml rhIL-3, and 20 ng/ml rhG-CSF (PeproTech). For colony formation assays, 3,000 undifferentiated CD34+ cells were seeded into MethoCult SF H4236 (Stem Cell Technologies) supplemented with the following cytokines: 50 ng/ml rhSCF, 10 ng/ml rhIL-3, 20 ng/ml rhIL-6 (PeproTech), 3 U/ml rhEPO, 20 ng/ml rhG-CSF, and 10 ng/ml rhGM-CSF. Cells were cultured for 10 days, followed by colony scoring and counting.

HUDEP-2 cells (Kurita, et al. 2013) were maintained in expansion medium composed of StemSpan (Stem Cell Technologies) supplemented with 50 ng/ml rhSCF, 3 U/ml rhEPO, 1 µM dexamethasone (Sigma-Aldrich), and 1 µg/ml doxycycline (Sigma-Aldrich). HUDEP-2 differentiation induction was achieved by culturing the cells for 48 hours in doxycycline-free medium. HEK293T cells (ATCC) were grown in DMEM (GIBCO BRL) supplemented with 2 mM L-glutamine, antibiotic-antimycotic (Thermo Fisher Scientific), and 10% FBS (GIBCO BRL).

Transfection and Transduction

For production of lentivirus, pCMV-dR8.74 (GAG POL TAT REV) and pMD2.G (VSV-G) were co-transfected into HEK293T with pLKO.1 shRNA vectors at a 3:1:4 mass ratio using the Clontech CalPhos mammalian transfection kit (Clontech 631312). pLKO.1 shRNA

plasmids were purchased from GE Dharmacon. After 12-16 hours, the transfection medium was replaced with Opti-MEM I (Thermo Fisher Scientific), and viral supernatant was collected 36 hours later, passed through a 0.45 µm filter, and stored at -80°C. HUDEP-2 cells were transduced via incubation for 24 hours in a 1:1 mixture of viral supernatant and StemSpan, supplemented with 50 ng/ml rhSCF, 3 U/ml rhEPO, 1 µM dexamethasone, and 1 µg/ml doxycycline. Transduced cells were selected by adding 2 µg/ml puromycin (Sigma Aldrich) for 72 hours. CD34+ cells, pre-expanded for 2 days, were transduced over two additional days in a 1:1 combination of viral supernatant and IMDM, supplemented with 100 ng/ml each of rhTPO, rhSCF, and rhFlt3-l, plus 10 ng/ml rhIL-3. These cultures were seeded onto retronectin-coated plates, prepared by pre-treatment with 40 µg/ml retronectin (Takara) in PBS at room temperature for 2 hours, followed by incubation with 2% Fraction V BSA (Sigma Aldrich) in PBS, and then a wash with 2.5% wt/wt HEPES in HBSS (GIBCO BRL). The transduction cultures were incubated at 37°C for 2 hours, spun at 500 rcf for 90 minutes at room temperature, then incubated at 37°C overnight. The spin procedure was repeated the following day with fresh viral medium. Cells were selected on the third day with 2 µg/ml puromycin until we observed total cell death in un-transduced control cells (a minimum of 24 hours), followed by unilineage cultures as described above.

Fluorescence Cytometry

Cells were washed once with PBS, followed by staining for 30 minutes on ice with PBS containing 1:500 Zombie Violet viability dye (BioLegend), and 3 µl fluorochrome-conjugated antibody per sample. After staining, cells were washed once with PBS, followed by fixation for 20 minutes on ice using IC Fixation Buffer (eBioscience). After fixation, cells were washed once with PBS with 1% FBS, and re-suspended PBS with 1% FBS. Cells were analyzed on a BD FACSCalibur, and data was analyzed using FlowJo version 8.8.7. For cell sorting, cells were stained as described above, re-suspended in 1% Fraction V BSA in PBS, and sorted into the serum-free expansion medium using a BD FACSAria Fusion. The PKH26 membrane staining was performed according to the manufacturer's protocol, and cells were cultured for 3 days before analysis.

Immunoblot

For lysis, cells were washed once with PBS, followed by resuspension in Laemmli buffer (60 mM Tris-HCl, pH 6.8, 2% SDS, 100 µM dithiothreitol, 10% glycerol, and 0.01% bromophenol blue) supplemented with cOmplete protease inhibitor cocktail (Roche Diagnostics 11836170001) and PhosSTOP phosphatase inhibitor cocktail (Roche Diagnostics 04906845001). Samples were passed through 27G needles to shear DNA, followed by 5-10 minutes incubation at 95°C. After SDS-polyacrylamide gel electrophoresis and Western transfer, nitrocellulose or PVDF membranes were blocked with 5% non-fat dried milk in Tris-buffered saline (50 mM Tris and 150 mM NaCl) with 0.1% Tween-20. For protein detection, primary antibodies were applied overnight at a dilution of 1:1000 at 4°C with shaking. HRP-conjugated secondary antibodies were applied for one hour at a dilution of 1:10,000 at room temperature with shaking. All antibodies were diluted in TBS with Tween-20 and 1% non-fat dried milk. HRP substrates consisted of Super Signal West Pico and Super Signal West Femto (Thermo Fisher Scientific). Immunoblot signal quantitation was performed using a BioRad GS-800 scanning densitometer.

Immunofluorescence

10^5 cells were cytospun onto glass slides, fixed for 15 minutes at room temperature with 4% paraformaldehyde, and permeabilized for one hour at room temperature with PBS containing 2% FBS, 2% BSA, and 0.03% Triton X-100. Mouse anti-human RUNX3 primary antibody (R&D Systems) was applied overnight at 20 μg/ml in staining buffer (PBS with 2% FBS, 2% BSA, and 0.03% Triton X-100) at 4°C. Goat anti-mouse Alexa Fluor 546 secondary antibody (Thermo Fisher) was applied at 1:300 in staining buffer at room temperature for one hour. The slides were washed three times with staining buffer and once with PBS before mounting coverslips with Vectashield medium (Vector Laboratories H-1000). Images were obtained with a Zeiss LSM-700 confocal microscope using the 63x objective, and image processing was performed with Fiji ImageJ v2 (National Institutes of Health).

