Published in final edited form as:

Exp Hematol. 2014 April ; 42(4): 317–327.e2. doi:10.1016/j.exphem.2013.11.015.

Clonal-Level Responses of Functionally Distinct Hematopoietic Stem Cells to Trophic Factors

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Abstract

Recent findings from several groups have identified distinct classes of hematopoietic stem cells (HSCs) in the bone marrow, each with inherent functional biases in terms of their differentiation, self-renewal, proliferation and lifespan. It has previously been demonstrated that myeloid- and lymphoid-biased HSCs can be prospectively enriched based on their degree of Hoechst dye efflux. In the present study, we used differential Hoechst efflux to enrich lineage-biased HSC subtypes and analyzed their functional potentials. Despite similar outputs *in vitro*, bone marrow transplantation assays revealed contrasting lineage differentiation *in vivo*. To stratify the molecular differences underlying these contrasting functional potentials at the clonal level, single-cell gene expression analysis was performed using the Fluidigm Biomark system and revealed dynamic expression of genes including *Meis1*, *CEBP α* , *Sfp1* and *Dnmt3a*. Finally, single-cell gene expression analysis was used to unravel the opposing proliferative responses of lineage-biased HSCs to the growth factor TGF β 1, revealing a potential role for the cell cycle inhibitor *Cdkn1c* as molecular mediator. This work lends further credence to the concept of HSC heterogeneity and presents unprecedented molecular resolution of the HSC response to trophic factors using single-cell gene expression analysis.

Keywords

Stem Cells; TGF β 1; protein; mouse; Bone Marrow Transplantation; Single-Cell Analysis

Recent studies from several independent laboratories have demonstrated that phenotypically indistinguishable hematopoietic stem cells (HSCs) can have contrasting functional outputs *in vivo*, leading to the concept of HSC heterogeneity or functional diversity [1–6]. Although

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Financial Disclosure Declaration

There are no financial conflicts of interest relating to this work to declare.

the nomenclature and functional classifications used by different groups varies, HSCs seem to cluster broadly into three operational classes based on their output in bone marrow transplantation assays. Compared to the normal peripheral blood composition of unmanipulated mice, myeloid-biased HSCs generate more myeloid progeny relative to lymphoid cells, lymphoid-biased HSCs produce more B- and T-cells relative to myeloid cells, while lineage-balanced HSCs turn out differentiated blood cells in the same ratio as the normal blood composition [1–3].

We have previously demonstrated that the degree of Hoechst dye efflux can be used to prospectively identify lineage-biased HSC subtype in the bone marrow [3]. While virtually all of the long-term repopulating activity resides in the Hoechst-effluxing “side population” (SP)[7], the gradation of dye staining allows for at least partial discrimination of HSC subtypes [3]. Cells towards the bottom of the SP, lower-SP cells, show a bias towards myeloid cell generation in bone marrow transplantation assays, while the cells from the top of the SP, upper-SP cells, preferentially differentiate into lymphoid progeny *in vivo* [3]. Given that other groups have identified the existence of a lineage-balanced HSC subtype, we sought to determine if closer scrutiny of Hoechst efflux potential could be used to prospectively enrich for myeloid-biased, lymphoid-biased and lineage-balanced HSCs.

In the present study, we show that the degree of Hoechst dye efflux strongly correlates with HSC lineage potential *in vivo*. To interrogate the molecular determinants of this differential lineage output, we performed single-cell gene expression analysis of clones from within each SP fraction on a total of 576 individual HSCs using the Fluidigm Biomark system. This represents a significant advance over transcriptional profiling of bulk cells, which report population averages and can mask inherent clonal variation. We provide the first information regarding the clonal responses of lineage-biased HSC subtypes to a trophic factor - TGFβ1. We thus provide further evidence for the HSC heterogeneity model and use novel molecular profiling techniques to delineate the molecular responses of individual lineage-biased HSC clones to extrinsic stimuli.

