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New insights into the endothelial origin of hematopoietic system inspired by "TIF" approaches

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Abstract

Hematopoietic stem progenitor cells (HSPCs) are derived from a specialized subset of endothelial cells named hemogenic endothelial cells (HECs) via a process of endothelial-to-hematopoietic transition during embryogenesis. Recently, with the usage of multiple single-cell technologies and advanced genetic lineage tracing techniques, namely, "TIF" approaches that combining transcriptome, immunophenotype and function/fate analyses, massive new insights have been achieved regarding the cellular and molecular evolution underlying the emergence of HSPCs from embryonic vascular beds. In this review, we focus on the most recent advances in the enrichment markers, functional characteristics, developmental paths, molecular controls, and the embryonic site-relevance of the key intermediate cell populations bridging embryonic vascular and hematopoietic systems, namely HECs and pre-hematopoietic stem cells, the immediate progenies of some HECs, in mouse and human embryos. Specifically, using expression analyses at both transcriptional and protein levels and especially efficient functional assays, we propose that the onset of Kit expression is at the HEC stage, which has previously been controversial.

Key Words: Endothelial-to-hematopoietic transition; Genetic lineage tracing; Hematopoietic stem progenitor cells; Hemogenic endothelial cell; Single-cell technology

1. INTRODUCTION

Lineage tracing and live imaging analyses in vertebrates show that hematopoietic stem progenitor cells (HSPCs) are derived from a subset of embryonic endothelial cells, named hemogenic endothelial cells (HECs), which undergo a process known as endothelial-to-hematopoietic transition (EHT).[1–3] Understanding how HSPCs are formed from their upstream endothelial precursors during embryogenesis will greatly shed light on the strategies for manipulating the de novo generation of HSPCs in dishes.[4–6] In recent years, the development of single-cell omics technologies and advanced genetic lineage tracing strategies have largely contributed to deciphering cellular heterogeneity, lineage relationships, and regulatory mechanisms during the establishment of vascular and hematopoietic systems.[7–18] Here, we focus on the recent progresses in the diversified embryonic endothelial specification, the transcriptomic and functional characteristics of HECs, and the molecular controls of EHT, and present a vision for the use of massive datasets and advanced technologies in the study of developmental hematopoiesis.

2. TRANSCRIPTOMIC, IMMUNOPHENOTYPIC, AND FUNCTIONAL IDENTIFICATION OF HECs

HECs are defined as a kind of specialized endothelial cells with the functional capacity to give rise to hematopoietic cells. In addition to this mostly important aspect, the definition of HECs also includes several other features: they must be endothelial cells in nature and usually express the transcription factor Runx1, localize in the vascular inner layer, and express endothelial rather than hematopoietic surface markers.[19] During embryonic development, multiple waves of hematopoiesis occur sequentially, which together build the principal hematopoietic system throughout the individual ontogeny.[20–24] In mammals, except for the primitive hematopoiesis, the cellular origin of which remains unclear that mesoderm progenitors or hemangioblasts are suggested to be the candidates,

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the occurrence of all the HSPCs once the primordial vasculature has been established, including hematopoietic stem cell (HSC)–dependent and HSC-independent hematopoiesis, no matter localized intra- or extra-embryonically, are believed to be derived from HECs.[25–31]

The formation of HSCs is a transient and rare event in mammalian embryos, occurring in the ventral part of dorsal aorta in aorta-gonad-mesonephros (AGM) region at embryonic day (E) 10.5 to E11.5, accounting for about 1 to 3 per embryo.[32,33] Efficient capture of the HSC-primed HECs has long been chased but has only recently been achieved, especially with the help of using the relevant high-precision single-cell transcriptomic profiling.[7,8,10–12,14] In addition to their AGM localization, the intra-embryonic HECs are readily recognized given their endothelial nature and the expression of Runx1 from the transcriptomic view. The number of the intra-embryonic HECs reaches the peak at E10.0 and almost becomes undetectable at E11.0.[7] Unambiguous arterial feature suggests the arterial endothelial origin of these HECs,[7,10,11,14] whereas they are more active in some biological processes including cell cycle and ribosome biogenesis compared to adjacent arterial endothelial cells.[7] Through the computational prediction and functional validation, a combination of surface markers ($CD31^+CD41^-CD43^-CD45^-Procr^+Kit^+CD44^+$, PK44, approximately 100 per embryo) is identified to efficiently enrich the HSC-competent HECs at E10.0.[7] Importantly, PK44 cells did not produce hematopoietic colonies in the CFU-C culture system, indicating they are not yet hematopoietic-committed.[7] PK44 cells are still molecularly and functionally heterogeneous and have the potential to differentiate into either endothelial or hematopoietic cells. Specifically, individual cells of a small proportion of PK44 cells display dual endothelial-hematopoietic potential, suggesting that the HECs represented by PK44 are undergoing hemogenic specialization, and have not been committed to either endothelial or hematopoietic cell fate.[7,8,34] Using an index-sorting strategy that can record the expression levels of multiple proteins of each sorted cell, the endothelial-biased population in PK44 cells can be further enriched by the marker combination of $CD93^{hi}Kit^{lo}$ or $CD146^{hi}Kit^{lo}$, while the hematopoietic-biased population can be enriched by $CD93^{lo}Kit^{hi}$ or $CD146^{lo}Kit^{hi}$.[34]