Immunohistochemistry

Immunohistochemistry was performed using a robotic platform (Ventana discover Ultra Staining Module, Ventana Co., Tucson, AZ, USA) on paraffin-embedded bone marrow clots of young (<10 years old) and aged (60-70 years old) subjects obtained according to IRB-reviewed protocols. A heat-induced antigen retrieval protocol was carried out using a TRIS–ethylenediamine tetracetic acid (EDTA)–boric acid pH 8.4 buffer (Cell Conditioner 1), with a 64 minute setting. Endogenous peroxidases were blocked with peroxidase inhibitor (CM1) for 8 min before incubating the tissues with RUNX3 antibody (Abcam, Cat # Ab 135248) at a 1:800 dilution for 60 min at room temperature. Antigen-antibody complex was then detected using DISCOVERY anti-mouse HQ HRP detection system and DISCOVERY ChromoMap DAB Kit (Ventana Co.). All the slides were counterstained with hematoxylin subsequently; they were dehydrated, cleared and mounted for the assessment. Slides were imaged using a NonoZoomer S360 (Hamamatsu Corporation) and the final images were prepared using NDP.view2 Plus Image viewing software U12388-02.

Mass Cytometry

Cells were washed once with PBS, then stained for 60 seconds with 100 μM cisplatin (Sigma Aldrich P4394) in PBS. 1% FBS in PBS was promptly added to quench the staining. After washing, cells were fixed with 0.2 μm filtered 1.6% paraformaldehyde in PBS for 10 minutes at room temperature. 1% FBS in PBS was promptly added to quench fixing, and after washing, cells were stored at -80°C. Upon thawing, each sample was washed once with cell staining medium consisting of 0.5% Fraction V BSA (Sigma Aldrich), and 0.02% w/v sodium azide (Sigma Aldrich S2002) in PBS. Subsequently, cells were washed twice with 0.02% w/v saponin (Sigma Aldrich S7900) and 0.02% w/v sodium azide in PBS to transiently permeabilize them. Each sample was then separately stained with a unique barcoding combination of palladium isotopes, shaking at room temperature for 15 minutes followed by washing three times with cell staining medium. The barcoded samples were pooled and simultaneously stained for surface antigens for 30 minutes at room temperature with shaking. Antibodies to CD45RA, CD11b, CD117, CD36, and CD71 were used at a dilution of 1:800. Antibodies to CD34, CD38, CD123, CD41, and CD235a were used at 1:400. Cells were washed twice with cell staining medium, then permeabilized in -20°C methanol for 10 minutes on ice with intermittent shaking. Cells were washed once with cell staining medium, followed by intracellular staining for 1 hour at room temperature with shaking. The Ki-67 antibody was used at 1:400, and antibodies to RUNX3 and cleaved Caspase-3 were used at 0.25 μg/ml. After staining, cells were washed twice with cell staining medium, then incubated for at least 15 minutes at room temperature in intercalator buffer consisting of 0.2 μm filtered PBS/1.6%

paraformaldehyde with a 1:5,000 dilution of CellID Ir-Intercalator (Fluidigm 201192A). Cells were washed once with cell staining medium, once with 0.05% Tween-20 (Sigma Aldrich) in water, and finally re-suspended in water. The sample volume was adjusted with water and normalization beads (Fluidigm 201078) to achieve a cell concentration of approximately 0.5 x 10^6/mL. Data acquired using the Fluidigm CyTOF 2 was bead-normalized and underwent barcode deconvolution using the debarcoding tool MATLAB standalone executable from Zunder et al. Nature Protocols 2015.

Data was inverse hyberbolic sine-transformed using a cofactor of 0.25. FlowSOM (Van Gassen et al. 2017) was used to construct a self-organizing map with a number of grid points equal to the square of the number of lineage markers. Each cell was assigned a phenocode for every lineage marker using flowType (Aghaeepour et al. 2014). Each grid point was then immunophenotyped using the aggregated phenocodes of the cells assigned to the grid point. For any given grid point to be assigned an immunophenotype for a particular marker (i.e. CD45RA+), 75 percent of the cells assigned to the gridpoint had to be labelled with the same phenocode for the particular marker. For each immunophenotype observed, number of cells were tabulated to form a hierarchical count table. Every level of the hierarchy was tested for differential abundance between conditions using edgeR (Robinson et al. 2010; McCarthy et al. 2012) with a quasi-likelihood framework as specified by the cydar™ package (Lun 2017).

RNA-sequencing

Cells were washed with PBS, followed by RNA extraction using the QIAgen RNeasy Plus Mini Kit (QIAgen 74134), with added DNA digestion. Briefly, cells were lysed using buffer RLT supplemented with β-mercaptoethanol, and lysates were applied to the gDNA eliminator column. The flow-through was applied to the RNeasy spin column, and the column was washed once with buffer RW1. Freshly prepared DNase solution (QIAgen 79254) was applied to the column and incubated at room temperature for 10 minutes. The column was washed once with buffer RW1, and twice with buffer RPE. RNA was eluted with nuclease-free water, and the eluate was re-applied to the column for a second spin to ensure complete elution of the RNA. RNA yield was assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific). Samples were sequenced by the Genomic Services Laboratory at HudsonAlpha Institute for Biotechnology using ribosomal reduction, 100bp, paired-end, and 50 million read-depth parameters on an Illumina HiSeq 2500 machine.

