When blood development meets single-cell transcriptomics

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Abstract

Blood cells arise during embryonic development by three temporally distinct waves. Belonging to the third wave, hematopoietic stem cells (HSCs) are generated from hemogenic endothelium via endothelial-to-hematopoietic transition in mid-gestational embryos. Recently, studies combined with single-cell transcriptomics have provided massive new insights into the molecular evolutions and the underlying mechanisms of distinct waves of hematopoietic specification. In this review, we discuss the current single-cell profiling techniques, the most recent novel findings involved in the generation of distinct waves of blood cells, especially the HSCs, using single-cell transcriptional profiling combined with functional evaluations, and the perspectives to use the accumulating huge single-cell transcriptional data sets to study developmental hematopoiesis.

Keywords: Development, Hematopoietic stem cell, Single-cell transcriptomics

1. INTRODUCTION

Hematopoietic development during embryogenesis occurs in mainly three waves. The first or primitive wave gives rise to primitive erythrocytes, megakaryocyte, and macrophages, starting from embryonic day (E) 7.5 in mouse exclusively in the yolk sac from Flk1$^+$ mesoderm. Shortly after, the second or transient definitive wave takes place in multiple embryonic sites, which generates multipotent hematopoietic progenitors, such as erythroid and myeloid progenitors (EMPs) and immune-restricted progenitors. Not until E10.5 the first self-renewing hematopoietic stem cells (HSCs) are detectable in mouse embryo in the aorta–gonad–mesonephros (AGM) region, marking the third wave or definitive HSC generation.[1] HSCs are most stringently and functionally defined as the ones having the long-term robust multilineage repopulating hematopoietic activity. It has now been acknowledged that the definitive waves of hematopoiesis are directly derived from a kind of specified vascular endothelial cells, namely, hemogenic endothelial cells (HECs) via endothelial-to-hematopoietic transition (EHT).[1,2] Due to the rapid dynamic molecular changes during hemogenic fate choice, the development of single-cell technologies would promisingly shed light on the precise identification of the extremely rare and pivotal cell populations during blood cell emergence.

Nowadays, several single-cell techniques can achieve single-cell transcriptional profiling, namely, single-cell quantitative PCR (sc-qPCR), well-based single-cell RNA sequencing (scRNA-seq), and droplet-based scRNA-seq, the choice of which depends on the biological question needed to be addressed. Sc-qPCR allows targeted analysis of known genes, which has been instrumental in studying specific signaling pathways or transcription factors. Nevertheless, genome-wide transcriptomics approaches are needed to identify novel factors. On comparing the two scRNA-seq techniques, the droplet-based one demonstrates high-throughput albeit higher risk of cell doublets, which could be used as a screening strategy for unbiased analysis of the whole tissue. To answer specific questions or study the minority cell population such as HSCs or HECs, the well-based scRNA-seq should be much more accurate.[3] Single-cell transcriptional profiles have not only provided a landscape of the molecular programs during different waves of blood emergence but also enabled the computational screening for the molecular mechanisms underlying these developmental processes (Figure 1).

2. HSC-INDEPENDENT WAVES OF HEMATOPOIETIC SPECIFICATION BY SINGLE-CELL TRANSCRIPTIONAL PROFILING

Based on the biological knowledge, several algorithms have been developed to reconstruct the specification path of blood cells from mesodermal progenitors.[4,5] The Flk1$^+$ or CD41$^+$ cells in mouse embryos ranging from E7.0 to E7.75 were profiled by well-based scRNA-seq and the proposed trajectory of the first wave blood fate commitment recapitulates the gradual upregulation of several known regulators such as Gata1 and Nfe2.[6] Furthermore, combined usage of dpath, self-organizing map, and random walk with restart algorithms allows the reconstruction of

