Edinburgh Research Explorer

The (intra-aortic) hematopoietic cluster cocktail: what is in the mix?

OPEN ACCESS

Experimental Hematology
The (intra-aortic) hematopoietic cluster cocktail: what is in the mix?
--Manuscript Draft--

Manuscript Number:	22-179R1
Article Type:	Review Article
Keywords:	intra-aortic hematopoietic cluster; IAHC; mouse; embryo; hematopoietic stem cells; HSC; development; hematopoiesis; endothelial-to-hematopoietic transition; EHT; HSC fate acquisition; heterogeneity; micro-niche
Corresponding Author:	Chris S. Vink, PhD The University of Edinburgh Centre for Inflammation Research Edinburgh, UNITED KINGDOM
First Author:	Chris S. Vink, PhD
Order of Authors:	Chris S. Vink, PhD
	Elaine Dzierzak, PhD
Abstract:	The adult-definitive hematopoietic hierarchy and hematopoietic stem cells (HSCs) residing in the bone marrow are established during embryonic development. In mouse, human and many other mammals, it is the sudden formation of so-called intra-aortic/arterial hematopoietic clusters (IAHCs) that best signifies and visualizes this de novo generation of HSCs and hematopoietic progenitor cells (HPCs). Cluster cells arise through an endothelial-to-hematopoietic transition and, for some time, express markers/genes of both tissue types, whilst acquiring more hematopoietic features and losing endothelial ones. Amongst several hundreds of IAHC cells, the midgestation mouse embryo contains only very few bona fide adult-repopulating HSCs, suggestive of a challenging cell fate to achieve. Most others are HPCs of various types, some of which have the potential to mature into HSCs in vitro. Based on the number of cells that reveal hematopoietic function, a fraction of IAHC cells is uncharacterized. This review aims to explore the current state of knowledge on IAHC cells. We will describe markers useful for isolation and characterization of these fleetingly-produced yet vitally-important cells and for the refined enrichment of the HSCs they contain, and speculate on the role of some IAHC cells that are as-yet functionally uncharacterized.

08-12-2022

Dear Dr Purton,

Many thanks for your time and forwarding the reviewers' comments. We are delighted to hear our review has been accepted in principle. Many thanks as well to the reviewers for their extremely helpful and insightful comments that reveal great expertise in the field.

I hereby would like to resubmit the revised Manuscript and attach separate files containing the Manuscript (textual changes in red font), Response to Reviewers and Highlights.

Additionally, I would also like to take this opportunity to submit a Cover Image for Exp Hematology that, we think, very nicely complements the Review Article. The image shows a live (unfixed) E10.5 WT mouse embryonic aorta, with CD31+cKit+ intra-aortic hematopoietic clusters marked in red and cyan, and the CD31+ endothelial lining in red. The vitelline artery and intersomitic vessels are visible too.

I would also like to request that, once formally accepted and passed on to the publication team, the Corresponding Author be changed to Elaine Dzierzak (details on Title Page). There were some issues with her account when submitting the paper, hence me taking on the role of corresponding author in the meantime.

We look forward to hearing from you.

Kind regards,

Chris Vink, PhD

EXPERIMENTAL HEMATOLOGY

AUTHORSHIP STATEMENT

Please include the signed authorship statement with your submission, or it can be mailed to exphem@elsevier.com.

MANUSCRIPT NUMBER:

TITLE OF ARTICLE: **The (intra-aortic) hematopoietic cluster cocktail: what is in the mix?**

(1) All authors have contributed significantly to the research described in the paper and have read and approved the final manuscript.

(2) The manuscript is the original work of the authors except for material in the public domain and such excerpts from other works as may be included with the prior written permission of the copyright owners.

(3) Neither this manuscript nor another manuscript with substantially similar content under our authorship has been published, has been accepted, or is being considered elsewhere for publication except as described in an attachment.

(4) All affiliations or financial involvement with any entity with a financial interest in the subject matter are completely disclosed and all financial and material support for this research and work are clearly identified in the manuscript.

Author Printed Name	Author Signature
Chris S. Vink, PhD	
Elaine Dzierzak, PhD	

Vink Review – Response to Reviewers

Reviewer #1:

1) It would be very helpful to Readers to have comments on the limitations of the approaches employed in the reviewed studies, how they complement each other and how particular approaches can heavily affect the perception of cellular heterogeneity. Authors already indicate potential issues with some approaches, such as low-labeling systems.