Several feature genes of HECs have been used to construct reporter mouse lines for the enrichment of HECs, represented by *Runx1*, *Gfi1*, and recently identified *Neurl3*.[7,8,35–37] Studies using a *Runx1+23* enhancer-reporter transgenic (*23GFP*) mouse model confirmed that hemogenic potential of the endothelial cells is restricted to the $23GFP^+$ population. At about E10, the proportion of $23GFP^+$ cells in AGM endothelial cells is approximately 10%. Interestingly, only 1/49 of the $23GFP^+$ cells have hemogenic potential, which is somewhat lower than expected. Consistently, *Runx1* transcripts are detected in less than half of $23GFP^+$ cells.[37] At E10.5, the hemogenic potential was reported to be restricted to Gfi1-$Tomato^+$ endothelial cells, which account for 2% to 3% of endothelial cells in the AGM region. However, as its expression is not detected at E9.5, it may not be sensitive enough for the study of HECs at earlier times.[36] Recent studies identified *Neurl3* as one of the signature genes of the in silico–identified intra-embryonic HECs,[7,8,11,18] and a *Neurl3-EGFP* knockin mouse model was further constructed.[7] Neurl3-$EGFP^+$ endothelial cells account for nearly half of the aortic endothelial cells in AGM region at E10, largely co-express Runx1 transcriptionally and histologically, and enrich the HSC competence. These characteristics make the *Neurl3-EGFP* mouse an ideal model for studying the biology of HECs.[7,8]

Along the developmental path from endothelial cells to HSC-primed HECs, a precursor cell population of HECs called pre-HECs has been proposed to exist. Transcriptomically, there was a developmental bottleneck separates pre-HECs from HECs.[10] Pre-HECs are characterized by the expression of ACE.[11,14] Compared to HECs, pre-HECs exhibited robust arterial characteristics, a more quiescent cell cycle state, and very low levels of Runx1 expression.[10,11,14] Considering the high similarity between the transcriptome of pre-HECs and the aortic endothelial cells prior to the emergence of HSCs, it is possible that these 2 differently named cells are essentially the same (**Fig. 1A**).

The inclusion of single-cell transcriptome data from endothelial cells at early stages of vascular development enables us to recognize the de novo origins of HECs and arterial endothelial cells. Intra-embryonic primitive endothelial cells from E8.0 experience 2-step fate choices to become HECs, namely an initial arterial fate choice followed by a hemogenic fate decision[9,38] (**Fig. 1B**). It is worth noting that during early vascular development in mammals, there exist 2 types of arterial endothelial cells with distinct transcriptomic profiles, anatomical distributions, and even cellular origins.[9,38] One type is the major artery endothelial cells, which express major artery marker CD44 in addition to the known arterial markers Dll4 and Unc5b, localized predominantly in the major arteries including aorta and intracranial arteries. Some of them continue to mature as major artery endothelial cells, while a subset of them specialize into HECs only in a short time window before E11. The other type is the arterial vascular plexus endothelial cells, which express Kitl, Ptp4a3, Nid2, and Vwa1 in addition to several known arterial markers such as Dll4 and Unc5b, and are widely distributed throughout the whole embryo in the form of small vessels. Of note, by genetic lineage tracing studies, they are recently shown to be derived from venous-featured endothelial cells via venous-to-arterial fate conversion, a process representing the first arteriogenesis in the mammalian embryo.[9,38] These arterial plexus endothelial cells seem not to be the direct upstream cell origin of HECs by computational prediction, and their future cell fates still need to be determined[9,38] (**Fig. 1B**). Understanding the characteristics, origins and fates of the 2 types of arterial endothelial cells is important for precisely resolving the de novo origin of HSCs, which would facilitate exploring the strategies of HSC regeneration in vitro.

HECs also exist in the extra-embryonic yolk sac besides the intra-embryonic AGM region.[39,40] The researchers found that the emergence of rounded hematopoietic cells from polygonal clusters of Kit^+ cells precedes the establishment of arborized arterial and venous vasculature in the yolk sac. In contrast to intra-embryonic HECs, which are only present in the arterial vascular bed, the flat $Runx1^+Kit^+$ presumptive HECs in the yolk sac can be detected in both arterial and venous vessels after remodeling.[39] Erythro-myeloid progenitors are thought to be derived from HECs present in both arterial and venous vascular bed of the yolk sac,[39] whereas in vivo and in vitro studies suggested that lymphoid potential is predominantly detected in the arterial vessels.[41,42] Recently, unbiased transcriptomic analyses have revealed that in general the arterial feature of HECs in yolk sac is much weaker than that in the AGM region[43,44] A study using Gja5-EGFP reporter as an arterial marker for functional assays showed that lymphoid potential can be detected in non-arterial yolk sac endothelial cells.[43] Recent studies have identified Neurl3-EGFP as a faithful marker for both intra-embryonic and yolk sac HECs. The combination of Neurl3-EGFP and Unc5b-Tomato reporters revealed that yolk sac HECs contain 2 subpopulations with different level of arterial characteristics, and the lymphoid potential in the yolk sac was enriched in Neurl3-$EGFP^+Unc5b\text{-}Tomato^+$ endothelial cells with an arterial-biased feature.[44] The different labeling efficiencies of arterial endothelial cells using different reporter mice and the distinct lymphoid induction strategies may underlie the seemingly conflicting conclusions.[43,44] In addition to Neurl3-EGFP, CD44 has been reported to enrich yolk sac endothelial cells with hemogenic potential at E10.0-E10.5.[43] The immunophenotypic PK44 cells in the yolk sac demonstrate short-term multilineage reconstitution capacity after in vitro incubation. The yolk sac PK44 cells show endothelial- or hematopoietic-biased characteristic similar to those in the AGM region, but display transcriptomic features