Raw fastq.gz data files were merged (fastq Merger; gsl.hudsonalpha.org) and uploaded to usegalaxy.org. Trimmomatic was used to eliminate low quality sequences from the reads, followed by alignment to the hg19 human reference genome using HISAT2. RmDup was used to eliminate PCR duplicates from the resulting bam files. Differential gene expression was determined with DESeq2 applied to data converted by featureCounts. The data was also processed using Cufflinks to estimate FPKM for each sample, followed by Student's t-test to assess statistical significance for specific transcripts. The Synergizer tool was used to convert UCSC gene identifiers into hgnc gene symbols.

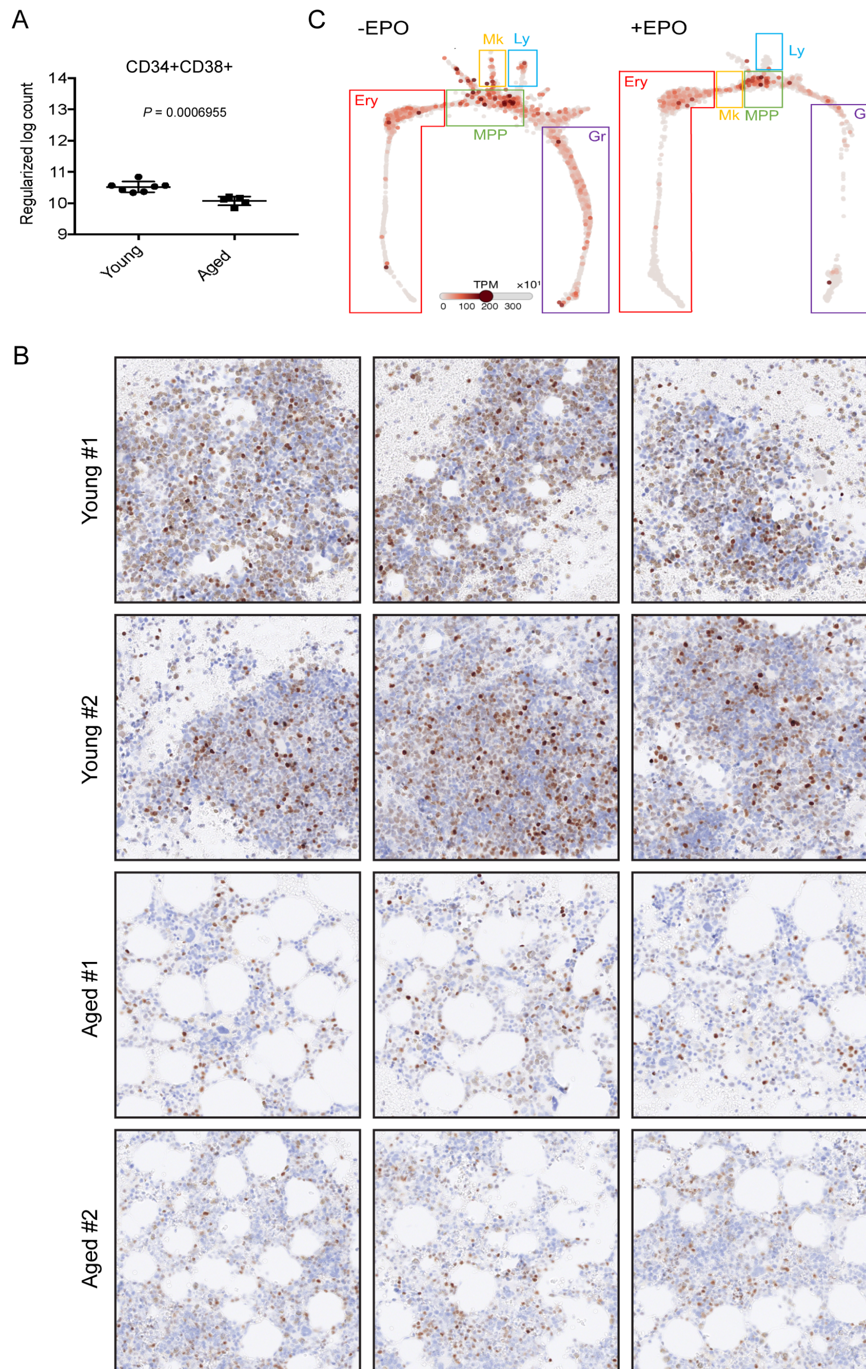
Unexplained Anemia of the Elderly Studies

Normal young, normal elderly, and UAE human bone marrow samples were obtained according to IRB-approved protocols. Mononuclear cells were prepared using Ficoll-Paque Plus (GE Healthcare), and stained with the appropriate antibodies and analyzed and sorted using a FACSAriaII cytometer (BD Biosciences). Cell types were defined as follows: HSC, Lin-CD34+CD38-CD90+ CD45RA-; MEP, Lin-CD34+CD38+CD123-CD45RA-; CMP, Lin-