However, the in vitro functional assays and in vivo transplantation studies of isolated IAC cells are just taking a snapshot of a developing tissue at a particular stage. If the detected cellular heterogeneity in an IAC is due to an evolving and continuous maturation process conducting to the same cell type rather than 'more definitive,' heterogeneity is difficult to disentangle, especially for cells transcriptionally transitioning among different states (endothelial to hematopoietic). These studies mostly indicate the ability of the sorted cells to behave, for instance, as an HSC (defined by engrafting multilineage ability) or HSPC at the time of isolation. But these approaches are not able to report fate and if the cell would have matured later into an HSC if to remain in the embryo, perhaps in another location as in the fetal liver. It would be very useful to clearly highlight these limitations to the reader in a more organized manner.

In addition, the difference between potential and fate and how this can affect the interpretation of detected cellular heterogeneity is barely considered in this manuscript and should be clearly discussed as it is very likely an important factor.

Thank you. We agree this is a valuable point to add. We have now added the following text to page 4: "As the "gold standard" to test HSC functionality, such transplantations trigger an HSC towards expansion and multilineage differentiation to ultimately reveal its true potential at the time of isolation. When examined in their biological undisturbed (non-transplant) environment, the actual in vivo cell fate of these de novo-generated HSCs might not necessarily be the same (Ganuza et al., 2022a; Haas et al., 2018; Jacobsen and Nerlov, 2019). The same might hold true for cells revealing progenitor function during in vitro hematopoietic assays. Similarly, other cells that are non-transplantable and do not reveal HSC potential at the time of isolation, might be fated to mature into HSCs (Ganuza et al., 2022a). Understanding de novo generation of a cell that can directly display HSC potential versus a cell that might be fated to become an HSC, will be of great importance to produce HSCs for clinical (transplantation) application. Though fate-mapping/lineage-tracing (genetic-barcoding) studies have revealed valuable information on contribution of various HSPCs to steady-state hematopoiesis (Ganuza et al., 2022a), the maturation of these HSC-fated cells into bona fide HSCs under homeostatic conditions will rely on variable factors unlikely to be easily translatable for regenerative purposes."

2) Authors mostly focused on mouse data. Yet, they indicate differences in the endothelial to hematopoietic transition (EHT) process with zebrafish where IACs are not formed. Although much less is known about the composition of human IACs it would be helpful if some comparisons on potential mechanisms related to IAC formation or EHT recently inferred from scRNAseq data in human tissues are

included (e.g., Calvanese et al., 2022; Crosse et al 2020). This will make the Review more comprehensive and relevant across species.

We have now added the following text to page 15: "In mice, IAHCs might function to support the many different types of HPCs, pre-HSPCs and developmentally-restricted HSCs before expansion and differentiation in the fetal liver microenvironment. The same could be true for human embryos that also generate ventral IAHCs. These arise between CS13 (27 dpc) and CS17 (39-42 dpc) and contain functional HSCs between CS14-17 (Ivanovs et al., 2017). Though much less is known about de novo HSC generation in human, recent scRNA-seq studies have started to carefully dissect the process to reveal a mix of overlapping and distinct players and markers as compared with mouse aortic HSCs (Calvanese et al., 2022; Crosse et al., 2020; Rix et al., 2022; Zeng et al., 2019)."

3) Authors provide an interesting discussion on whether IAC size could relate to HSC function and if there may be different types of EHT. But, I think the literature supporting this is still quite limited and should perhaps be toned down, although I do agree that the data in Bennett et al., 2022 is provocative and complements studies recently performed by the authors (Vink et al., 2020).

We have modified the conclusive sentence to which this comment relates into: "This suggests an uncoupling of IAHC growth and HSC generation and a direct HEC-to-HSC transition, however this will need further investigation."

