Published in final edited form as:

Exp Hematol. 2016 June ; 44(6): 447–450. doi:10.1016/j.exphem.2016.03.003.

Understanding hematopoiesis from a single-cell standpoint

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Abstract

The cellular diversity of the hematopoietic system has been extensively studied, and a plethora of cell surface markers have been used to discriminate and prospectively purify different blood cell types. However, even within phenotypically identical fractions of hematopoietic stem and progenitor cells or lineage-restricted progenitors, significant functional heterogeneity is observed when single cells are analyzed. To address these challenges, researchers are now using techniques to follow single cells and their progeny to improve our understanding of the underlying functional heterogeneity. On November 19, 2015, Dr. David Kent and Dr. Leïla Perié, two emerging young group leaders, presented their recent efforts to dissect the functional properties of individual cells with a webinar series organized by the International Society for Experimental Hematology. Here, we provide a summary of the presented methods for cell labeling and clonal tracking and discuss how these different techniques have been employed to study hematopoiesis.

Cellular heterogeneity within defined populations is becoming increasingly evident, while examination of cellular cohorts at the population level may obscure unique properties of individual cells. For example, hematopoietic stem and progenitor cells (HSPCs) are defined as multipotent cells able to give rise to all hematopoietic (myeloid, lymphoid, and thrombo-erythroid) lineages. However, there is growing evidence that subpopulations with inherent lineage bias exist. In addition, it has been postulated that committed progenitor populations may be inherently heterogeneous. Given the heterogeneity of those cellular compartments, single-cell analysis is essential to define their functional potential.

Single-cell sorting has been employed by the stem cell field to address function of individual cells through either in vivo transplantation or in vitro culture experiments. With advances in sequencing technology, single cells can be assayed for their entire DNA sequence (genome) [1], RNA expression (transcriptome) [2], DNA methylation and chromatin structure

(epigenomes) [3], and most recently, the combination of both epigenome and transcriptome [4,5]. Evaluation of genome-wide information at the single-cell level provides unique insights into the potential of individual cells, but requires destruction of the starting cell, and thus, functional output cannot be performed in tandem [6–8]. However, several tools have been developed to address this problem. First, flow cytometric index sorting allows for retrospective analysis by collecting and comparing parameters (light scattering properties, cell surface marker expression levels) from each of the individual sorted cells from the same experiment. Second, viral barcoding provides a powerful way to assay multiple single cells in the same assay, but is limited by the genetic manipulation of starting cells. In tandem, such powerful methods can provide novel insights into the cellular heterogeneity of defined hematopoietic cell types. On November 19, 2015, Dr. David Kent and Dr. Leïla Perié highlighted techniques employed by their groups to study the functional properties of individual cells with a webinar series organized by the International Society for Experimental Hematology (ISEH) [9,10] and moderated by Dr. Claudia Waskow. (The webinar can be viewed at the ISEH website [11].) Here, we present an overview of this webinar together with advantages and limitations of the main techniques used to identify functional differences between hematopoietic populations: index sorting and viral barcoding (Fig. 1).

Linking genome-wide expression data with functional properties in single cells—David Kent

One long-standing challenge in stem cell biology is the identification of distinct molecular markers that would allow isolation of pure, functional hematopoietic stem cells (HSCs). Over the last decades, a number of laboratories have developed different cell surface marker combinations or used reporter gene constructs to prospectively isolate HSCs with achieved purities ranging 20%–50% [12–16]. Although some transplantation failures may be partially attributed to the technical challenges of single-cell transplants, a sizable fraction of analyzed cells do not appear to have stem cell properties. These “contaminating cells” within the isolated HSC population therefore obscure subsequent functional or gene expression analyses. As mentioned above, a variety of functional assays have revealed vast heterogeneity within the HSC pool, as single stem cells exhibit differences in lineage output [17–19], repopulation kinetics [20,21], and response to extrinsic factors [22].