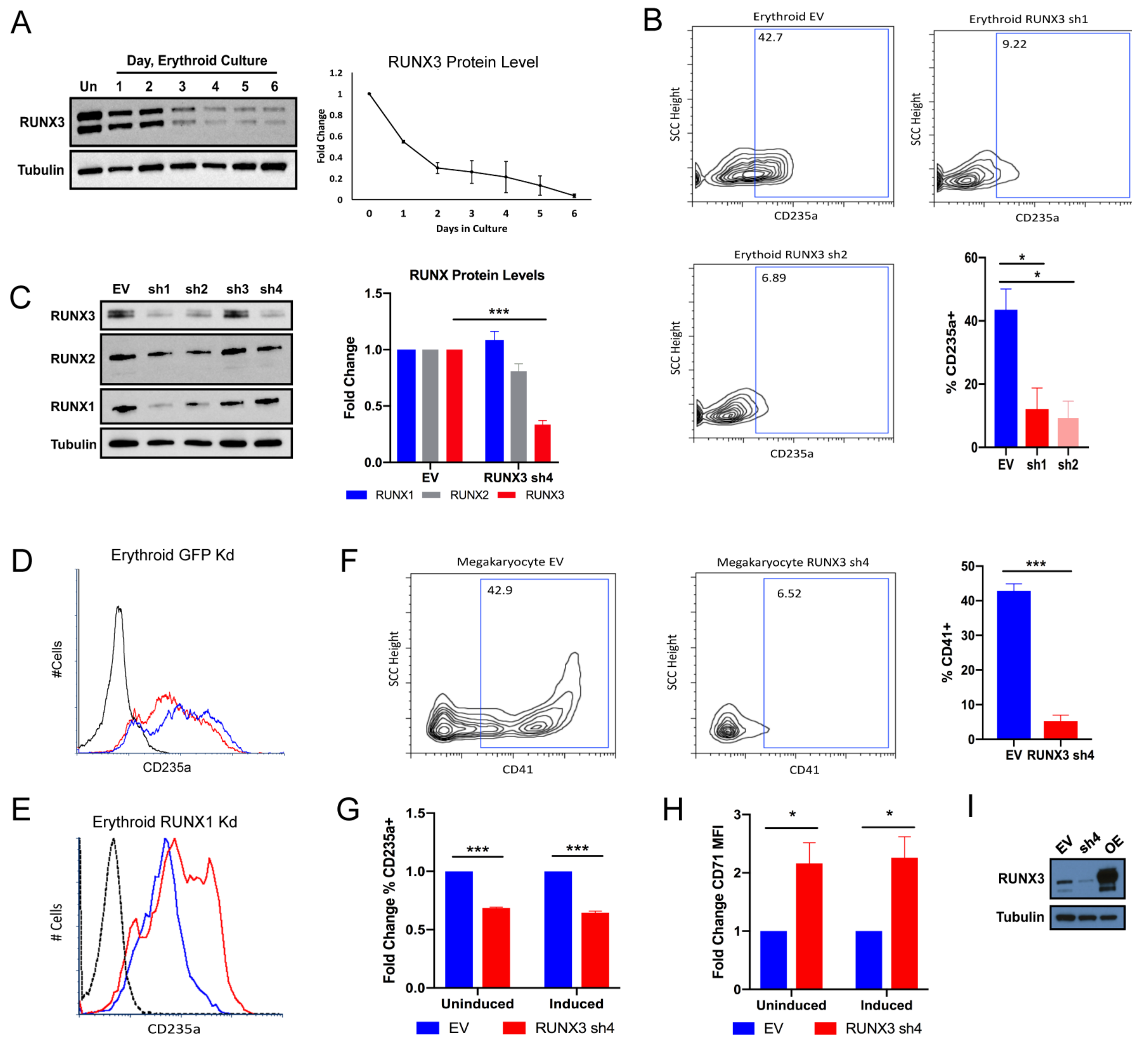
CD34+CD38+CD123+CD45RA-. Methylcellulose colony formation was assayed by sorting cells into individual wells of a 6-well plate, each containing 3 ml of complete methylcellulose (Methocult GF+ H4435, Stemcell Technologies). Plates were incubated for 12-14 days at 37°C, and colonies then scored based on morphology.

For microarray analysis, total RNA was extracted using TRIzol (Invitrogen) or Ambion RNA Isolation Kit (Applied Biosystems by Life Technologies) according to the manufacturer's protocols and treated with DNase I (Qiagen). All RNA samples were quantified with the RiboGreen RNA Quantitation Kit (Molecular Probes), subjected to re- verse transcription, two consecutive rounds of linear amplification, and production and fragmentation of biotinylated cRNA (Affymetrix). Fifteen micrograms of cRNA from each sample was hybridized to Affymetrix HG U133 Plus 2.0 microarrays. Hybridization and scanning were performed according to the manufacturer's instructions (Affymetrix). Data was analyzed using the gene expression commons platform (Seita J, Sahoo D, Rossi DJ, Bhattacharya D, Serwold T, Inlay MA, Ehrlich LI, Fathman JW, Dill DL, Weissman IL. PLoS One. 2012;7(7):e40321. doi: 10.1371/journal.pone.0040321. PMID: 22815738).

