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Single-cell RNA-seq technology lends a hand into HSC ontogeny

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Hematopoietic stem cells (HSCs) are rare populations in the bone marrow of adult mammals which produce millions of blood cells for daily circulation by balancing self-renewal and multi-lineage differentiation. In vertebrates, a subset of specialized mesodermal precursor cells commit to hematopoietic cells after gastrulation, which are separated into two major waves. The initial wave, termed primitive hematopoiesis, mainly exists in the mammalian yolk sac, which produces early erythroid cells to facilitate tissue oxygenation during the rapid growth of embryos. The second wave, termed definitive hematopoiesis, gives rise to HSCs with long-term reconstitution capability that arise from hemogenic endothelium (HE) in the aorta-gonad mesonephros (AGM) region, as well as the placental labyrinth and vitelline and umbilical arteries, and subsequently colonize the fetal liver (FL), spleen, thymus, and eventually occupy the bone marrow (Orkin and Zon, 2008). In past decades, several surface markers have been reported to identify the precursor of HSCs, so-called pre-HSCs, including CD41, CD45, CD31, c-Kit, vascular endothelial cadherin (VE-cadherin), etc. However, due to the rarity and dual-potential (both hematopoietic and endothelial) of pre-HSCs, their heterogeneity and unique surface markers remain elusive. In a study recently published on *Nature*, Zhou F et al for the first time captured the nascent pre-HSCs using a new combination of markers including CD201 (endothelial protein C receptor, EPCR) (Zhou et al., 2016). Zhou F et al further developed the single-cell initiated serial transplantation, and validated that the $CD31^{+}CD45^{-}CD41^{low}$c-$Kit^{+}CD201^{high}$ and $CD31^{+}CD45^{+}$c-$Kit^{+}CD201^{high}$ subsets in the E11 AGM region could highly enrich T1 and T2 pre-HSCs at frequencies of 1:2.3 and 1:2.1, respectively, indicating the high purity of pre-HSCs.

Accumulated evidence has documented that heterogeneity is a feature of HSC proliferation, self-renewal, and differentiation, which is finely-tuned by myriad intrinsic and extrinsic mechanisms, and is believed to play a role in disease evolution. Due to the rapidly evolving next-generation sequencing technology, several groups have developed single-cell RNA sequencing (RNA-seq) techniques which could analyze the transcriptome at single-cell and single-base resolutions (Tang et al., 2011). Together with the advances in flow cytometry allowing isolation of HSCs for single-cell transplantation assays of cellular fate choice, the molecular and functional heterogeneity as well as the cellular hierarchy have been demonstrated by several groups in both murine and human adult HSCs. These studies have identified key molecules that correlate with long-term self-renewal and have revealed single-cell molecular circuits that are linked to functionality of adult HSCs. However, single-cell RNA-seq analyses in pre-HSCs in mouse mid-gestation embryos have not yet been fully explored.

In their recent report, Zhou F et al made great efforts by performing multiple experiments, including single-cell co-culture/transplantation assays and single-cell RNA-seq, in five cell types related to HSC ontogeny, endothelial cells (ECs), T1 pre-HSCs, T2 $CD41^{low}$ HSCs, E12 and E14 FL HSCs. Principal component analysis (PCA) and unsupervised hierarchical clustering analyses revealed that T2 $CD41^{low}$ HSCs contained two subpopulations, with one be-

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ing similar to T1 pre-HSCs and the other exhibiting myeloid lineage signatures, indicating the presumed T2 $CD41^{low}$ HSCs were heterogeneous. Later, $CD31^{+}CD45^{+}c\text{-}Kit^{+}CD201^{high}$ cells were proven to be functional T2 pre-HSCs and were used to replace T2 $CD41^{low}$ HSCs. Further, single-cell RNA-seq analyses suggested that the T1 and T2 pre-HSCs were close to each other but distinct from ECs and FL HSCs, providing strong evidence to support the previous hypothesis that embryonic hematopoiesis develops from embryonic ECs to T1 and T2 pre-HSCs, and on to FL HSCs. Pre-HSCs have both hematopoietic and endothelial potential, but it is controversial whether HSCs originate from ECs either in major arteries or in veins (Ditadi et al., 2015). The single-cell RNA-seq data showed that T1 pre-HSCs expressed arterial markers but much lower levels of venous markers compared with ECs, suggesting that pre-HSCs might stem from arterial rather than venous ECs.

More interestingly, Zhou F et al observed a 4.4-fold increase in the average mRNA copy number, and a 6.9-fold increase in the absolute expression levels of 290 ribosome-associated genes in T1 pre-HSCs compared to ECs, respectively. These findings suggested that during ECs differentiation into pre-HSCs, there is a tremendous increase in both transcriptional activity and ribosome-based translational machinery. Mechanistically, gene set enrichment analysis (GSEA) revealed that both oxidative phosphorylation and glycolysis pathways were hyperactivated in the T1 pre-HSCs, implying that the entire mitochondrial metabolism was specifically activated at this stage. mTOR pathway has been reported to enhance mitochondrial biogenesis, activity and energy metabolism in HSCs and other systems (Qian et al., 2016). Indeed, mTOR pathway, especially the mTORC2 complex, was found highly enriched in T1 pre-HSCs compared with ECs. When Rictor, core component of mTORC2, was conditionally disrupted by endothelial Tie2-cre, HSCs from mutant AGM exhibited strikingly lower repopulating capacity than controls, suggesting mTORC2 pathway was essential for HSC emergence from endothelial cells. Moreover, T1 pre-HSCs in S/G2/M phase had the highest reconstitution capacity, which was lower in G0 phase and almost nonexistent in G1 phase. These data are different from adult HSCs which are in G0 phase, and from E14 FL HSCs which are in G1 phase, indicating the heterogeneity in cell cycle status of T1 pre-HSCs. The mechanisms underlying correlation between pre-HSC cell cycle status and functionality merit future investigation.

Although hematopoietic stem cell transplantation (HSCT) has been widely used for treatment of congenital and acquired hematopoietic malignancies for decades, donor availability and allogenicity remain unresolved issues. Compared to the strategies of using transcriptional factors to reprogram somatic cells into HSCs, or to differentiate ES/iPS into HSCs, it might be safer to mimic the HSC ontogeny *in vivo* and induce ES/iPS cells into HSCs *ex vivo* by use of cytokines and growth factors. However, such attempts have been largely unsuccessful due to our limited understanding of embryonic hematopoiesis. In this study, Zhou F et al comprehensively analyzed the transcriptomes of ECs, pre-HSCs, and FL HSCs at the single cell level, and revealed the underlying mechanisms for HSC emergence, providing extremely important information about signaling pathways and molecules by which the arterial endothelial cells give rise to the first definitive pre-HSCs. Moreover, since definitive HSCs arise not only from the AGM region, but also from other sites including head, placental labyrinth, and vitelline and umbilical arteries, it is worthwhile to perform single-cell RNA-seq in these regions and to compare the differences of molecular regulation network among these distinct sites.

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

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