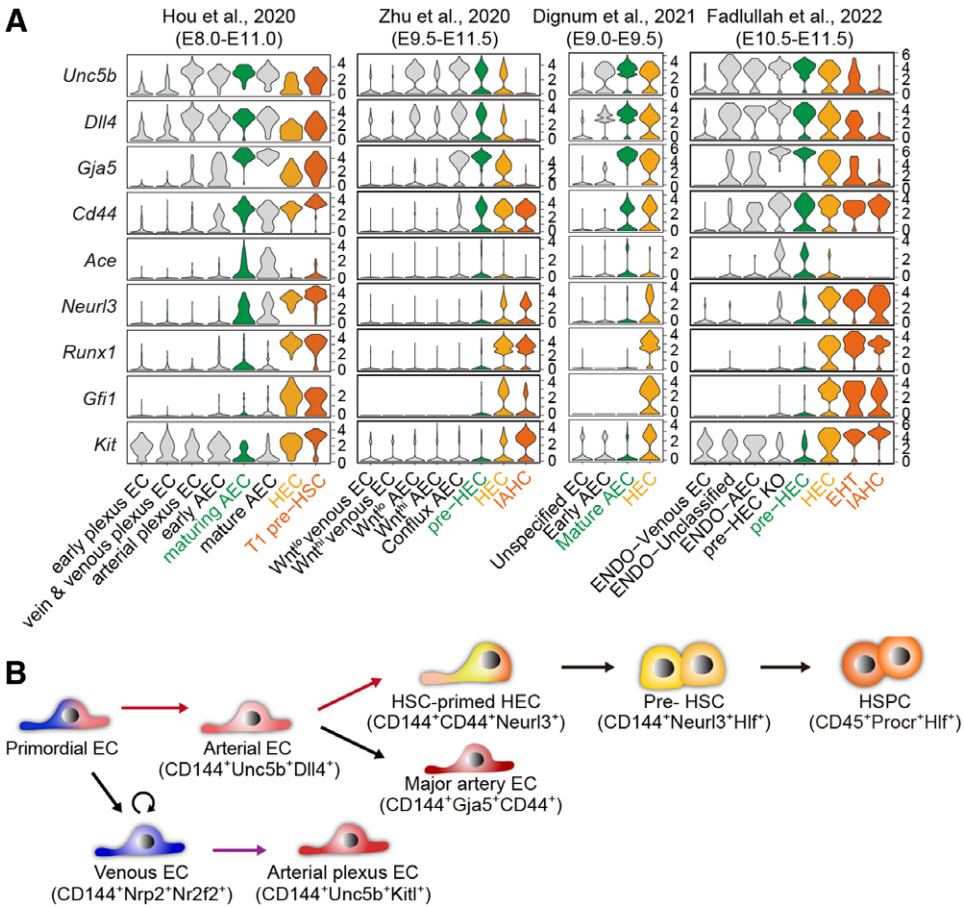


Figure 1. Cellular evolution and marker genes along arteriovenous and hemogenic specification during early vascular development in mouse embryos. (A) Violin plots showing the expression levels of indicated genes during the EHT process in multiple single-cell transcriptomics datasets. Note that *Kit* mRNA expression is initiated at the HEC stage. (B) Schematic diagram of cellular evolution and marker genes along arteriovenous and hemogenic specification during early vascular development. The red arrows represent the 2 fate choices experienced from primitive endothelial cells to HECs and the purple one represents the fate conversion from venous to arterial ECs. AEC = arterial endothelial cells, EC = endothelial cells, HEC = hemogenic endothelial cells, HSC = hematopoietic stem cell, HSPC = hematopoietic stem progenitor cell, IAHC = intra-aortic hematopoietic clusters.

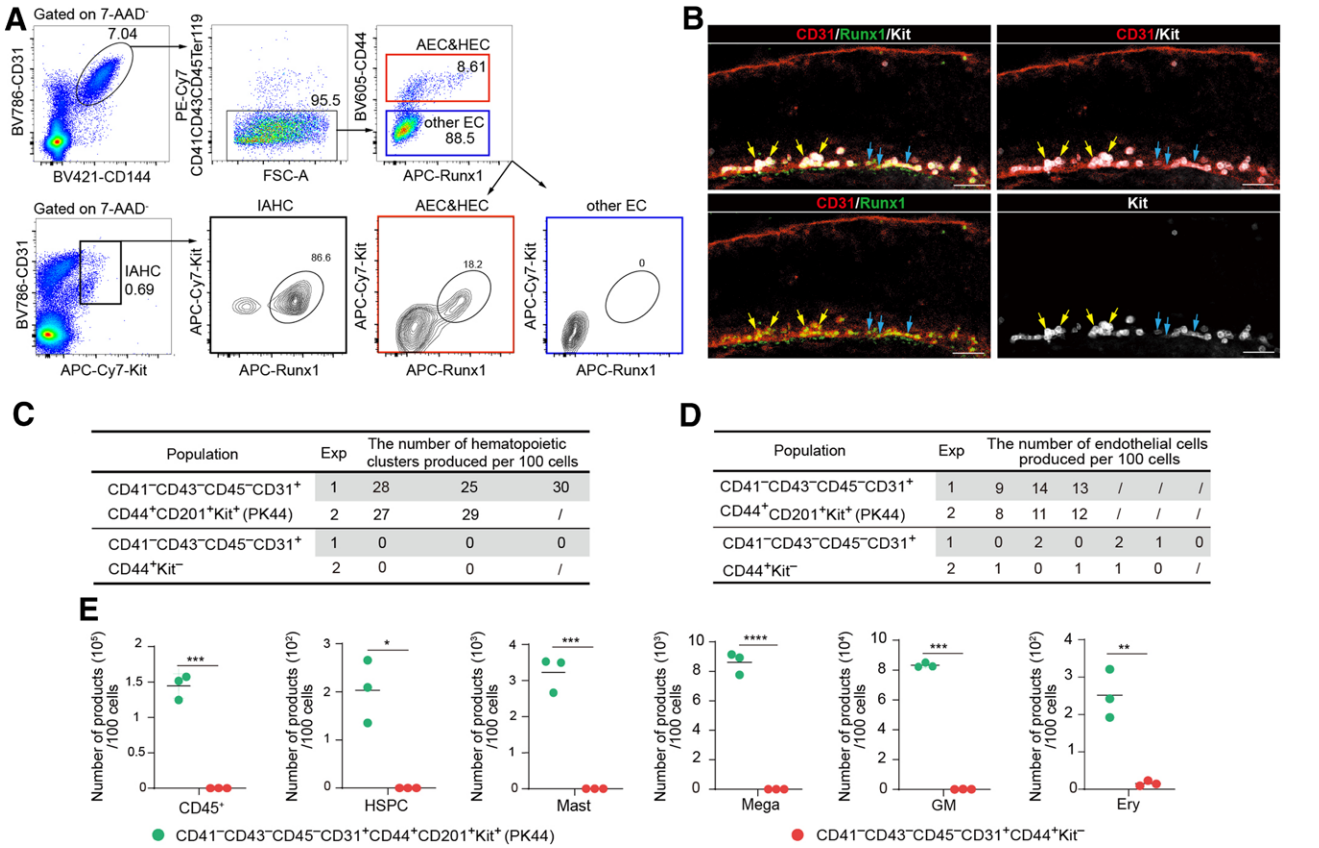
distinct from those in the AGM region, expressing yolk sac but not aortic endothelial cell markers such as Lyve1 and Stab2.[34]