Reviewer #2:

1. I suggest introducing the concept of pre-HSCs earlier in the manuscript, with a description of how they are defined.

The paper starts with historic observations, leading up to discovery of HECs, and the EHT that gives rise to HSCs and IAHCs. We then discuss cell markers to isolate IAHC cells, and only after that discuss and speculate on the different cellular components of IAHCs and their functions and/or potentials. In the "IAHC micro-niche" section, we introduce pre-HSCs. The hypothesis of a direct HEC-to-HSC transition vs pre-HSC maturation is explained later.
While we have tried to introduce the concept earlier, this led to confusion and shifted the focus of the introductory section. We have therefore added more detail and phenotypic descriptions of pre-HSCs to the "IAHC micro-niche" section (p11) where we feel it is more appropriate.

2. Page 2. I was unfamiliar with the term "rami" with respect to the vasculature. A quick Google search describes it as an abnormally branching coronary artery in the heart.

"Rami" is the term used in the original Emmel 1916 publication. In the drawings used from this publication, these branches are referred to as "aortic rami" and abbreviated as "ar." We have kept one example in the figure legend (to clarify "ar") and have deleted "rami" in main text.

3. Page 2. What are extravascular cluster/islands? A little more description here would be helpful.

Now explained in more detail on page 2. "…and the UA was shown to have a strong and direct effect on intra- and even extravascular cluster formation and blood-forming capacity of this artery (Yzaguirre and Speck, 2016a; Zovein et al., 2010). The extravascular clusters of hematopoietic and/or endothelial cells, also referred to as mesenteric blood islands, were derived intravascularly from both the VA and UA (Yzaguirre and Speck, 2016a; Zovein et al., 2010)."

4. Page 3. The authors suggest that the levels of Notch signaling are likely responsible for IAHCs forming in the artery. Differences in flow could also contribute, which I think is worth mentioning.

Added to page 3: "and differences in blood-flow associated hemodynamic forces (Horton et al., 2021)."

5. Page 4. Are there really 1-2 HSCs at E10.5? I believe it is lower than that at E10.5.

Deleted "E10.5" here. 1-2 between E10.5-11.5 is more accurate.
Changed into: "Mouse embryos contain around 500-700 phenotypic IAHC cells at embryonic day (E)10.5-11.5 (Figure 2A), with only 1-2 of these being *bona fide* HSCs at this time of development, as determined by long-term serial multilineage repopulation of lethally-irradiated adults."

6. Page 5. CD44 is cited as a marker of HECs, but it is also a well known marker of arterial endothelial cells.

Indeed, the reviewer is correct. This was already mentioned in the text: "The hyaluronan receptor CD44 (Cd44) was shown recently to be a functional cell surface marker of mouse HECs at the very moment when they change morphology and express both an endothelial and hematopoietic program (Oatley et al., 2020). Onset of expression is slightly earlier than CD41 and cKit, and continues on most IAHC cells (Oatley et al., 2020), as seen in human (Watt et al., 2000). Others reveal wider and in fact pan-endothelial and -IAHC expression for CD44 (Hou et al., 2020; Wheatley et al. 1993)."
We have added the Wheatley 1993 publication to make this clearer.

7. Page 5-6, sentence beginning "The fact that slightly more functional HSCs . . " The authors indicate that there is a correlation between IAHC size and HSC functionality. I think it is more intuitive to refer to this as an anti-correlation.

The reviewer is correct. It might indeed be more intuitive to refer to an increase in HSC-functionality and a decrease in IAHC size as an inverse correlation.

8. Page 6. What is the middle segment of the aorta? Can the authors provide the boundaries, or whatever defines the middle?

This has now been clarified in text: "..in the middle segment of the aorta at the junction with the VA (2 somites cranial and 5 caudal of junction) at E10.5..."

9. Page 7. The authors describe mRNA and reporter markers of IAHCs. I suggest also including protein markers, as good antibodies exist for some of the markers they mention.

We have added a new introductory sentence to this section: "While good antibodies exist for intracellular molecules (e.g. Runx1 (Rybtsov et al., 2014; Sa da Bandeira et al., 2022; Yzaguirre and Speck, 2016a; Yzaguirre and Speck, 2016b)), the isolation and characterization of live (non-fixed/non-permeabilized) cells is more easily performed with fluorescent reporter genes."

10. Page 8. Ly6a-GFP marks more endothelial cells than HECs, so the statement that the Ly6A transgene specifically marks HSC-generating HE is not quite accurate.

Changed into "with the Ly6a transgene is highly enriching for "HSC-generating" HE in vivo."