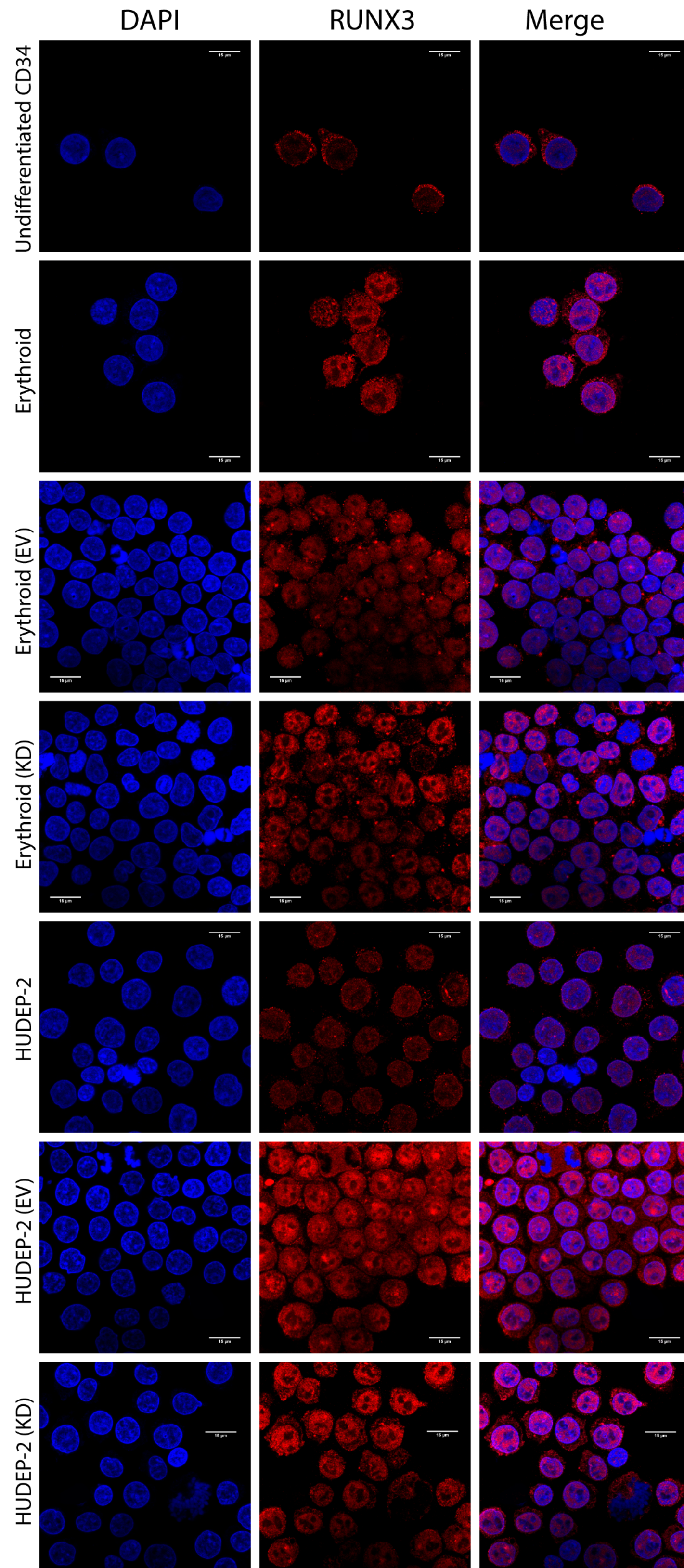
SUPPLEMENTARY FIGURES



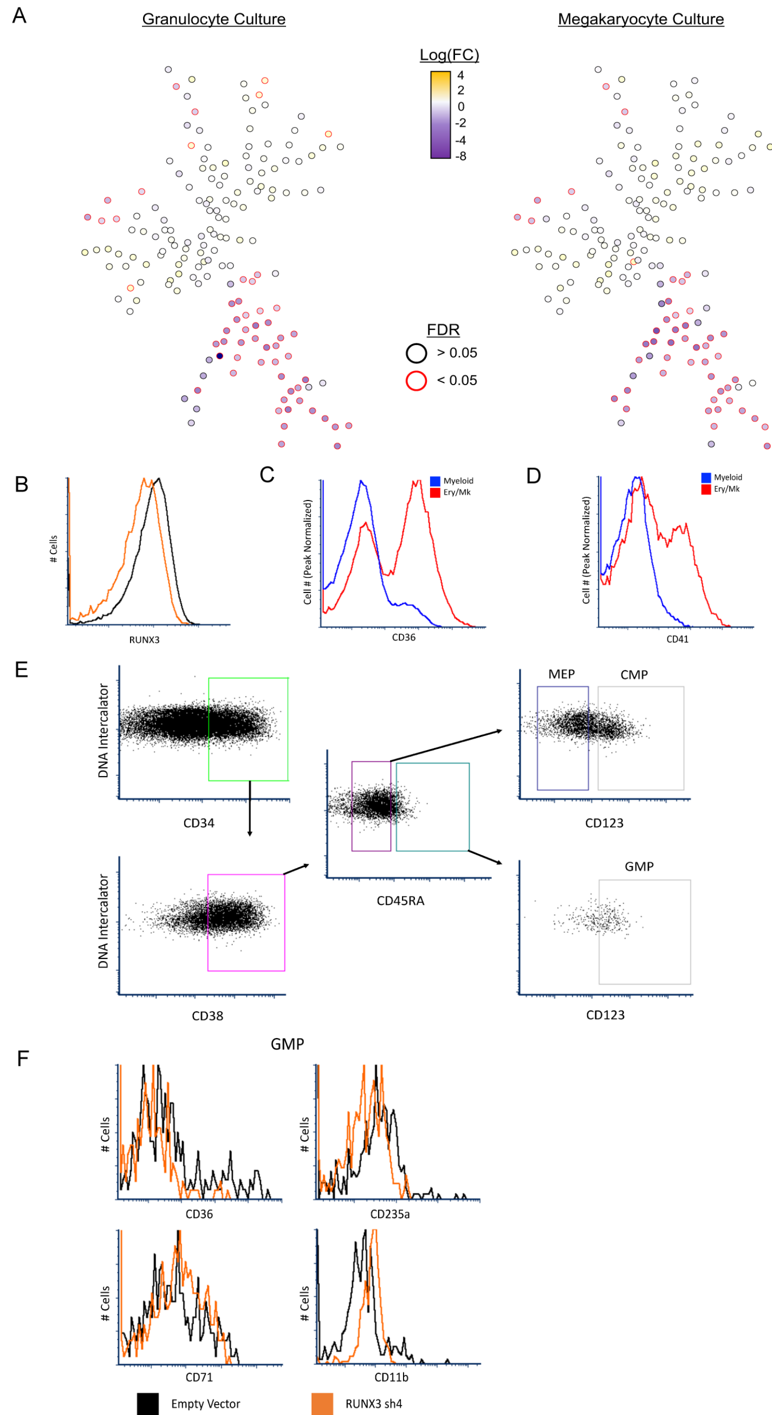
Supplementary Figure 1. HSPC *RUNX3* Levels Decline With Aging
(A) Human *RUNX3* mRNA levels from RNA-seq analysis of Lin-CD34+CD38+ bone marrow HSC obtained from healthy young and aged individuals. (B) Immunostaining of RUNX3 protein in bone marrow clots of two young (<10 years) and two aged (60-70) subjects shown at 20X magnification. (C) Mouse *Runx3* mRNA expression heat-map overlaid on a multi-lineage hematopoietic SPRING plot derived from single cell RNA-seq on Kit+ marrow cells (Ery: erythroid; MPP: multipotent progenitor; Mk: megakaryocyte; Ly: Lymphoid; Gr: granulocyte. Gene visualizer from Tusi et al. 2018 is available at https://kleintools.hms.harvard.edu/paper_websites/tusi_et_al/).