3. KIT EXPRESSION ENRICHES THE FUNCTIONAL HECs IN MOUSE EMBRYOS

Along the procedure of EHT, Kit expression is usually thought to be initiated from the hematopoietic progenies of HECs located in the intra-aortic hematopoietic clusters (IAHCs) rather than in the HEC stage based predominantly on histological analysis.[2,11,35,45–47] In contrast, other groups showed that some flat cells located in the endothelial layer do have Kit expression, albeit at lower levels than IAHC cells.[48] Additionally, it has been shown that endogenous HECs are enriched in the Kit$^+$ population,[49] and recently, many research teams have begun to acknowledge that HECs with a Kit^{lo} or Kit$^+$ phenotypes.[50,51] These controversies are not only due to the different sensitivity of detection means, but also reflect the ambiguity of HEC definition given the difficulties in efficiently evaluating the blood-forming capacity of HECs in previous studies, while this functional aspect should be highly emphasized.

A combined analysis of 4 recently published single-cell transcriptomic datasets on the EHT process showed that *Kit* expression is clearly elevated at the HEC stage[7,10–12] and continues to increase in the pre-HSC and IAHC populations (**Fig. 1A**). It is widely acknowledged that HECs should express Runx1.[52] At the protein level, Runx1-positive endothelial cells are CD44-positive, consistent with the fact that the intra-embryonic HECs are predominantly distributed in the aortic endothelial layer (**Fig. 2A**). Importantly, Runx1-positive immunophenotypic endothelial cells in the aorta are essentially Kit-positive, albeit at relatively lower levels than IAHCs (**Fig. 2A**), in line with the transcriptomic findings (**Fig. 1A**). In situ immunostaining showed that some flat Runx1$^+$ cells located at the endothelial layer co-express Kit, although the expression level is lower than that in the IAHCs (**Fig. 2B**). Taken together, Kit is indeed expressed in HECs at protein levels, facilitating the use of Kit as a sorting marker of HECs for functional assays.

Using PK44 population as a positive control, the hemogenic potential of the aortic Kit$^-$ endothelial population (CD41$^-$CD43$^-$CD45$^-$CD31$^+$CD44$^+$Kit$^-$) has been assessed in parallel, showing that they hardly form typical hematopoietic clusters in the in vitro co-cultures (**Fig. 2C**). Interestingly, the endothelial tube forming capacity of the aortic endothelial Kit$^-$ population is also significantly lower than that of PK44 population (**Fig. 2D**). Further flow cytometric analysis validated that these Kit$^-$ aortic endothelial cells produce very few CD45$^+$ hematopoietic progenies, let along hematopoietic lineage cells (**Fig. 2E**). Taken together, we propose that functional HECs are essentially Kit$^+$ rather than Kit$^-$. This finding also emphasizes the functional evaluation as a crucial basis for the definition of HECs.



Population	Exp	The number of hematopoietic clusters produced per 100 cells		
$CD41^-CD43^-CD45^-CD31^+$	1	28	25	30
$CD44^+CD201^+Kit^+$ (PK44)	2	27	29	/
$CD41^-CD43^-CD45^-CD31^+$	1	0	0	0
$CD44^+Kit^-$	2	0	0	/

Population	Exp	The number of endothelial cells produced per 100 cells					
$CD41^-CD43^-CD45^-CD31^+$	1	9	14	13	/	/	/
$CD44^+CD201^+Kit^+$ (PK44)	2	8	11	12	/	/	/
$CD41^-CD43^-CD45^-CD31^+$	1	0	2	0	2	1	0
$CD44^+Kit^-$	2	1	0	1	1	0	/

Figure 2. Kit is expressed in HECs at the transcriptomic, protein, and functional levels. (A) Representative FACS plots showing the expression of Kit and Runx1 in the endothelial population (AEC and HEC, other EC) and IAHCs in E10.0 AGM region. (B) Representative whole-mount confocal image of the E10.0 AGM region stained with CD31, Kit, and Runx1. Blue arrows indicate $CD31^+Runx1^+Kit^{+/lo}$ endothelial cells distributed in the endothelial layer of the aorta. Yellow arrows indicate $CD31^+Runx1^+Kit^{+/hi}$ bulging and bulged cells. Scale bar: 50 μm. (C, D) Table showing the number of hematopoietic and endothelial progenies produced per 100 indicated cells after 7 d of co-culture. The "/" means inoculated without cells. (E) Graph showing the absolute number of hematopoietic products generated per 100 indicated cells after co-culture with OP9-DL1-GFP stromal cells. HSPC ($GFP^-CD45^+Sca1^+Kit^+Procr^+$); mast cells (Mast: $GFP^-CD45^+Kit^+T1/ST2^+$); megakaryocytes (mega: $GFP^-CD45^-CD41^+$); granulo-monocytes/macrophages (GM: $GFP^-CD45^+F4/80^+Gr1/Mac1^+$); erythrocytes (Ery: $GFP^-CD45^-Ter119^+$). Data are means ± SD from 3 independent experiments. *.01 < $P \le .05$; **.001 < $P \le .01$; ***.0001 < $P \le .001$; ****$P \le .0001$. AEC = arterial endothelial cells, AGM = aorta-gonad-mesonephros, EC = endothelial cells, FACS = fluorescence-activated cell sorting, HEC = hemogenic endothelial cells, HSPC = hematopoietic stem progenitor cell, IAHC = intra-aortic hematopoietic cluster, SD = standard deviation.