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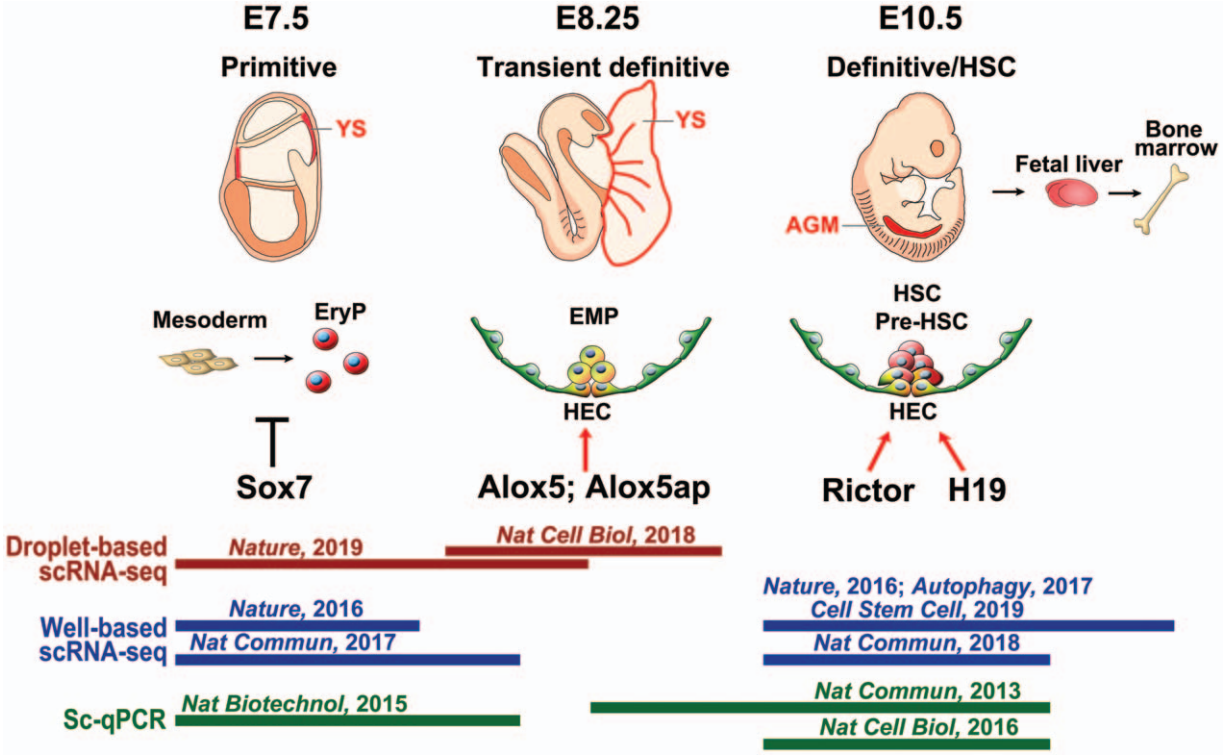

Figure 1. Decoding distinct waves of hematopoietic specification by single-cell transcriptional profiling. Three waves of blood cell generation in mouse embryos at temporally distinct developmental stages: primitive (E7.5), transient definitive (E8.25), and definitive/HSC (E10.5). The representative sites for different waves of hematopoiesis are shown in red. Blood cells are generated from mesoderm progenitors in the primitive wave in the yolk sac (YS) to form primitive erythrocytes (EryPs) and from HECs in the definitive waves either in the YS to form erythroid-myeloid progenitors (EMPs) or in the AGM region to form pre-hematopoietic stem cells (pre-HSCs) and HSCs. Later in the embryos, HSCs migrate to the fetal liver for expansion and subsequently colonize the bone marrow for maintenance through adulthood. The reported data sets (each for one line) and related references based on the three kinds of single-cell profiling techniques to decode the molecular programs underlying these developmental processes are shown, including single-cell quantitative PCR (sc-qPCR, green), well-based single-cell RNA sequencing (scRNA-seq, blue), and droplet-based scRNA-seq (red). Combined with functional investigations, the roles of Sox7, Alox5 and Alox5ap, Rictor, and lncRNA-H19 in regulating distinct waves of blood generation have been revealed as indicated.