Methods

Flow Cytometry

Whole bone marrow (WBM) was isolated from femurs, tibias and iliac crests. SP staining was performed with Hoechst 33342 (Sigma) as previously described [7]. Briefly, whole bone marrow was resuspended in staining media at 10^6 cells/mL and incubated with 5 mg/mL Hoechst 33342 for 90 minutes at 37°C. Magnetic enrichment was performed with anti-mouse CD117 microbeads (Miltenyi Biotec, Auburn, CA, USA) on an AutoMACS (Miltenyi Biotec). Post-enrichment, the positive cell fraction cells were resuspended at 10^8 cells/mL and incubated on ice for 20 minutes with the following antibodies – Gr1-PEcy7 (RB6-8C5), Mac1-PEcy7 (M1/70), B220-PEcy7 (RA3-6B2), Ter119-PEcy7 (TER119), CD4-PEcy7 (RM4-5), CD8-PEcy7 (53-6.7), Sca-1-APC (E13-161.7), CD150-PE (TC15-12F12.2) and c-Kit-FITC (2B8). For analysis of transplanted mice, SP staining and antibody labeling was performed as above with the addition of antibodies to discriminate competitor- (CD45.1+) and donor- (CD45.2+) derived cells. All antibodies were obtained from Becton Dickinson (Franklin Lakes, NJ, USA), BioLegend (San Diego, CA, USA) or

eBioscience (San Diego, CA, USA) and used at 1:100 dilutions. Cell sorting was performed on a MoFlo cell sorter (Dako North America, Carpinteria, CA) and analysis performed on a LSRII (Becton Dickinson).

Mice and Bone Marrow Transplantation

All animal procedures were IUCAC-approved and conducted in accordance with Washington University in St. Louis institutional guidelines (Animal Studies Committee Approval No. 20110264). Mice were housed in a specific pathogen-free facility and fed autoclaved acidified water and mouse chow ad libitum. All mice were C57Bl/6 background distinguished by CD45.1 or CD45.2 alleles. For bone marrow transplantation, recipient C57Bl/6 CD45.1 mice were transplanted by retro-orbital injection following a split dose of 10.5 Gy of lethal irradiation. 200 donor HSCs (CD45.2) were competed against 2.0×10^5 unfractionated WBM cells with the opposite CD45 allele (matched to the recipient).

Peripheral Blood Analysis

Mice were bled retro-orbitally, the red blood cells were lysed, and each sample was incubated with the following antibodies on ice for 20 minutes – CD45.1-FITC (A20), CD45.2-BV421 (104), CD3e-APC (145-2C11), B220-APC (RA3-6B2), Gr1-PEcy7 (RB6-8C5), Mac1-PEcy7 (M1/70), B220-PEcy7 (RA3-6B2). Cells were then spun down, resuspended in a propidium iodide solution, and analysis was performed using either an LSRII (BD) or FACScan (BD) instrument.

in vitro Differentiation Assays

To assess myeloid differentiation, single cells were sorted into 96-well plates containing Methocult 3434 medium (Stem Cell Technologies, Vancouver, Canada) supplemented with penicillin / streptomycin and cultured *in vitro* at 37°C for 14-days. OP9 and OP9-DL1 cells were maintained and cultured as previously described [8]. For B-cell differentiation, 250 cells were sorted directly into wells of 24-well plates seeded with 5×10^4 OP9 cells. Cells were cultured for 14-days in the presence of recombinant mouse Flt3L (10 ng/mL) and IL-7 (10 ng/mL) (both from Miltenyi Biotec) with half media changes every fourth day. For T-cell differentiation, 250 cells were sorted directly into wells of 24-well plates seeded with 5×10^4 OP9-DL1 cells. Cells were cultured for 14-days in the presence of recombinant mouse Flt3L (5 ng/mL) and IL-7 (1 ng/mL) with half media changes every fourth day. Following the culture period, cells were stained with markers for B-cells (CD19, B220) or T-cells (CD4, CD8, CD44, CD25) and analyzed with an LSRII (BD).

Single-Cell Gene Expression Analysis

Single cells were sorted into individual wells of 96-well plates where lysis and reverse-transcription/specific-target amplification (RT-STA; 18 cycles) was performed with the CellsDirect One-Step qRT-PCR Kit (Invitrogen, Grand Island, NY, USA). The resulting RT-STA reactions were diluted 1:3 in DNA Suspension Buffer (TEKnova, Hollister, CA, USA) and used as the template cDNA. High throughput real-time PCR was performed using the Fluidigm Biomark system. 48×48 gene expression chips were primed using the IFC (Integrated Fluidic Circuit) Controller MX. Amplified specimens were loaded into the

sample inlets of the chips mixed with Quanta PerfeCTa qPCR Fast Mix, low ROX (Quanta Biosciences, Gaithersburg, MD, USA) and 20X GT loading reagent. The 20X Taqman assays-on-demand (AODs; Life Technologies, Grand Island, NY, USA) were loaded with 2X assay loading reagent in the assay inlets. The samples and assays were loaded in the chips using the IFC MX and the chips were cycled using the Fluidigm BioMark. The data was loaded into Fluidigm Real-Time PCR Analysis Software and exported to csv files after analysis of the data was complete. Following quality control analysis, final normalized gene expression values, principal component analysis, violin plots and hierarchical clustering were generated with the Singular Analysis Toolset (Fluidigm) in the programming environment R. The Taqman AODs used are listed in Supplementary Table 1.