4. ENRICHMENT MAKERS AND HETEROGENEITY OF THE NASCENT HSCs IN MOUSE EMBRYOS

The enrichment markers of nascent HSCs in embryos have been explored for more than 2 decades. Nascent HSCs in AGM region still express endothelial cell markers such as CD34, CD144, and CD31,[47,53,54] and have also begun to express the hematopoietic marker CD45,[54] as well as the stem cell marker Kit.[47] *Ly6a-GFP* transgene and *Hlf-Tomato* knockin reporter mouse models have also been constructed to enrich nascent HSCs.[55–57] All AGM HSCs express Ly6a-GFP$^+$ and the percentage of Ly6a-GFP$^+$ cells in E11.0 AGM is about 1.7%.[55] $CD45^+$ E10.5 nascent HSCs exist exclusively in Hlf-Tomato$^+$ cells, while the percentage of $CD45^+$Hlf-Tomato$^+$ cells in AGM region is less than 0.1%.[57] Studies using the reporter mouse mentioned above and dorsal aorta perfusion with fluorescent dye confirmed that the nascent HSCs are localized in IAHCs.[55–58] In recent years, $CD31^+Kit^+Procr^+$ has been shown to enrich functional nascent HSCs in AGM region, and the efficiency was approximately 61-fold than that of $CD31^+Kit^+Procr^{lo}$ population.[59] Through iterative single-cell analyses, the immunophenotype of $CD31^{hi}SSC\text{-}A^{lo}Kit^{hi}Gata2^{med}CD27^{med}$ (~20 per AGM) has been recently identified to enrich all functional HSCs in IAHCs, which account for about 0.01% of cells in the AGM region and are localized to aortic clusters containing one to two cells.[60]

HSCs are derived from a type of precursors called pre-HSCs, which cannot directly repopulate irradiated adult recipients, but can reconstitute more permissive recipients (eg, busulfan-conditioned neonates, immune-deficient adult) and mature into transplantable HSCs in vivo or in vitro.[58,61,62] Although pre-HSCs still co-express endothelial cell markers and manifest certain arterial features,[61] they are not endothelial cells in nature as they are primed with a hematopoietic CD41 marker.[58] According to the expression of CD45, pre-HSCs in E10.5-E11.5 AGM region could be separated into 2 consecutive subsets type I ($CD144^+CD45^-$) and type II ($CD144^+CD45^+$).[58] Unlike nascent HSCs, the pre-HSCs have been reported to be distributed more broadly both in the endothelial and subendothelial cell layers.[58] The Kit$^+$ IAHCs are reported to contain pre-HSCs or HSC precursors, and their number increases from 2 to 12 per aorta from early E10 to mid-E10, far exceeding the estimated number of HSCs at this stage (~0.1 HSC).[62] Unlike nascent HSCs, pre-HSCs in IAHCs are not restricted to a specific phenotype at E10 based on Ly6a-GFP and CD45 expression, but acquire Ly6a-GFP and CD45 expression as they mature into an HSC fate.[62] Subsequently, a study using the surface marker combinations $CD31^+CD45^-CD41^{low}Kit^+Procr^{hi}$ (~11 per embryo) and $CD31^+CD45^+Kit^+Procr^{hi}$ (~18 per embryo) captured high purity type I and type II pre-HSCs in E11 AGM region, respectively, as rigorously validated by single-cell–initiated serial transplantation.[61] Hlf-Tomato can enrich $CD45^+$ but not $CD45^-$ pre-HSCs,

suggesting that the cells should acquire Hlf expression before or together with CD45 to give rise to functional HSCs.[57] Importantly, lineage tracing studies confirmed that Hlf- and Procr-labeled cells contribute to the HSC pool and maintain their size in the adult bone marrow.[57,59] Type I pre-HSCs in both G0 or S/G2/M phase exhibit reconstitution potential,[61] differing from HSCs in adult bone marrow and E14 fetal liver, which are mostly in G0 and G1 phase, respectively.[63]

Through single-cell–initiated serial transplantation experiments, researchers detected the functional heterogeneity in emerging HSCs during embryonic development, starting from pre-HSC stage. These earliest HSCs show predominantly the myeloid-deficient (γ) type and the lymphomyeloid-balanced (β) type, the latter has a stronger capacity for reconstruction and self-renewal.[64] Together with previous findings, the data suggest that functional heterogeneity of HSCs exists from the very beginning of embryonic HSC emergence and throughout whole lifespan.[65,66] Whether the pre-HSCs are fated to become a specific subtype intrinsically at the HEC stage, or they are in response to extrinsic cues from the microenvironment remains to be further investigated. Transcriptomics and clonal analysis of HECs in mouse embryos showed that Cxcr4 expression can distinguish between HSC-competent and multipotent progenitor-competent HECs, suggesting that the fate of HSCs or non-HSC progenitors is committed at the HEC stage.[12]