Supplementary Figure 2. RUNX3 Participates in Human Erythroid and Megakaryocyte Differentiation (A) RUNX3 protein levels by immunoblot in CD34+ HSPC, undifferentiated and at indicated durations of erythroid culture. Representative immunoblot of whole cell lysates from 3 independent experiments. (B) Flow cytometry plots for erythroid differentiation of CD34+ cells transduced with empty vector (EV) or RUNX3-targeting (RUNX3 sh1 and sh2) lentiviral shRNA constructs and subjected to unilineage erythroid culture for 3 days. Graph summarizes the quantitation of erythroid differentiation from 3 independent experiments. (C) RUNX family protein expression by immunoblot in CD34+ cells transduced with lentiviral empty vector (EV) or shRNA expression constructs targeting *RUNX3*. Graph summarizes mean fold change in tubulin-normalized protein levels associated with RUNX3 knockdown. N=3. (D) Histogram overlay for erythroid differentiation of un-transduced CD34+ cells (blue), CD34+ cells transduced with a GFP-targeting lentiviral shRNA construct (red), and unstained cells (black). (E) Histogram overlay for erythroid differentiation of CD34+ cells transduced with empty vector (blue), CD34+ cells transduced with a RUNX1-targeting lentiviral shRNA construct (red), and unstained cells (black). (F) Flow cytometry plots for megakaryocyte differentiation of CD34+ cells transduced with empty vector (EV) or RUNX3-targeting (RUNX3 sh4) lentiviral shRNA constructs and subjected to unilineage megakaryocytic culture for 3 days. Graph summarizes the quantitation of megakaryocyte differentiation from 3 independent experiments. (G-I) Summary of differentiation status by flow cytometry on transduced HUDEP-2 cells -/+ differentiation induction, and immunoblot of RUNX3. Graphs show mean fold changes in CD235a+ percentage or CD71 mean fluorescence intensity associated with RUNX3 knockdown. N = 3. B: one-way ANOVA with Tukey's post hoc test. C, F, and G: Two-way ANOVA with Bonferroni's test. E: two-tailed Student t test. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.005$. Error bars: +/-SEM.



Supplementary Figure 3. Cellular localization of RUNX3
Immunofluorescent detection of RUNX3 in untransduced, empty vector transduced, and RUNX3 sh4 transduced CD34+ HSPC and HUDEP-2 erythroblasts. HSPC were analyzed undifferentiated and at day 3 of erythroid culture, and HUDEP-2 were uninduced.



Supplementary Figure 4. RUNX3 Levels Influence Progenitor Lineage Output Balance
(A) Minimum spanning tree plots depicting cell population-nodes identified by CyTOF on indicated cultures of transduced CD34+ progenitors. Heatmap coloration of nodes reflects $\log_2$(fold changes) in their frequency associated with RUNX3 knock-down. (B) Histogram overlay comparing RUNX3 levels in cells transduced with empty vector (black) or RUNX3 sh4 construct (orange). (C-D) CyTOF histogram overlays from control EV-transduced progenitors cultured in erythroid medium, comparing expression of CD36 and CD41 between myeloid (blue) and erythro-megakaryocytic (Ery/Mk, red) compartments as depicted in Figure 3A. (E) Gating strategy used to determine CMP, MEP, and GMP population frequencies. (F) Histogram overlay comparing CD36, CD235a, CD71, and CD11b expression between control (EV, black) and RUNX3-deficient (RUNX3 sh4, orange) GMP.

SUPPLEMENTARY TABLE

Populations in erythroid culture identified by CyTOF to be significantly affected by RUNX3 deficiency.

Population Phenotype (diminished frequency)	log(fold change abundance)	FDR
CD117- CD235a+ CD34+ CD36hi CD71+	-3.719262064	0.020801921
CD117- CD34+ CD36hi	-2.93570212	0.020801921
CD117- CD34+	-2.927367589	0.020801921
CD36hi	-2.026476318	0.020801921
CD235a+ CD36hi	-1.962454631	0.020801921
CD117+ CD36hi	-1.94043204	0.020801921
CD36hi CD71+	-1.924672676	0.020801921
CD117+ CD235a+ CD36hi	-1.906223603	0.020801921
CD235a+ CD36hi CD71+	-1.87934004	0.020801921
CD34+ CD36hi CD45RA- CD71+	-5.527807648	0.021172785
CD36hi CD38-	-3.20231726	0.021172785
CD117+ CD123- CD235a+ CD36hi CD71+	-2.324952873	0.021172785
CD123- CD36hi	-2.295056993	0.021172785
CD34+ CD36hi	-2.272313596	0.021172785
CD34+ CD36hi CD71+	-2.169146303	0.021172785
CD117+ CD235a+ CD34- CD71+	-2.112339189	0.021172785
CD117+ CD235a+ CD36hi CD71+	-1.821118771	0.021172785
CD117+ CD235a+ CD71+	-1.623566908	0.021172785
CD235a+ CD71+	-1.336391726	0.021172785
CD117+ CD71+	-1.068179401	0.021172785
CD117+ CD123- CD235a+ CD34- CD71+	-4.221637154	0.02181571
CD123+ CD235a+ CD34+ CD36hi CD45RA+ CD71+	-2.424672903	0.02181571
CD117+ CD34+ CD36hi	-2.183237624	0.02181571
CD235a+ CD34+ CD36hi CD71+	-1.911194053	0.021826715
CD117+ CD235a+ CD34-	-1.741000304	0.021826715
CD117+ CD34- CD71+	-1.546220087	0.021826715
CD117+ CD123- CD71+	-1.37695306	0.021826715
CD117+ CD38- CD71+	-2.62508303	0.022251608
CD117+ CD235a+ CD34+ CD36hi CD45RA+	-2.464415391	0.022251608
CD235a+ CD34+ CD36hi CD45RA+	-2.378683681	0.022251608
CD235a+ CD34+ CD36hi	-2.154723865	0.022251608
CD235a+ CD34+ CD36hi CD45RA+ CD71+	-2.044416618	0.022251608
CD117+ CD123- CD235a+ CD71+	-1.505742441	0.022251608
CD117+ CD123-	-1.289467248	0.022251608
CD117+ CD235a+	-1.064811503	0.022251608
CD71+	-0.7540356	0.022251608
CD34- CD36hi	-1.839183625	0.022768574
CD117+ CD235a+ CD34+ CD45RA- CD71+	-4.18452121	0.023049541