TGFβ1 and Proliferation Assays

Recombinant TGFβ1 (R&D Systems, Minneapolis, MN, USA) was reconstituted according to the manufacturer's recommendations. For assessment of TGFβ1-induced proliferative effects, HSCs were sorted into tubes containing pre-labelled B220⁺ carrier cells as previously described [9]. Cells were cultured overnight in Stempro-34SFM (Life Technologies) supplemented with 100 ng/mL TPO, 100 ng/mL SCF, 50 ng/mL Flt3L and 10 ng/mL IL-3 ± 20 pg/mL TGFβ1. Following the incubation, cells were analyzed for proliferation index using the Ki67-FITC Flow Kit (BD). For *in vivo* experiments, mice were administered 0.1 μg recombinant TGFβ1 (in 200 mL PBS) or the same volume of PBS (control) via intraperitoneal injection for three consecutive days [10].

Cdkn1c Immunostaining

24-hours after the last injection, HSCs were sorted directly onto microscope slides and processed for Cdkn1c immunostaining. Cells were fixed with 4% paraformaldehyde and permeabilized with 1% Triton-X 100 (Sigma). Cells were stained with anti-rabbit Cdkn1c primary antibody (Abcam ab-75974; 1:250 dilution) and counterstained with AlexaFluor-488 anti-rabbit secondary antibody (Invitrogen; 1:500 dilution). Cells were washed and mounted in Vectashield with DAPI (Vector Laboratories). Images were captured on Nikon 80i fluorescent microscope with a Photometrics ES2 mono cooled CCD camera. Intensity of Cdkn1c fluorescent staining was quantified using ImageJ software.

Statistics

Student *t* test and 1-way ANOVA's were used for statistical comparisons where appropriate. Significance is indicated on the figures using the following convention: **p*<0.05, ***p*<0.01, ****p*<0.001. Error bars on all graphs represent the SEM.

Results

Degree of Hoechst Efflux Discriminates Lineage-Biased HSC Subtypes

Single-cell transplantation studies have shown that the degree of Hoechst dye efflux is a powerful discriminator of lineage-biased HSC activity [3]. Re-analysis of our previously compiled single-cell transplant data extrapolated from the Dykstra and Eaves [2] classification of HSC subtype output (GM:(B+T)>2.0 = myeloid-biased; GM:(B+T) 0.25–2.0 = lineage-balanced; GM:(B+T)<0.25 = lymphoid-biased) showed that while there is

enrichment of myeloid-biased HSCs and lymphoid-biased HSCs in the lower-SP and upper-SP respectively, even these highly selected cell populations contained a mixture of lineage-biased clones (Fig. 1A). Analysis of donor-derived peripheral blood GM:(B+T) ratios for lower-SP^{KLS} clones at 16-weeks post-transplant classified 18/41 as myeloid-biased, 14/41 as lineage-balanced and 9/41 as lymphoid-biased. Similarly for upper-SP^{KLS} cells, 3/31 were myeloid-biased, 5/31 were lineage-balanced and 23/31 were lymphoid-biased.