5. NEXT-GENERATION OMICS FACILITATES DECIPHERING THE PRECISE REGULATION OF EHT IN MOUSE EMBRYOS

Runx1 is one of the most pivotal transcription factors for embryonic hematopoiesis, which has been reported to be required for the EHT and consequent HSC formation but not thereafter.[67–70] However, the exact stage of crucial involvement of Runx1 in this multi-step EHT process remains unclear. The finding that Runx1 deficiency does not preclude formation of VE-cad$^+$CD45$^-$CD41$^+$ (pre-HSC Type I) cells but blocks transition to the subsequent CD45$^+$ stage (pre-HSC Type II) suggests that Runx1 only works after T1 pre-HSC stage.[71] Recently, a single-cell transcriptome dataset involving the whole EHT process in AGM region demonstrated that insufficient Runx1 dosage leads to a block in cellular identity at the pre-HEC stage, preventing the pre-HECs from transitioning to HECs.[10] Consistently, the other single-cell RNA sequencing (scRNA-seq) atlas of EHT continuum deciphers the precise cellular and molecular changes that are caused by the deficiency of the pivotal transcription factor Runx1b, and also reveals that *Runx1b* deletion leads to obstruction of pre-HECs to HECs differentiation.[11,14]

The technology of scRNA-seq provides clues to study the regulation of hematopoietic development. Combining computational and functional screening of dynamic long non-coding RNA (lncRNA) profiles of HSC development, lncRNA-H19 is identified as a key regulator of in vivo HSC emergence in AGM region. LncRNA-H19 promotes pre-HSC and HSC specification via demethylation of a series of master hematopoietic transcription factors such as Runx1 and Spi1.[15] Full-length scRNA-seq enables the construction of an isoform-based transcriptional atlas of HSC development, which allows the identification of stage-specific RNA alternative splicing events. EHT process is accompanied by a significant alternative splicing modality switch, which is mainly orchestrated by the splicing regulator Srsf2. Loss of Srsf2 from the endothelial stage affected the splicing pattern of several master hematopoietic regulators and significantly impaired HSC generation.[16]

Recently, the global DNA methylation dynamics of HSC development have been revealed at the genome scale. Interestingly, during HEC specification from upstream arterial endothelial cells, the differentially methylated region–associated genes are involved in hematopoiesis- but not endothelial cell–related biological processes, suggesting that DNA methylation modification participates in the hematopoietic fate priming but not endothelial feature extinguishing during this process.[17] Paired scRNA-Seq and scATAC-Seq reveals that pre-HEC stage is characterized by increased accessibility of chromatin enriched for SOX, FOX, GATA, and SMAD motifs.[10] Recently, with optimized low-input itChIP-seq and Hi-C assays, HSC regulatory regions are found already pre-configurated with active histone modifications as early as arterial endothelial cell stage, preceding chromatin looping dynamics within topologically associating domains. Half of Runx1 binding-mediated chromatin looping structures between enhancers and promoters are observed prior to HEC stage.[72] Furthermore, using a low cell number ChIP-seq protocol to profile the genomic locations of 4 histone-modification marks over the course of HSC ontogeny from HEC to adult HSCs, 2 broadly expressed transcription factors, SP3 and MAZ, are shown to play an important role in the formation of HECs.[18]

In summary, single-cell/low-input multi-omics provides additional information on the regulation of hematopoietic development, which, combined with the previously established discovery of transcription factors that play a key role in hematopoietic development, will shed light on the study of efficient generation of functional intact HSCs.

6. EMERGING HSCS AND THEIR ENDOTHELIAL ORIGIN IN HUMAN EMBRYOS

There are many similarities in the EHT process between humans and mice. It has been widely proposed that human HSCs are also derived from HECs, based on the expression of multiple endothelial markers in emerging HSCs, their spatiotemporally intimate relationship with vascular endothelial cells,[73] and the hierarchy established by human pluripotent stem cell in vitro differentiation models.[74] Functional assay with precise dissection of the dorsal aorta revealed that human HSCs are principally distributed in the ventral part of the dorsal aorta in the AGM region.[75,76] Histologically, similar to mice, HSCs are thought to be mature in IAHCs budding from the ventral wall of the dorsal aorta.[73,76] In human embryos, IAHCs begin to appear on the ventral floor of the dorsal aorta at 4 weeks post conception (PCW)/Carnegie Stage 12 (CS 12).[73,76] Thereafter, in 5 PCW/CS 14, the first functional HSCs of human embryos are detected at the AGM, expressing surface markers such as CD34, CD45, CD144 (VE-cadherin), KIT, THY-1, Endoglin, and RUNX1 whose homologs are also expressed in mouse HSCs.[75,77] Human HSCs also appear in the yolk sac, liver, and placenta later.[75,77] Limiting dilution assays demonstrated that the frequency of HSCs is ~0.8 per AGM region in 5 to 6 PCW (CS 14–17) human embryos, which is close to the number of HSCs in the mouse AGM region. They can generate more than 300 daughter HSCs and reconstruct all blood lineages in the primary mouse recipients.[32,77] Nevertheless, the reconstitution dynamics and differentiation behaviors of these human nascent HSCs in primate recipients remain to be determined.

Given the limited accessibility of human embryonic tissues and the poor investigating methods that can be used on human embryos, the study of human HSC development is lagging far behind compared to that in animal models. In recent years, with the help of single-cell transcriptome technologies, molecular characteristics of the emerging HSCs in human embryos come to be well recognized. The expression profile of nascent HSCs in the AGM region of early human embryos is defined as RUNX1$^+$HOXA9$^+$MLLT3$^+$MECOM$^+$HLF$^+$SPINK2$^+$, which could be used to distinguish HSCs from lineage-restricted progenitors throughout gestation.[78] These transcriptomically identified nascent HSCs could be found in 5 PCW (CS14) AGM, placenta and yolk sac, some in umbilical and vitelline vessels, but minimal in the liver, head and heart, implying that HSCs populate extra-embryonic tissues before colonizing the liver.[78]