the lineage hierarchies from Etv2-expressing precursors to the hematopoietic and endothelial lineages.[5] Recently, an atlas based on droplet-based scRNA-seq has been reported, in which mouse embryos at nine sequential time points ranging from E6.5 to E8.5 were collected. The constructed trajectory toward the primitive erythroid lineage does not include the endothelial clusters, further validating the origin of the first wave of hematopoiesis not from mature endothelial cells.[2,7]

Based on the transcriptional identification of distinct cell populations, the function of key hematopoietic transcription factors could be reevaluated. When cells were collected from E7.5 to E8.25, *Scl* knockout led to the downregulation of several hematopoietic regulators but an unchanged expression of cardiac markers in the transcriptional-defined endothelial population, in addition to the expected lacking of primitive erythrocytes.[6] When using chimeric mouse models and embryos were obtained at E8.5, $Scl^{-/-}$ cells assigned to a given endothelial population demonstrated an upregulation of cardiac-related genes, consistent with previous reports that $Scl^{-/-}$ yolk sac endothelial cells can adopt a cardiac fate.[7,8] On the other hand, several novel factors involved in these HSC-independent hematopoiesis waves have been computationally predicted and functionally validated, including the need of Sox7 downregulation for blood generation[4] and the regulatory role of leukotriene pathway in the EMP development in the yolk sac.[9]

3. ENDOTHELIAL-TO-HEMATOPOIETIC TRANSITION IN AGM REGION BY SINGLE-CELL TRANSCRIPTIONAL PROFILING

Several single-cell transcriptional profiling studies on HECs, cells undergoing EHT, intra-aortic hematopoietic cluster (IAHC) cells, and hematopoietic stem progenitor cells in the AGM region have been reported in recent years, with the aim of precisely delineating the process of EHT, which is required for HSC emergence.[10–12]

Using the GFP transgenic reporter under the control of *Runx1* +23 enhancer ($23GFP^+$) as the marker of putative HECs, several HEC-related cell populations in PAS/AGM region from E8.5 to E11.5 mouse embryos, including $23GFP^-$ ECs, $23GFP^+$ HECs, and $CD41/CD45^+$ hematopoietic progenitor cells, were transcriptionally profiled using Fluidigm sc-qPCR for 18 hematopoietic and endothelial featured genes. Combined with single-cell in vitro endothelial–hematopoietic potential evaluation, an early-onset model of EHT was proposed: that the hematopoietic program initiates at E9.5 accompanied by an earlier loss of endothelial potential.[10]

The expression of *Gfi1*, a downstream target of Runx1, has been shown to specifically define the HECs that generate emerging HSCs.[11] Combined with previous notion that Kit is expressed in the IAHCs but not endothelial layer, the $Cdh5^+Gfi1^+Kit^-$ cells are therefore defined as HECs and

Cdh5$^+$Gfi1$^+$Kit$^+$ cells as IAHCs.[11,13] The Gfi1$^-$ ECs, Gfi1$^+$ HECs, and IAHCs were then transcriptionally profiled using either sc-qPCR of scRNA-seq.[11,12] Interestingly, very similar transcriptomes between Gfi1$^-$ ECs and Gfi1$^+$ HECs are revealed by scRNA-seq, in contrast to an obvious difference between the two populations by Fluidigm sc-qPCR analysis of 70 hematopoietic and endothelial genes.[11,12] By mechanically picking up single whole IAHCs (wIAHCs) in the aortas around HSC emergence, the cellular components of IAHCs were investigated at single-cell level, showing pre-HSCs are predominantly involved. Ventral and dorsal wIAHCs are very similar in both cellular composition and transcriptomes. Moreover, larger wIAHCs contain more heterogeneous cells but do not enrich a specific cell type.[12]