Given that even these highly purified bone marrow populations contained a mixture of lineage-biased HSC subtypes, we speculated that these populations could be better resolved by further selection according to Hoechst dye efflux. To this end, we performed transplantation of 200 CD45.2 lower-, mid-, or upper-SP^{KLS} cells competed against 200,000 CD45.1 whole bone marrow (WBM) cells into lethally irradiated CD45.1 recipient mice. SP sorting gates were defined by initially segregating the SP into equal thirds based on cell frequency, then contracting the gates by approximately 5% in each direction to minimize cross-contamination (Fig. 1B). Monthly analysis of peripheral blood reconstitution kinetics showed lower-SP^{KLS} cells generating a higher proportion of myeloid cells, and upper-SP^{KLS} cells generating a higher proportion of T-cells. Cells from the mid-SP^{KLS} fell into intermediate levels between lower- and upper-SP^{KLS} cells both for overall peripheral blood chimerism (Fig. 1C), and in terms of their myeloid/T-cell differentiation profile (Fig. 1D). B-cell output from all transplanted cell populations was not different long-term post-transplant (16-weeks). Analysis of the SP profile of recipient mice post-primary transplant showed that while all transplanted fractions were able to regenerate the entire SP, each preferred to regenerate the population from which they were initially isolated (Fig. 1E). To test if these lineage-biases were stable over serial transplantation, 200 donor-derived HSCs (CD45.2⁺SP^{KLS}) were purified from the bone marrow of primary recipients 18-weeks post-transplant (Fig. 1E) and competed against fresh WBM competitor cells in secondary transplantation (Fig. 1F). Analysis of short-term reconstitution (4-weeks) highlighted the lymphoid-bias of upper-SP^{KLS} cells with a significantly higher B-cell output (Fig. 1G). Reconstitution at 16-weeks post-secondary transplant recapitulated the myeloid-bias of lower-SP^{KLS} cells and T-cell bias of upper-SP^{KLS} cells observed following primary transplantation (Fig. 1G). These findings emphasize the fact that these lineage-biases are inherently stable properties to individual HSCs. The output of mid-SP^{KLS} cells again fell into an intermediary profile, suggesting this cell fraction was enriched for lineage-balanced HSCs. Mid-SP^{KLS} showed a trend towards increased B-cell output compared to both lower- and upper-SP^{KLS}, although this was not statistically significant.

HSCs from all SP Fractions Possess Robust Tri-Lineage Potential *in vitro*

To further identify biases in the hematopoietic output from SP fractions, a series of *in vitro* assays were performed for differentiation potential. For these and all further experiments presented, additional selection for CD150⁺ cells was included in the purification strategy (SP^{KLS150}). For myeloid differentiation capacity, single lower-, mid-or upper-SP^{KLS150} cells were sorted into individual wells of 96-well plates containing Methocult M3434 media and colony formation was scored after 14-days. From four independent experiments, no differences were observed in the total numbers of myeloid colonies formed from the different SP fractions or the proportions of granulocyte (CFU-G), macrophage (CFU-M),

granulocyte/macrophage (CFU-GM) and granulocyte/erythroid/macrophage/megakaryocyte (CFU-GEMM) colonies (Fig. 2A). For B-cell differentiation, 250 lower-, mid- or upper-SP^{KLS150} cells were sorted into wells of 24-well plates seeded with OP9 stromal cells in media supplemented with IL-7 and Flt3L. After 14-days of co-culture, we analyzed the entire contents of each well by flow cytometry to enumerate the number of B220⁺CD19⁺ cells generated from each SP fraction. There were no significant differences in the *in vitro* B-cell potential of each SP fraction (Fig. 2B), although a subtle trend for enhanced B-lymphopoiesis from mid-SP^{KLS} cells was seen, similar to secondary transplantation experiments. For T-cell maturation, 250 lower-, mid- or upper-SP^{KLS150} cells were sorted into wells of 24-well plates seeded with OP9-DL1 stromal cells in media supplemented with lymphoid cytokines (Flt3L and IL-7). T-lineage development was assessed after 14 days of co-culture by flow cytometric analysis of mature and progenitor T-cell populations (Fig. 2C). Again, no differences in the proportions or numbers of different T-cell populations were observed (Fig. 2D). Collectively, these *in vitro* assays highlight the fact that all of the SP fractions contain multi-lineage differentiation potential given the appropriate stimuli (cytokines, stromal support), but the inherent biases of each subtype are only elucidated by the complex signaling interactions and cellular composition of the hematopoietic niche *in vivo*.

Gene Expression Analysis of Lineage-Biased HSC Clones

The transplantation data suggested that Hoechst dye efflux can be a powerful prospective discriminator of lineage-biased HSC subtypes with the lower-SP enriched for myeloid-biased HSCs, the mid-SP enriched for lineage-balanced HSCs, and the upper-SP enriched for lymphoid-biased HSCs. However, the possibility exists that mid-SP^{KLS} cells behave in this way because this cell fraction contained a mixture of more functionally distinct cells, rather than being inherently lineage-balanced. The apparent balanced blood cell output of bulk-transplanted mid-SP^{KLS} cells could be observed if the transplanted bolus contained an equivalent number of myeloid- or lymphoid-biased HSCs.