11. Page 10. The authors refer to a pro-HSC stage but don't define it. This could be addressed in the suggested pre-HSC section that would be earlier in the manuscript.

On p10 changed into "the transition of CD41$^+$CD43$^-$ IAHC cells ("pro-HSCs") into.." We have added more details on pro-/pre-HSCs in the next section.

12. Figure 1. Is it possible to get permission from the journals to increase the size of the font in some of the figures, specifically 1Bi and 2B?

We have now increased the font of Figure 1B1. Unfortunately, it was not possible to increase the font of the numbers (of cells per cluster) in Figure 2B without compromising the appearance and clarity of the figure. The figure was originally published like this.

13. Page 17. Same comment as #2. I don't think many people know what an aortic "ramus" is.

We have deleted the word "ramus" in the main text. Given that in Emmel 1916 this was abbreviated as "ar" for "aortic ramus", we retain the description of Bii) as "the entrance of an aortic branch/ramus (ar).."

The (intra-aortic) hematopoietic cluster cocktail: what is in the mix?

Chris S. Vink[1], Elaine Dzierzak[1*]

[1]The University of Edinburgh, Centre for Inflammation Research, Edinburgh, Midlothian, Scotland, EH16 4TJ, UK

Word count: 4597 (excluding Abstract, Figure Legends and in-text References)

Key words: intra-aortic hematopoietic cluster; IAHC; mouse; embryo; hematopoietic stem cells; HSC; development; hematopoiesis; endothelial-to-hematopoietic transition; EHT; HSC fate acquisition; heterogeneity; micro-niche

*Corresponding author and lead contact:
Prof. Elaine Dzierzak
University of Edinburgh
Centre for Inflammation Research
Queens Medical Research Institute
47 Little France Crescent
Edinburgh, EH16 4TJ, UK
Tel: +44 10 242 9163
Email: Elaine.Dzierzak@ed.ac.uk

Highlights

- Marker combinations identify hematopoietic cells emerging from the embryonic aorta
- IAHC cells are phenotypically, transcriptionally and functionally heterogeneous
- HSC fate acquisition in the aorta can occur directly without gradual maturation
- Most EHT events lead to accumulation of immature pre-HSCs and HPCs in large IAHCs
- Distinct IAHC cells lacking direct hematopoietic function may act as a micro-niche

Twitter Abstract:

Here we explore the current state of knowledge on the vitally important intra-aortic hematopoietic cluster cells that arise during development and contain the earliest functional hematopoietic stem cells.

Abstract

The adult-definitive hematopoietic hierarchy and hematopoietic stem cells (HSCs) residing in the bone marrow are established during embryonic development. In mouse, human and many other mammals, it is the sudden formation of so-called intra-aortic/arterial hematopoietic clusters (IAHCs) that best signifies and visualizes this *de novo* generation of HSCs and hematopoietic progenitor cells (HPCs). Cluster cells arise through an endothelial-to-hematopoietic transition and, for some time, express markers/genes of both tissue types, whilst acquiring more hematopoietic features and losing endothelial ones. Amongst several hundreds of IAHC cells, the midgestation mouse embryo contains only very few *bona fide* adult-repopulating HSCs, suggestive of a challenging cell fate to achieve. Most others are HPCs of various types, some of which have the potential to mature into HSCs *in vitro*. Based on the number of cells that reveal hematopoietic function, a fraction of IAHC cells is uncharacterized. This review aims to explore the current state of knowledge on IAHC cells. We will describe markers useful for isolation and characterization of these fleetingly-produced yet vitally-important cells and for the refined enrichment of the HSCs they contain, and speculate on the role of some IAHC cells that are as-yet functionally uncharacterized.