4. DECODING PRE-HSC AND HSC DEVELOPMENT BY SINGLE-CELL TRANSCRIPTOMICS

Pre-HSCs are important intermediate cell populations between HECs and HSCs, the efficient isolation of which is pivotal for the subsequent transcriptional and functional studies. Through a set of screening with rigorous single cell-initiated serial transplantations, functional type I (T1) and T2 pre-HSCs both have been identified with high purity by the specific high expression of surface marker CD201 (coded by *Procr*).[14] Using polyA$^+$ enriched full-length RNA sequencing, the molecular characteristics of six populations related to HSC ontogeny were analyzed, namely, endothelial cell, T1 and T2 pre-HSCs in E11 AGM region, and HSCs in E12 and E14 fetal liver and adult bone marrow. These single-cell transcriptomic data further revealed a series of previously unknown features of developing HSCs. In contrast to the proliferative property of fetal liver HSCs and quiescent state of adult HSCs, heterogeneity exists in the functional pre-HSCs regarding the cycling status. Moreover, surface marker CD47 has been computationally screened out and functionally identified to enrich all the HSC-competent cell populations in AGM regions, including endothelial cells, pre-HSCs, and mature HSCs.[14] Notably, signaling pathway analysis combined with functional evaluation revealed the specific role of mTORC2 in HSC emergence but not thereafter.[14] In addition, the autophagy signaling has been predicted to play a potential role during HSC emergence, which needs further functional verification.[15]

Long non-coding RNAs (lncRNAs) function via multiple ways to modulate the biological processes throughout embryonic development and adult homeostasis.[16,17] Recently, the single-cell dynamic lncRNA profiles during HSC ontogeny are depicted.[18] In total, 401 unannotated lncRNAs have been identified, including the ones previously defined as the adult HSC-specific lncRNAs,[19] which exhibit different dynamics during HSC ontogeny.[18] Of note, lncRNAs show advantages as compared with mRNAs to distinguish different HSC-competent populations, especially for the very alike E12 and E14 fetal liver HSCs.[18] Combining bioinformatics analysis with in vitro functional screening by knockdown, six out of ten candidate lncRNAs are found influencing embryonic hematopoiesis. Among them, lncRNA H19 deficiency leads to retarded EHT with the consequently severely impaired HSC generation in AGM region. Further scRNA-seq and DNA methylation sequencing revealed that lncRNA H19 deficiency results in the promoter hypermethylation and the consequent downregulation of several key hematopoietic transcription factors such as Runx1 and Spi1. The role of lncRNA H19 in embryonic hematopoiesis is independent of its processed product mir-675, which is required for the maintenance of adult HSC homeostasis.[18,20]

5. FUTURE PERSPECTIVES

The advance in single-cell transcriptional profiling technologies have made great contribution to decode embryonic hematopoiesis, especially the ontogeny of HSCs. Combining computational screening with functional evaluation, accumulating novel regulatory mechanisms, such as unannotated lncRNAs or other RNA modification factors, would be promisingly uncovered in the near future. Meanwhile, the genomic level analyses are imminent. With scATAC-seq, scChIP-seq, or even single-cell integrated multi-omics,[21–24] the molecular programs and underlying mechanisms of blood development would be revealed in multiple dimensions. Prospectively, a series of novel genetic lineage tracing mouse models could be constructed based on the newly transcriptional-identified specific markers. Combined with the high-resolution single-cell time-lapse live imaging technologies,[25,26] the spatiotemporal development of blood cells would be elucidated in unprecedented detail. Most importantly, progresses of these technologies will provide a prospective research paradigm for decoding the occurrence of human hematopoietic system, which still remains elusive as compared to that being intensively studied in mouse. All these detailed knowledge of blood development would be promising to instruct the generation of any given type of hematopoietic cells from pluripotent stem cells in vitro in the future.

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