Single cell transplantation remains the only true functional assay for discriminating between these possibilities [11, 12]. However, such experiments are laborious, costly and time-consuming. To discern if the mid-SP truly represented a distinct lineage-balanced HSC subpopulation, we attempted to reconcile molecular differences between the SP fractions at the clonal level as an alternative. Previous profiling studies of purified SP fractions highlighted remarkable similarity in gene expression patterns between lower- and upper-SP^{KLS} cells [3], but these analyses were performed on bulk populations which mask the variability of individual clones. Individual HSCs can differ by size, protein levels and expressed mRNA transcripts, even within nominally homogeneous cell populations and taking averages of pooled cells can mask the dramatic variations in gene expression among cells. Recognizing cellular variations in what appear to be homogenous populations has become crucial to advancing stem cell research, and we utilized Fluidigm single-cell genomics microfluidic technology to examine gene expression in SP fraction clones.

We profiled gene expression in single cells from different SP fractions using the Fluidigm Biomark system. 96 lower-, mid- or upper-SP^{KLS150} cells were sorted directly into

individual wells of PCR plates where lysis and RT-STA was performed. Gene expression measurements of 47 HSC-relevant genes as well as assay controls 18S, Ubc, Polr2a and c-Kit were assessed with Taqman AODs. Single-cell expression data were analyzed with the Singular package in the programming environment R.

To determine if probe multiplexing caused inappropriate signal amplification, we compared Fluidigm 1-HSC delta CTs (relative to 18S) to 500-HSC samples sorted independently and prepared for traditional Taqman real-time PCR. There was good correlation between the methods, and although there was an increase in the delta CTs for some probes using the RT-STA multiplexing, it was consistent for the samples analyzed on the same platform (Supplementary Fig. 1A). The Fluidigm system was highly consistent with linear regression analysis showing approximately 95% correlation co-efficient for technical replicates of the same cell on the same chip (Supplementary Fig. 1B). To set the limits of detection, we noted that samples in which less than three of the four control housekeeping genes were not detected often expressed few other genes, likely resulting from poor target amplification in that sample reaction. Clones which were positive for less than three control genes or in which fewer than ten AODs were detected were filtered out of the final analysis to remove amplification bias (Supplementary Table 2). AODs which were not detected in any cells (e.g. Ebf1, Lef1, Gata1) were excluded from clustering analyses as we could not discriminate failure of detection versus true absence of expression in these instances.

Most clones from all three SP fractions expressed high levels of classical HSC-associated genes such as Nr4a2 [13], Nr4a3 [14], Hmga2 [15] and Lmo2 [16], although other genes with known roles in HSC function from mouse mutant studies or identified as highly / specifically HSC-expressed from previous profiling studies [17] were not detected in all clones (Fig. 3). For example, another recent single-cell profiling report identified Runx1 and Gata2 as being highly expressed in nearly all HSCs [18], but only approximately 50% of all HSCs were positive for either of these two genes in our study. These differences may be due to the different purification strategies used for HSC isolation. Other HSC-related genes with sporadic expression such as Prdm16, HoxA9, HoxA10 and Bmi1 have roles in different leukemias suggesting that while they may also have important functions in HSCs [19–22], their expression must be tightly regulated to prevent them from acting as proto-oncogenes. Given their functional outputs *in vivo*, it was anticipated that significant variation between the different SP fractions would be observed in critical regulators of myeloid (e.g. Gfi1) and lymphoid differentiation (e.g. Ikzf1, Scl). Surprisingly, most clones from all three SP fractions showed low expression of most of these factors. Of the clones that did express some of these genes, they often expressed multiple lineage regulators in concert.

Performing transcriptional analysis on individual cells permits interrogation not possible from analysis of bulk cells. For example, we sought to determine if the mid-SP^{KLS150} cell fraction was predominantly composed of lineage-balanced HSCs, or a mixture of myeloid- and lymphoid-biased HSCs. Principal component analysis was performed to generate two-dimensional clustering of SP fraction clones based on their gene expression profile (Fig. 4A). This analysis showed cells from the different fractions could be loosely grouped into distinct but overlapping clusters. The mid-SP^{KLS150} cluster intersected both the lower- and upper-SP^{KLS150} clusters (Fig. 4A). Violin plots, which show the probability density of the

expression data at different values, were useful for discriminating population-based patterns. Examples of genes which discriminate the three cell populations included *Meis1* (more positive clones in lower-SP^{KLS150}), *C/EBPα* (more positive clones in upper-SP^{KLS150}) and *Sfp1* (fewer positive clones in mid-SP^{KLS150}). There were other genes for which clones from all three SP fractions were almost uniformly positive, but differed in expression level. As has been previously reported [23], the *de novo* DNA methyltransferase enzyme *Dnmt3a* was one of the most highly expressed genes in all SP fractions. However, in terms of expression level in individual clones, *Dnmt3a* was significantly higher in lower-SP^{KLS150} cells than mid- or upper-SP^{KLS150} clones (Fig. 4C).