CD235a+	-0.919235293	0.024478996
CD235a+ CD36hi CD45RA+	-1.687778826	0.025456838
CD123- CD71+	-1.231364348	0.025456838
CD123- CD34- CD71+	-3.149913022	0.026118862
CD117+ CD235a+ CD34+ CD36hi	-2.011961177	0.026152719
CD36hi CD45RA- CD71+	-5.575943728	0.027394542
CD117+ CD235a+ CD36hi CD45RA+	-1.633671867	0.027394542
CD117+ CD235a+ CD36hi CD45RA- CD71+	-5.334345011	0.027678568
CD36hi CD45RA+	-1.578658274	0.027777726
CD34- CD71+	-1.477818478	0.028036592
CD117+ CD235a+ CD34+ CD36hi CD45RA+ CD71+	-1.932433149	0.030212605
CD36hi CD71-	-2.038562279	0.030255547
CD123- CD34+ CD36hi	-1.506923466	0.034311297
CD235a- CD34+ CD36hi	-1.880093946	0.034318267
CD117+ CD235a+ CD45RA- CD71+	-5.034412802	0.03624554
CD117+ CD235a+ CD34+ CD36hi CD71+	-1.495710107	0.03624554
CD235a+ CD36hi CD45RA+ CD71+	-1.475378789	0.039628627
CD117+ CD235a+ CD34- CD36hi CD71+	-1.655453922	0.041382437
CD117+ CD235a+ CD36hi CD45RA+ CD71+	-1.447625685	0.041382437
CD117+ CD34+ CD45RA- CD71+	-4.390262537	0.045572809
CD117+ CD34-	-1.054308414	0.045705551
CD123- CD34+	-1.274233859	0.047666676

Population Phenotype (increased frequency)	log(fold change abundance)	FDR
CD45RA+	0.961620903	0.021172785
CD36lo CD45RA+	1.949999308	0.021172785
CD117+ CD36lo CD45RA+	2.0199171	0.021172785
CD123+ CD36lo CD45RA+	2.079819864	0.021172785
CD123+ CD38+ CD45RA+	2.093917809	0.021172785
CD117+ CD123+ CD36lo CD45RA+	2.170353906	0.021172785
CD117- CD38+ CD45RA+	2.35114823	0.021172785
CD117+ CD123+ CD38+ CD45RA+	2.370327689	0.021172785
CD123+ CD36lo CD38+ CD45RA+	2.52321586	0.021172785
CD117+ CD123+ CD36lo CD38+ CD45RA+	3.168493405	0.021172785
CD117+ CD123+ CD36lo CD38+ CD45RA+ CD71-	3.486666794	0.021172785
CD123+ CD45RA+	1.690158574	0.021176596
CD36lo CD38+ CD45RA+	2.272647679	0.021176596
CD117+ CD123+ CD34+ CD36lo CD45RA+	2.283017298	0.021176596
CD117- CD123+ CD38+ CD45RA+	2.353692155	0.021176596
CD117+ CD36lo CD38+ CD45RA+ CD71+	2.535769836	0.021176596
CD117+ CD36lo CD38+ CD45RA+	2.600259996	0.021176596
CD117+ CD123+ CD36lo CD45RA+ CD71-	2.680126994	0.021176596

CD117+ CD123+ CD235a- CD45RA+ CD71+	2.687459827	0.021176596
CD45RA+ CD71-	2.016784542	0.021268162
CD117+ CD36- CD45RA+ CD71+	2.299899769	0.021268162
CD117+ CD123+ CD34+ CD45RA+	1.704345953	0.02181571
CD117+ CD45RA+ CD71-	1.986693782	0.02181571
CD117+ CD36lo	1.620452606	0.021826715
CD38+ CD45RA+	1.644879664	0.021826715
CD117+ CD123+ CD45RA+	1.671525396	0.021826715
CD123+ CD34+ CD45RA+	1.684655878	0.021826715
CD117+ CD123+ CD36lo CD71-	1.966847719	0.021826715
CD117+ CD123+ CD45RA+ CD71+	1.97001232	0.021826715
CD36lo CD38+ CD45RA+ CD71+	1.975230993	0.021826715
CD117+ CD36- CD38+ CD45RA+ CD71+	2.134898349	0.021826715
CD117+ CD123+ CD34+ CD36- CD45RA+ CD71+	2.186756531	0.021826715
CD117+ CD123+ CD45RA+ CD71-	2.192298028	0.021826715
CD117+ CD123+ CD34+ CD45RA+ CD71-	2.277775625	0.021826715
CD117+ CD123+ CD235a- CD36lo CD38+ CD45RA+ CD71+	2.649605266	0.021826715
CD117+ CD123+ CD235a- CD34+ CD36- CD45RA+ CD71+	2.734785211	0.021826715
CD117+ CD123+ CD34+ CD36lo CD45RA+ CD71-	2.839704884	0.021826715
CD117+ CD45RA+	0.820382787	0.022251608
CD117+ CD123+ CD34+ CD36lo	1.57550775	0.022251608
CD117+ CD123+ CD36lo	1.578013625	0.022251608
CD117+ CD71-	1.601521438	0.022251608
CD123+ CD45RA+ CD71+	1.70375139	0.022251608
CD117+ CD123+ CD71-	1.753618176	0.022251608
CD123+ CD38+ CD45RA+ CD71+	1.780140967	0.022251608
CD123+ CD36lo CD38+ CD45RA+ CD71+	1.827312586	0.022251608
CD235a- CD45RA+	1.905278528	0.022251608
CD117+ CD235a- CD45RA+	1.92979801	0.022251608
CD117+ CD123+ CD38+	2.058814479	0.022251608
CD117+ CD235a- CD38+ CD45RA+	2.109723532	0.022251608
CD235a- CD36lo CD45RA+	2.141941418	0.022251608
CD117+ CD123+ CD235a- CD34+ CD36- CD45RA+	2.184052312	0.022251608
CD117+ CD36lo CD38+	2.24701988	0.022251608
CD117+ CD235a- CD36lo CD45RA+	2.270590293	0.022251608
CD117+ CD235a+ CD36lo CD38+ CD45RA+ CD71+	2.36368958	0.022251608
CD117+ CD123+ CD36lo CD38+	2.517235551	0.022251608
CD117+ CD34- CD36- CD38+ CD45RA+ CD71+	2.74466792	0.022251608
CD117+ CD123+	1.395585034	0.022509853
CD117+ CD123+ CD34+	1.425052707	0.022694075
CD235a- CD36lo	1.940196737	0.022768574
CD117+ CD38+ CD45RA+	1.503418954	0.025427647