To address these challenges, Dr. Kent presented his recent work in the first part of the webinar. In collaboration with Bertie Gottgens’ laboratory, Dr. Kent hypothesized that comparing gene expression profiles of HSCs isolated with different strategies would reveal a conserved/overlapping molecular profile between HSCs that would not be shared by various contaminating cell fractions. Excluding contaminating cells based on the expected purity of each HSC population sorted would thus reveal the molecular signature of “true” stem cells and lead to identification of markers, enabling isolation of HSCs with higher purity.

To test this hypothesis, they combined single-cell gene expression techniques with single-cell in vivo assays and bioinformatic analysis. Initially, they isolated HSCs through four different immunophenotypic strategies (CD34⁺Flt3⁺CD48⁺CD150⁺KSL, CD45⁺EPCR

⁺CD48⁻ CD150⁺, CD34⁻Flt3⁻KSL, and SP CD150⁺ KSL) as well as five types of progenitor cells. The expression of 43 genes was compared across those nine cell populations (1,800 single cells total) by single-cell quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) [23]. Using multidimensional mathematical analysis (t-distributed stochastic neighbor embedding analysis [t-SNE]), they presented data in single-cell plots confirming that most cells of the same population clustered together. As initially hypothesized, they identified a region where differently sorted HSC populations overlapped, thus sharing a common gene expression profile (termed *molecular overlapping* [MoLo]) compared with those outside that region (no molecular overlap [NoMo]).

Taking advantage of flow cytometric index sorting, a technique that quantifies the intensity for all parameters used for the isolation of single cells, they were able to retrospectively link the cell surface marker expression of sorted cells with their outcome in downstream assays [24,25]. This permitted Dr. Kent and colleagues to associate gene expression data of MoLo HSCs with the expression levels of all fluorescence markers used for their isolation. By doing so, they revealed that 28 of 43 genes were differentially expressed between HSCs located in the overlapping region and those in the non-overlapping region. Interestingly, MoLo HSCs exhibited higher expression for Sca1 and CD150 and lower expression for CD48 than NoMo. To functionally test these results, CD48⁻CD150⁺Sca1⁺ HSCs were divided between Sca1 high-expressing (SLAM Sca1^{hi}) and low-expressing cells (SLAM Sca1^{lo}), and their HSC potential was assessed in both in vitro and in vivo assays [23]. Monitoring the cell cycle profile, colony size, and immunophenotype after in vitro culture revealed that SLAM Sca1^{hi} cells were enriched for behaviors typically associated with stem cells (slow division kinetics, small colony size, retaining cell surface marker expression in culture). SLAM Sca1^{hi} HSCs led to higher donor chimerism while producing all hematopoietic lineages compared with the myeloid-deficient SLAM Sca1^{lo} cells in bulk transplantation experiments. In addition, single-cell transplants illustrated that this strategy based on three markers (CD48, CD150, and Sca1) yields HSCs with at least 50% purity, comparable to previous schemes using three markers (CD150, CD48, CD41) [13].

To further investigate the underlying HSC heterogeneity at the transcriptome level, single-cell RNA sequencing was performed and identified differential expression of 4,533 genes between single CD34⁻Flt3⁻CD48⁻CD150⁺KSL cells. Going one step further, Dr. Kent presented plots that could link the single-cell RNA sequencing data with single-cell transplantation data, as populations used in both assays were index sorted for the exact same flow cytometry parameters. Bioinformatic analysis of those data identified EPCR as a marker positively correlating with retention of functional stem cell properties, while negatively correlating with differentiation. Indeed, isolating SLAM Sca1^{hi} EPCR^{hi} cells improved HSC purity to almost 70% as shown by single-cell transplantations [23], providing a novel strategy for functional HSC isolation.

Cellular barcoding following multiple single-cell lineages in vivo—Leïla Perié

In her seminar, Dr. Perié presented the methods used by her laboratory to perform barcoding and lineage tracing of hematopoietic progenitors. Her data indicate that lymphoid-primed multipotent progenitors and common myeloid progenitor populations are highly heterogeneous, containing lineage-restricted cells of different commitment potential.