Molecular Basis of Opposing Proliferative Responses to TGFβ1 by HSC Subtypes

We had previously noted differential effects of the growth factor TGFβ1 on lineage-biased HSC subtypes. Exposure to low concentrations of TGFβ1 stimulated proliferation of myeloid-biased lower-SP^{KLS} cells *in vivo* and *in vitro* whilst proving inhibitory to lymphoid-biased upper-SP^{KLS} cells [3]. To extend these studies here, lower, mid- and upper-SP^{KLS150} cells were purified from the bone marrow of wild-type mice and cultured for 18-hours in the presence of hematopoietic cytokines SCF, TPO, Flt3L and IL-3, with or without 20 pg/mL recombinant TGFβ1. After the incubation, cells were processed for analysis of proliferative index by Ki67 staining. These studies confirmed our previous results indicating TGFβ1 was stimulatory for myeloid-biased lower-SP^{KLS150} cells while being inhibitory for lymphoid-biased upper-SP^{KLS150} cells (Fig. 5A). However, bulk sorted and analyzed mid-SP^{KLS150} cells did not show any significant differences in Ki67 staining upon exposure to TGFβ1 (Fig. 5A). We attempted to use other measures to stratify the differential proliferative responses of these HSC populations to TGFβ1, but quantification of TGFβ1 signaling by measures such as phosphorylation levels of Smad2/3 by intracellular flow cytometry did not resolve any differences (data not shown). This suggested our methods were not sensitive enough to detect subtle responses to TGFβ1 in HSC populations using cell populations in bulk.

Given functional roles for TGFβ1 signaling have been implicated in HSC hibernation [24] and regeneration following genotoxic stress [25], we thought it was important to evaluate the molecular response of lineage-biased HSCs to TGFβ1 on a clonal level. Using the Fluidigm Biomark system, we analyzed the gene expression responses of 48 lower-, mid- and upper-SP^{KLS150} cells exposed to TGFβ1. The HSC populations were purified by flow cytometry and cultured for 18-hours in hematopoietic cytokines as above with or without the addition of recombinant TGFβ1 (“TGF” or “Ctl”, respectively). Following the culture period, cells were re-sorted using the same HSC flow cytometry markers for single-cell gene expression analysis (Supplementary Table 3).

The gene with the most divergent expression profile amongst SP fraction clones in response to TGFβ1 was *Cdkn1c* (p57^{Kip2}). This cell cycle inhibitor is degraded through the ubiquitin-proteasome pathway in cells stimulated by TGFβ1 [26], and is induced in primitive human hematopoietic cells during cell cycle arrest [27]. We observed the increased proliferative activity of myeloid-biased lower-SP^{KLS150} cells in response to TGFβ1 to be associated with a decrease in *Cdkn1c* gene expression, both in terms of the number of *Cdkn1c*⁺ HSCs and

the overall gene expression level (Fig. 5B). Conversely, cell cycle inhibition of lymphoid-biased upper-SP^{KLS150} cells in response to TGFβ1 was associated with increases in Cdkn1c gene expression (Fig. 5B). This suggested that the opposing proliferative responses of lineage-biased HSCs exposed to TGFβ1 may be mediated by the cell cycle inhibitor Cdkn1c.

To determine if this response occurred *in vivo*, mice were administered recombinant TGFβ1 or control PBS on three consecutive days by intraperitoneal injection. 24-hours after the last dose, SP fraction cells were isolated and quantified for Cdkn1c protein expression by immunofluorescence microscopy (Fig. 5C). Baseline Cdkn1c protein expression in control mice was higher in lower-SP^{KLS150} cells compared to mid- and upper-SP^{KLS150} cells (Fig. 5D), as was observed for single cell mRNA expression. While cells from all three SP fractions showed increased Cdkn1c protein levels after TGFβ1 exposure, the average increase in Cdkn1c was significantly reduced in lower-SP^{KLS150} cells (1.43-fold increase) compared to mid- and upper-SP^{KLS150} cells (2.25- and 2.28-fold increase respectively). This clonal analysis implicates Cdkn1c as a molecular mediator of the opposing proliferative responses of lineage-biased HSCs in response to TGFβ1.