CD117+ CD123+ CD38+ CD45RA+ CD71+	1.744456412	0.025427647
CD117- CD123- CD36lo CD71-	2.374625197	0.025456838
CD34- CD45RA+ CD71-	2.235289254	0.025725544
CD34- CD36- CD71+	2.557835468	0.025956549
CD36- CD45RA+ CD71+	1.931527814	0.026118862
CD123+ CD34+ CD36lo	1.477841918	0.026152719
CD117+ CD123+ CD34+ CD36lo CD71-	1.77349216	0.026355272
CD123+ CD34+	1.332506425	0.026857217
CD34+ CD36lo	1.415031769	0.027394542
CD34- CD45RA+	1.971837004	0.027394542
CD117+ CD123+ CD34+ CD45RA+ CD71+	1.710120145	0.027678568
CD34- CD36- CD45RA+	2.515739011	0.027678568
CD123+ CD235a+ CD38+ CD45RA+ CD71+	1.610692758	0.028659103
CD117+ CD36- CD38+ CD45RA+	1.747189368	0.028659103
CD117- CD34- CD36lo	2.228242427	0.028659103
CD38+ CD45RA+ CD71+	1.439757948	0.028790802
CD117+ CD123+ CD34+ CD71-	1.615420271	0.028790802
CD123+ CD34+ CD45RA+ CD71+	1.644404536	0.028790802
CD117- CD38+	1.684508468	0.028790802
CD123+ CD235a+ CD34+ CD38+ CD45RA+ CD71+	1.741804463	0.028790802
CD123+ CD235a- CD36lo CD71+	1.737054092	0.031330165
CD235a+ CD36lo CD38+ CD45RA+ CD71+	1.632398539	0.03159593
CD123+ CD38+	1.46944081	0.031910071
CD117+ CD235a-	1.446718275	0.032742922
CD117- CD123+ CD38+	1.667976719	0.03293287
CD117+ CD123+ CD235a- CD36lo CD45RA+	1.942988443	0.034318267
CD117+ CD123+ CD34+ CD71+	1.506950682	0.034606909
CD117+ CD38+	1.367833291	0.03624554
CD34- CD36- CD38+	2.217660354	0.03624554
CD117+ CD36lo CD38+ CD71+	1.882729378	0.036964803
CD123+ CD36lo CD71-	1.306104029	0.038056128
CD117+ CD235a- CD36- CD45RA+	1.727856868	0.038056128
CD123+	1.01407299	0.038141191
CD36lo	1.088420266	0.039628627
CD117+ CD38+ CD45RA+ CD71+	1.296671901	0.041382437
CD34+ CD71-	1.29931619	0.041382437
CD123+ CD235a+ CD45RA+ CD71+	1.368995023	0.041382437
CD117+ CD123+ CD71+	1.468879417	0.041382437
CD36- CD38+ CD45RA+ CD71+	1.731075573	0.041382437
CD117+ CD34- CD45RA+	1.855595825	0.041382437
CD123+ CD71-	1.249028359	0.041539258
CD71-	1.130962136	0.042454287

CD117+ CD123+ CD235a+ CD34+ CD36- CD38+ CD45RA+ CD71+	1.578437851	0.042454287
CD34- CD38+ CD71+	2.490096029	0.043667741
CD38+	1.277308649	0.043784112
CD123+ CD36lo CD38+	1.578960511	0.043823071
CD235a-	1.219792524	0.044528442
CD117+ CD123+ CD235a- CD45RA+	1.654594197	0.044528442
CD123+ CD235a+ CD34+ CD45RA+ CD71+	1.428613685	0.044983013
CD123+ CD235a+ CD34+ CD45RA+	1.232751363	0.045322654
CD117+ CD235a+ CD36- CD38+ CD45RA+ CD71+	1.592963585	0.045572809
CD123+ CD34+ CD36- CD45RA+ CD71+	1.669095408	0.045572809
CD117- CD235a+ CD36- CD38+ CD45RA+ CD71+	1.836492088	0.045572809
CD123+ CD36lo	0.977073229	0.045705551
CD36lo CD71-	1.230438919	0.045705551
CD123+ CD235a- CD71+	1.624765909	0.045705551
CD123+ CD235a+ CD45RA+	1.137180858	0.047381762
CD117+ CD36- CD45RA+	1.286272205	0.047381762
CD34- CD38+	2.047977524	0.049929175