Early observations of intra-aortic clusters

Understanding *de novo* hematopoietic cell generation, i.e. transition or differentiation of a non-hematopoietic precursor to a hematopoietic cell identity, has long been a goal in the field of developmental hematopoiesis. Over a century ago, before functional assays existed, histological studies on many species of early-stage vertebrate embryos (Figure 1A) revealed the existence of clusters of hematopoietic-like cells tightly associated to the ventral aortic endothelium, frequently near the divergence of vascular branches (Figure 1B) (Dantschakoff, 1907; Emmel, 1916; Jordan, 1916; Jordan, 1917; Maximow, 1909; Minot et al., 1912; Sabin, 1920; Stricht, 1899). Given the cross-species conservation of intra-aortic hematopoietic clusters (IAHCs), it was thought that the biomechanical process(es) giving rise to them would also be conserved. Rather than an incidental accretion and accumulation of IAHCs from the circulating blood, the acquisition of a spheroidal shape and exclusive localization to the vascular wall, implicated an active transition state of endothelial cells (ECs) with a unique hemogenic capacity (Emmel, 1916; Jordan, 1916; Jordan, 1917; Sabin, 1920). Similar intra-arterial masses/clusters were observed in the umbilical (UA) and vitelline arteries (VA), and other temporary ventral aortic branches, leaving several of these studies to postulate a causal relationship between IAHC formation and vascular remodeling/atrophy (Emmel, 1916; Jordan, 1916; Jordan, 1917). Indeed, the remodeling of the VA (first being connected to the aorta via the "vessel of confluence" (Daane and Downs, 2011), then via a plexus of small ventral aortic branches, to forming a singular arterial connection) and the UA was shown to have a strong and direct effect on intra- and even extravascular cluster formation and blood-forming capacity of this artery (Yzaguirre and Speck, 2016a; Zovein et al., 2010). The extravascular clusters of hematopoietic and/or endothelial cells, also referred to as mesenteric blood islands, were derived intravascularly from both the VA and UA (Yzaguirre and Speck, 2016a; Zovein et al., 2010).

The early 20th century observations, combined with later functional proof of the definitive hematopoietic potency of hematopoietic cluster-containing embryonic arteries (Ciau-Uitz et al., 2000; de Bruijn et al., 2000; Dieterlen-Lievre, 1975; Lassila et al., 1978; Medvinsky and Dzierzak, 1996; Turpen et al., 1981), strongly suggested a correlation between cluster formation and hematopoietic stem and progenitor cell

(HSPC) generation. Additionally, these studies shed light on the unique spatiotemporal relationship between the hematopoietic and (cardio)vascular systems.

The first experimental evidence that endothelial cells could adopt blood cell morphology and form IAHCs came from the avian model (Jaffredo et al., 1998). Specific dye-labelling of endothelial cells in chick embryos prior to the onset of hematopoiesis and IAHC formation, resulted in the later appearance of dye-positive IAHCs expressing hematopoietic surface markers (Jaffredo et al., 1998) indicating a cell transition or transdifferentiation. The ECs endowed with hemogenic capacity were called "hemogenic endothelium" (HE). This term was proposed in the early 80s when electron microscopy surprisingly revealed ultrastructural similarities and tight junctions between IAHCs and the underlying endothelial cells (Smith and Glomski, 1982) (Figure 1C). Disjointedness of the endothelial layer and basal lamina was revealed where IAHCs form/emerge, with the most lumenal IAHC cells lacking a basal lamina (Garcia-Porrero et al., 1995; Jaffredo et al., 1998; Tavian et al., 1996). Real-time *in vitro* and *in vivo* imaging ultimately demonstrated that IAHCs arise via an endothelial-to-hematopoietic transition (EHT) event (Bertrand et al., 2010; Boisset et al., 2010; Eilken et al., 2009; Kissa and Herbomel, 2010; Lancrin et al., 2009).

These events and resulting hematopoietic clusters are found predominantly (if not exclusively) in the arterial (as opposed to venous) vasculature. This is likely due to the requirement for Notch signaling in both the acquisition of arterial fate and generation of hematopoietic clusters (Kumano et al., 2003; Robert-Moreno et al., 2005; Robert-Moreno et al., 2008; Thambyrajah and Bigas, 2022), and differences in blood-flow associated hemodynamic forces (Horton et al., 2021).

Isolation and characterization of IAHC cells and the first adult-repopulating HSCs

While the developmental landscapes of HECs, IAHCs and HSPCs are highly similar, these individual populations are phenotypically overlapping and functionally heterogeneous, and therefore challenging to isolate and characterize. Cells undergoing EHT still express varying levels of many endothelial genes and surface

markers for some period, while already starting to display hematopoietic features (Swiers et al., 2013) (Figure 2C).