Discussion

The implications for the HSC heterogeneity model are profound for experimental and clinical hematology. A greater understanding of the molecular determinants of lineage-bias may ultimately allow for selective isolation or expansion of specific HSC subtypes for bone marrow transplantation. While previous gene expression profiling studies of bulk HSCs have yielded insights into processes such as proliferation [28] and aging [29], more recent studies have focused on single-cell profiling to uncover the inherent variability of HSC clones which has uncovered a previously unrecognized Gata2, Gfi1 and Gfi1b regulatory network [18]. We have used degree of Hoechst dye efflux sorting coupled with single-cell gene expression profiling to examine the molecular determinants of HSC subtype clonal diversity. It should be noted that while this sorting strategy enriches for lineage-biased HSCs (Fig. 1A), like other purification schemes it does not represent homogeneous HSC populations.

Given their contrasting lineage outputs in bone marrow transplantation assays, we expected to find higher expression of T-cell determinants (e.g. Notch1, Gata3) in upper-SP^{KLS150} clones and increased levels of pro-myeloid factors in lower-SP^{KLS150} clones. In general, there was low expression for most master regulators of lineage determination across clones from all three SP fractions. As the cells were isolated from a resting state, this could indicate the quiescent HSCs were not “primed” for differentiation [30], but is also consistent with the observation of “transcriptional bursting” in single cells, a model suggesting eukaryotic transcripts are produced in short but intense bursts interspersed with intervals of inactivity [31, 32]. Alternatively the functional biases (and subsequent gene expression changes) of lineage-biased HSCs may only be artificially elucidated under the guise of a transplant setting which forces differentiation. From this work and others, it is clear that all HSC subtypes have the potential to fulfill the criteria used as standard definitions of HSC functional capacity (long-term self-renewal, tri-lineage differentiation). But in the natural

setting *in vivo*, it is likely they exist as highly-biased clones when in communication with the other HSC populations, as has been witnessed from co-transplantation of HSC subtypes in combination [3]. These HSC subtypes do not act in isolation, but rather act in concert to sustain the organism's needs.

Single-cell profiling allows resolution of cell populations which is masked by the average output of bulk populations. Transplantation of bulk mid-SP^{KLS} cells produced a lineage-balanced output. Principal component analysis of single-cell gene expression data showed that clones from each of the three SP fractions weakly cluster together, indicating there are molecular differences that would suggest the mid-SP^{KLS150} cell fraction is enriched for an intermediate lineage-balanced HSC population rather than a mixture of myeloid- and lymphoid-biased HSCs. This is most clearly illustrated by the expression pattern of genes such as *Sfp1* (Fig. 4B), which is expressed at lower levels in mid-SP^{KLS150} clones compared to both lower- and upper-SP^{KLS150} clones, suggestive of a distinct population. This would be consistent with findings from other groups that have identified lineage-balanced HSCs [1, 2], although single-cell bone marrow transplantation remains the only true measure of a cell's inherent potential and must be performed to confirm this.

HSCs display a biphasic response to different concentrations of TGFβ1, with higher concentrations being inhibitory and lower concentrations stimulatory [33, 34]. We show here that this discrepancy can be at least partly explained by the differential responses of lineage-biased HSC clones to TGFβ1. While myeloid-biased HSCs showed enhanced proliferation in response to TGFβ1, lymphoid-biased HSCs were inhibited at the same concentration (Fig. 5A). Clonal gene expression profiling revealed an opposing response of the cell cycle inhibitor *Cdkn1c* in lineage-biased HSCs exposed to TGFβ1, which was confirmed at the protein level *in vivo*. While this implicates *Cdkn1c* as a major mediator of TGFβ1 signaling in HSCs, further pharmacological or genetic experiments are needed to confirm the mechanism.

TGFβ1 is a trophic factor important for HSC quiescence [24] and regeneration [25], and *Cdkn1c* is the only canonical cyclin-dependent kinase inhibitor (CDKI) with high expression in HSCs [35] and the only CDKI induced in HSCs during the window of TGFβ1 activation [25]. Identification of a trophic factor which seems to differentially regulate the activity of lineage-biased HSC subtypes may help explain some of the biology underlying HSC heterogeneity. For example, lymphoid-biased HSCs predominate in the fetal liver [12] while myeloid-biased HSCs accumulate with age [3–5], although it may be more appropriate to refer to aged HSCs as “lymphoid-deficient” [12, 36]. Perhaps the development of these HSC subtypes during ontogeny is reflective of changes in TGFβ1 levels in the niche, or alterations in the proportions of TGFβ1 producing cells in the bone marrow. Exploitation of the TGFβ1-*Cdkn1c* axis may have therapeutic potential in bone marrow transplant by stimulation of HSCs prior to transplant, a scenario in clinical trial for prostaglandin E₂ [37], or “rejuvenation” of aged HSCs.