In addition, the cellular evolution and molecular programs underlying the generation of the first HSCs from HECs in human have been finely investigated recently.[78,79] The human HECs are captured at 4 to 5 PCW (CS 12–14) in an unbiased way, showing an unambiguous feature of arterial endothelial cells and high expression of RUNX1, MYB, and ANGPT1. Consistent with mice, the biological processes enriched in human HECs are related to ribosome biogenesis.[7,79] CD44 is screened out as a surface marker to enrich HECs in endothelial cells more than 10-fold.[79] Transcriptomic and immunohistological assays showed that ALDH1A1$^+$KCNK17$^+$ could represent HECs, which arise from an IL33$^+$ALDH1A$^+$ arterial endothelial cells termed pre-HECs.[78] Moreover, WT1$^+$, DLK1$^+$, and FBLN5$^+$ mesenchymal cells in the AGM region are predicted to act as niche cells to contribute the signals required for the development of HECs, including Notch, bone morphogenetic protein, and transforming growth factor beta signals.[79] Interestingly, at earlier CS 10, prior to the detection of the HSC-primed HECs, another molecularly distinct intra-embryonic HEC population has been uncovered, which lacks the arterial feature and appears to be formed independently of HSC-primed HECs.[79]

7. CONCLUSIONS AND PERSPECTIVES

In recent years, advances in single-cell omics technology have greatly expanded our understanding of the cellular and molecular evolution and regulation mechanisms underlying the EHT process in mouse and human embryos. ScRNA-seq has been widely used for marker screening and definition and for the identification of heterogeneous populations. In addition to scRNA-seq, the most promising protocols for surface marker screening and definition are CITE-seq[80] and REAP-seq,[81] which combine scRNA-seq and surface marker profiling and can help to resolve discrepancies between the transcriptome and protein expression and facilitate marker screening and definition, although they have not yet been reported to be used in the study of process of the EHT. Other multi-omics technologies performed in the same single cell also hold promise for more accurate definition and identification of heterogeneous populations from different perspectives, such as CoTECH (combined assay of transcriptome and enriched chromatin binding),[82] Paired-Tag (joint analysis of gene expression and histone modifications),[83] Ribo-RNA-lite,[84] and T&T-seq[85] (includes both transcriptome and translatome sequencing). However, these technologies are not yet widely used, and studies using them to elucidate the process of HSC generation are highly anticipated.

The use of massive data resources provides multi-dimensional imaginations and possibilities for future researches. For example, by accurately understanding heterogeneous populations through transcriptomics, the cell type- or tissue-specific signature genes could be screened out to construct new mouse models. The in-depth application of these mouse models can help us explore the functions of new candidate genes or the biological processes previously uninvestigated during HSC development. For instance, as one of the signature genes of HSC-primed HECs, Nupr1 is further identified to be a negative regulator of EHT process to generate HSCs.[86]

In addition to exploring gene function, novel Cre mouse models can be established to trace the fate of certain cell populations newly identified. This noninvasive genetic lineage tracing strategy is convenient for studying the origin and fate determination of HSPCs under physiological conditions. During embryonic development, the occurrence of HSC-independent hematopoiesis precedes the generation of HSCs, as the former can more rapidly differentiate into functional mature blood cells to meet the needs of blood supply, oxygen exchange, and early immune surveillance of early embryos.[23,24,87,88] Of note, the important contribution of HSC-independent hematopoiesis in embryonic development and postnatal stages has been increasingly uncovered and valued. Traditionally, the HSC-progenitor hierarchy in the fetal liver is believed to be established through the differentiation of fetal HSCs in a manner similar to adult bone marrow.[89–92] Recently, lineage tracing experiments using *Hlf-CreERT2* and *Evi1-CreERT2* mouse models demonstrated that fetal HSCs minimally contribute to the generation of progenitors and functional blood cells before birth.[93] Generated within a limited time window for EHT, which is presumably due to the transient nature of embryonic endothelial cells to choose a hematopoietic fate, fetal HSCs might be similar to adult HSCs regarding the differentiation latency, and their main value is as an important reserve of blood production. Using an barcoding strategy and fate mapping with *Flt3-CreER* mice, it was shown that embryonic multipotent progenitors (eMPPs), independent of traditional HSCs, predominantly drive hematopoiesis in the young adult and are the predominant source of lymphoid output.[13] Subsequent study using inducible *Fgd5-Cre* and *Cdh5-Cre* to trace the fate of HSC-derived and endothelial cell–derived (HSC-independent) hematopoietic products, respectively, have demonstrated that HSC-independent fetal MPPs are derived from endothelial cells as early as E7.5.[21] The sequential and overlapping occurrence of both HSC-independent and HSC-dependent hematopoiesis during embryogenesis makes it difficult to identify the specific functions of HSC-independent hematopoietic products, and there are still many open questions about their exact roles, origins, and contributions.

Lineage tracing starting with endothelial cells can more clearly identify the cellular origin of blood birth than tracing studies starting with hematopoietic cells. Specifically, hematopoietic products can be detected to be associated with vascular beds in distinct anatomical sites such as yolk sac, placenta, head, and heart, in addition to aorta.[39,41,94–97] Whether and to what extent these presumed different waves of embryonic hematopoiesis contribute to the hematopoietic system are not exactly known. Therefore, lineage tracing studies starting with specific sub-population of vascular endothelial cells would have unique advantages in distinguishing hematopoietic events in different anatomical sites and distinct vascular beds, which will certainly bring new insights and discoveries in the field of developmental hematopoiesis.

On the other hand, considering the ethical limitations of reverse genetics research on human studies, non-human primates can serve as a suitable model to study the molecular regulation of HSC development in vivo as they are highly conserved with humans. In addition, although Cre-mediated tracing and gene barcoding technologies are not applicable to human studies, lineage tracing technology represented by endogenous genomic mutations is expected to be an important tool in future for studying the developmental paths of diversified HSPCs in humans.[98,99]

8. MATERIALS AND METHODS

8.1. Mice

All mice used in the experiment were raised in the Laboratory Animal Center, Military Medical Academy. Mouse experiments were performed with the approval of the Institute's Animal Care and Use Committee (approval no.: IACUC-DWZX-2021-056). All mice were of C57 background. The morning of the detection of the vaginal plug was defined as E0. For E10.0 embryos (31–35 somite pairs), the caudal half was dissected under the heart with the limbs removed.