The Perié laboratory uses a library of small, noncoding DNA sequences as barcodes. These are cloned into lentiviral vectors that also express a fluorescence reporter for easy isolation of transduced cells. For lineage tracing, cultured hematopoietic progenitors are transduced with the lentiviral library. Those sequences will integrate into the genome of transduced cells, allowing identification of their progeny from the presence of unique DNA barcodes. The transduced progenitors are then injected into myelo-ablated recipients, and the lineage contribution of each barcode is assessed by purifying specific hematopoietic populations at different time points after transplantation and performing nested PCR amplification and next-generation sequencing.

During her seminar, Dr. Perié emphasized some important aspects of cellular barcoding. For this method to be successful, validating the ability of the viral vectors used to transduce the cells of interest while keeping transduction efficiency low (between 5% and 10%) is essential to ensure a single DNA barcode per progenitor. The size of the library is also important: the number of cells to be transduced should be several orders of magnitude smaller than the diversity of the library (to ensure that each progenitor has a unique barcode). Also, the length of the barcodes will affect sequencing costs. Dr. Perié recommended sequencing the full library before any experiment to create a reference library and facilitate bioinformatic analysis of the data generated. Another important consideration is controlling the number of different progenitors transduced with the same barcode. To do so, Dr. Perié suggested transplanting the pool of transduced cells into at least two separate recipients and checking whether the same barcode appears in both mice.

It is also important to be aware of the limitations of cellular barcoding. The first limitation is that it provides no information on the exact time of commitment; if a progenitor gives rise to two different cell types, it is impossible to determine whether this occurs early or late during cell maturation. It also provides no information on whether the transduced progenitor underwent trans-differentiation or de-differentiation instead of commitment to one or more lineages. An important technical limitation is that the technique requires in vitro culture, use of lentivirus, and lengthy transplantation of the transduced progenitors into myelo-ablated recipients. All those steps can affect lineage commitment decisions and not reflect actual lineage differentiation during homeostasis.

Using the methods described above, Dr. Perié presented data indicating that early murine hematopoietic progenitors are highly heterogeneous and, in most cases, already committed to specific lineages. Transplanting lentivirally barcoded CD 16/32⁻CD127⁻CD 117^{hi}Sca1⁺CD 135^{hi} lymphoid-primed multipotent progenitors (LMPPs) into myelo-ablated recipients revealed that LMPPs are extremely heterogeneous in their lineage output. Most

LMPPs were already committed to dendritic, myeloid, or B-cell lineage, and only a small fraction was capable of multilineage reconstitution [26]. These results indicated that LMPPs could generate dendritic cells directly without passing through a common myeloid progenitor (CMP) or common lymphoid progenitor (CLP) stage [26]. These, together with mathematical modeling, suggested that the classic hematopoietic differentiation tree should be revised to include new LMPP subtypes [27]. Using the same technique, Dr. Perié and her colleagues examined lineage commitment to erythroid and myeloid lineages from the CMP stage. They found that the CMP population was also heterogeneous, with most CMP already committed to either myeloid or erythroid lineages, whereas only 5% of CMPs are bipotent [28]. These studies revealed the heterogeneity of hematopoietic progenitors defined by cell surface markers and highlighted the power of cellular barcoding to investigate progenitors' potential with single-cell resolution.

Conclusions

Recent studies using these powerful single-cell methods have provided insights into the heterogeneity of the hematopoietic compartment, including committed progenitor cells and not just the early stem cell compartment, and have rigorously defined a cell surface marker combination for HSCs. Also, the power of single-cell studies was highlighted and will soon become the norm for evaluating cell function and potential, as further optimization and enhancement of current methods to analyze genome-wide information generated from single cells are ongoing.

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Figure 1.

Single-cell methods used to define properties of individual cells that are masked in population-based experimental paradigms. Index sorting allows for the retrospective analysis of fluorescence-activated cell sorting (FACS) data post-experiment (i.e., after RNA sequencing, single-cell transplant, clonal culture assays). Lentiviral barcoding allows tagging of a plethora of single cells (after purification or enrichment of a population) that can then be used to track the potential of individual cells. There are benefits and drawbacks to each method, but both have been used to establish more in-depth appreciation of the heterogeneity in primitive hematopoietic cell potential.