In summary, we provide further evidence in support of the HSC heterogeneity concept supported by clonal analysis of gene expression profiles. Such studies are essential for determining the responses of lineage-biased HSC subtypes to different growth factors and

stress conditions, which may be missed by averaging the analysis of bulk cell populations. Further identification of additional markers to stratify lineage-biased HSC subtypes is crucial for meaningful interpretation of forthcoming next-generation sequencing platforms such as single-cell RNA-SEQ and epigenetic profiling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank members of the Challen and Dr. Margaret Goodell (Baylor College of Medicine) labs for helpful discussions and suggestions. Cell sorting and analysis was performed at the Washington University in St. Louis Siteman Cancer Center and Department of Pathology and Immunology flow cytometry cores. OP9-DL1 cells were a kind gift of Dr. JC Zúñiga-Pflücker (University of Toronto). Single-cell gene expression analysis was performed in collaboration with the Washington University in St. Louis Genome Technology Access Center (GTAC).

Support

This work was supported by grants awarded to G.A.C. from the National Institutes of Health (DK084259), Alex's Lemonade Stand, and the Children's Discovery Institute.

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Figure 1. Hoechst dye efflux can discriminate lineage-biased HSC subtypes

(A) Classification of the outputs of single lower- or upper-SP^{KLS} cells in bone marrow transplantation assays according to Dykstra and Eaves classification. (B) Cell sorting gates used for isolation of cells from different SP fractions by flow cytometry. (C) Peripheral blood chimerism from transplantation of 200 lower-, mid- or upper-SP^{KLS} cells (n = 16, 10, 17 respectively). (D) Proportional blood cell output of transplanted cell fractions short-term (4-weeks) and long-term (16-weeks) post-transplant. (E) Cell distribution of donor-derived (CD45.2⁺) SP^{KLS} cells following primary transplant. (F) Peripheral blood chimerism in secondary recipients from transplantation of 200 lower-, mid- or upper-SP^{KLS} cells post-primary transplant (n = 13, 9, 12

respectively). (G) Proportional blood cell output of transplanted cell fractions in secondary recipient mice. Mean $\pm$ SEM values are shown, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Figure 2. Differentiation potential of SP fractions *in vitro*

(A) Myeloid colonies formed after 14-days growth in Methocult M3434 ($n = 4$). (B) Numbers of B-cells formed per well after 14-day culture in IL-7 and Flt3L from initial seeding of 250 cells ($n = 8$). (C) Flow cytometric gating scheme for analysis of T-cell populations. (D) T-cell maturation of SP fraction cells after 14-days culture in lymphoid cytokines on OP9-DL1 stromal cells ($n = 4$). Mean $\pm$ SEM values are shown.

Figure 3. Gene expression of SP fraction clones at the single-cell level

Supervised gene expression clustering of lower-, mid- and upper-SP^{KLS150} clones into gene functional categories. Red arrows indicate genes of emphasis.

Figure 4. Clonal analysis of gene expression patterns can segregate SP fraction cells

(A) Principal component analysis of single-cell gene expression data shows weak clustering of SP fraction cells into discrete cluster (enclosed ovals). (B) Violin plots showing distribution of gene expression amongst SP fraction clones showing differential expression of Meis1, C/EBPα and Sfpi1 between the three populations. (C) While almost all clones from the three SP fractions were positive for Dnmt3a expression, Dnmt3a was expressed at higher levels in lower-SP^{KLS150} clones compared to mid- and upper-SP^{KLS150} cells. *** = $p < 0.001$.

Figure 5. Clonal analysis of the molecular response of SP fraction cells to TGFβ1

(A) Proliferative response of SP fraction cells to TGFβ1 (n = 4; LSP = lower-SP^{KLS150}; MSP = mid-SP^{KLS150}; USP = upper-SP^{KLS150}; Ctl = PBS control; TGF = +20pg/mL TGFβ1). (B) Expression of Cdkn1c in lower-, mid- and upper-SP^{KLS150} clones exposed to TGFβ1. (C) Cdkn1c immunofluorescent staining of lower-, mid- and upper-SP^{KLS150} cells following exposure to TGFβ1 *in vivo*. (D) Tukey box and whisker plot showing distribution of Cdkn1c protein expression in lower-, mid- and upper-SP^{KLS150} cells from control (PBS) and TGFβ1 treated mice. * = p<0.05.