8.2. Single-cell suspension preparation

Embryonic cells were prepared for flow cytometric sorting as follows: embryos were digested with 0.1% type I collagenase

(Sigma-Aldrich, Burlington, Massachusetts) for 20 to 30 minutes at 37°C, 20% fetal bovine serum (FBS) in phosphate buffer saline (PBS) was added to samples to deactivate the collagenase, then the samples were triturated and filtered to obtain a single cell suspension.

8.3. Flow cytometry and antibodies

Cells were sorted and analyzed by flow cytometers fluorescence-activated cell sorting (FACS) Aria II, the data were analyzed by FlowJo software (BD Biosciences, San Jose, California). Cell surface staining was performed with the following antibodies: Kit (eBioscience, San Diego, California, 2B8), CD201 (eBioscience, eBio1560), CD44 (Biolegend, San Diego, California, IM7), CD31 (BD Biosciences, MEC13.3), CD41 (BD Biosciences, MWReg30), CD43 (BD Biosciences, S7), CD45 (eBioscience, 30-F11), Sca-1 (BD Biosciences, D7), T1/ST2 (Biolegend, DIH9), CD41 (BD Biosciences, eBioMWReg30), Ter119 (eBioscience, TER-119), Gr1 (Biolegend, 1A8), Mac1 (eBioscience, M1/70), F4/80 (Biolegend, BM8), Runx1 (Abcam, Cambridge, UK, ab92336). 7-Amino-actinomycin D (7-aad; eBioscience) was used to exclude dead cells.

8.4. OP9-DL1-GFP co-culture

The 3×10^4 OP9-DL1-GFP cells were cultured per well in a 24-well plate (Corning) with α-minimum essential medium (MEM) (Gibco) containing 20% FBS (Gibco) for 1 day before co-culture. To compare the hematopoietic and endothelial potential of the PK44 and the remaining Kit$^-$ (CD41$^-$CD43$^-$CD45$^-$CD31$^+$CD44$^+$Kit$^-$) aortic endothelial populations during hematopoietic development, these cell populations were sorted and plated on OP9-DL1-GFP stromal cells, and induced with hematopoietic and endothelial induction medium, respectively. (Hematopoietic induction medium: 10% FBS/α-MEM, L-glutamine [2 mM], penicillin/streptomycin [100 U/m], IL-3 [100 ng/mL], stem cell factor [100 ng/mL] and FIt3L [100 ng/mL]; Endothelial induction medium: 15% FBS/improved minimum essential medium [IMEM], vascular endothelial growth factor [100 ng/mL].) After 7 days of co-culture, cells in the hematopoietic induction medium were used for flow analysis, and cells in the endothelial induction medium were used for immunohistochemical staining with CD31 (BD Pharmingen, MEC13.3).

8.5. Staining intracellular antigens

Prepare a single-cell suspension; Stain for cell surface markers (the antibodies used were as described earlier). After the final wash, discard the supernatant and fix cells in 300 μL of 2% paraformaldehyde for 30 minutes; Add 300 μL of Foxp3 Fixation/Permeabilization working solution to resuspend cells, and incubate for 60 minutes at room temperature; add 1× permeabilization buffer and centrifuge samples at 700 *g* for 5 minutes at room temperature, discard the supernatant; resuspend pellet in residual volume with 1× permeabilization buffer; without washing, add the recommended amount of directly conjugated antibody for detection of intracellular antigen(s) to cells and incubate for at least 30 minutes at room temperature. Add 500 μL of 1× permeabilization buffer to each well and centrifuge samples at 700 *g* for 5 minutes at room temperature, discard the supernatant; transferred to the flow tubes, and analyzed by flow cytometer.

8.6. Whole-mount immunofluorescence

The caudal half region was fixed in 2% PFA/PBS for 20 minutes on ice and dehydrated in graded concentrations of methanol/PBS (50%, 100%; 10 minutes each). Samples were bleached in 5% H_2O_2 for 1 hour on ice to block endogenous peroxidase. Then samples were incubated for 1 hour in blocking solution (0.2% bovine serum albumin, 1% milk, 0.4% [v/v] Triton X-100 in PBS), and incubated overnight at 4°C with designed primary antibody CD31 (Cell Signaling technology, Danvers, Massachusetts, 1:100) in blocking solution. After at least 3 times washes for 1 hour each in PBS-MT (1% milk, 0.4% [v/v] Triton X-100 in PBS), samples were stained with specific secondary antibody IgG (1:2000, Zhongshan golden bridge) in PBS-MT overnight at 4°C. After 3 times washes for 20 minutes each in PBS-T (0.4% [v/v] Triton X-100 in PBS), samples were stained with TSA Plus Fluorescein Kit (Histova, NEON 4-color IHC Kit for Wholemount/Cytometry, 1:500, 20 minutes). Then samples were rinsed 3 times with PBS-T to stop the enzymatic reaction. The staining method for the other antibody was the same as described earlier (the dilution ratio was 1:200 and 1:250 for primary antibody Runx1 and Kit, respectively). Finally, the samples were dehydrated in 100% methanol, and soaked in graded concentrations of BABB (phenylcarbinol and benzyl benzoate, 1:2)/methanol (50%, 100%; 1 minute each), and stored at −20°C until photographed.

8.7. Statistical analysis

Flow data were analyzed by FlowJo_V10. The remaining experimental data were processed with GraphPad Prism 8.3.0. The *t* test was used to test for statistically significant differences between groups. $P < .05$ was considered statistically significant. *$.01 < P < .05$; **$.001 < P \leq .01$; ***$.0001 < P \leq .001$; ****$P \leq .0001$.

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AUTHOR CONTRIBUTIONS

S.H. and Y.L. wrote the manuscript, Y.L. and B.L. revised the manuscript, X.G. performed the experiments with the help from X.D., X.N., H.W., and H.C. J.D. performed bioinformatics analysis, all authors reviewed the manuscript.

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