Despite their transitional and potentially "metastable" state, as indicated by co-expression of counteracting/cross-antagonistic (endothelial and hematopoietic) transcriptional programs (Graf and Enver, 2009; Olsson et al., 2016), useful markers to enrich for HECs, HSCs and IAHCs exist (Figure 2C). Mouse embryos contain around 500-700 phenotypic IAHC cells at embryonic day (E)10.5-11.5 (Figure 2A), with only 1-2 of these being *bona fide* HSCs at this time of development, as determined by long-term serial multilineage repopulation of lethally-irradiated adults (Kumaravelu et al., 2002; Muller et al., 1994; Yokomizo and Dzierzak, 2010). As the "gold standard" to test HSC functionality, such transplantations trigger an HSC towards expansion and multilineage differentiation to ultimately reveal its true potential at the time of isolation. When examined in their biological undisturbed (non-transplant) environment, the actual *in vivo* cell fate of these *de novo*-generated HSCs might not necessarily be the same (Ganuza et al., 2022a; Haas et al., 2018; Jacobsen and Nerlov, 2019). The same might hold true for cells revealing progenitor function during *in vitro* hematopoietic assays. Similarly, other cells that are non-transplantable and do not reveal HSC potential at the time of isolation, might be fated to mature into HSCs (Ganuza et al., 2022a). Understanding *de novo* generation of a cell that can directly display HSC potential versus a cell that might be fated to become an HSC, will be of great importance to produce HSCs for clinical (transplantation) application. Though fate-mapping/lineage-tracing (genetic-barcoding) studies have revealed valuable information on contribution of various HSPCs to steady-state hematopoiesis (Ganuza et al., 2022a), the maturation of these HSC-fated cells into *bona fide* HSCs under homeostatic conditions will rely on variable factors unlikely to be easily translatable for regenerative purposes.

The rarity of functional HSCs within the heterogeneous mix of IAHC cells, together with the lack of an exclusive HSC phenotypic signature, are amongst some of the challenges that must be overcome in attempts to uncover how *de novo* acquisition of HSC identity is achieved.

Unique extracellular markers

All mouse IAHCs and the entire aortic endothelial lining can be phenotypically characterized and isolated (by FACS) based on expression of endothelial surface markers like CD31 (*Pecam1*) and CD34 (*Cd34*) (Garcia-Porrero et al., 1998; Yokomizo and Dzierzak, 2010) (Figure 3). Additionally, all ECs and IAHC cells express VE-Cadherin (CD144, *Cdh5*), Tie2 (CD202B, *Tek*), Endoglin (CD105, *Eng*), and Esam (*Esam*) (Chen et al., 2011; Liakhovitskaia et al., 2009; Roques et al., 2012; Takakura et al., 1998; Yokomizo and Dzierzak, 2010). Flk1 (VEGFR-2, *Kdr*) and mature pan-hematopoietic marker CD45 (*Ptprc*) show mutually exclusive expression, with Flk1 expressed on all ECs and the most basal IAHC cells, and CD45 mainly on the outer/lumenal cells of IAHCs (Bertrand et al., 2005; Yokomizo and Dzierzak, 2010). This expression pattern is like that observed in chick ECs and IAHCs (Jaffredo et al., 1998). In contrast to the more broadly expressed surface markers, CD41 (*Itga2b*) is observed on some IAHC cells (presumably those transitioning from Flk1$^+$ to CD45$^+$) but not on ECs (Bertrand et al., 2005; Ferkowicz et al., 2003; Mikkola et al., 2003; Robin et al., 2011; Yokomizo and Dzierzak, 2010) and thus is generally considered the earliest marker of definitive hematopoietic fate commitment. CD43 (*Spn*) is another marker expressed only on some IAHC cells, and specifically on those with potential to mature into definitive HSCs upon *in vitro* culture ("T[ype]1/2 pre-HSCs") (Rybtsov et al., 2014; Rybtsov et al., 2016). During IAHC maturation, its expression comes on slightly later than CD41 and increases before the onset of CD45 expression. CD201 (*Procr* or *Epcr*) expression likely initiates slightly before pan-IAHC marker cKit (CD117, *Kit*) during/before EHT, with expression sustained on HSCs (Baron et al., 2018; Hou et al., 2020; Zhou et al., 2016). Expression of pro-inflammatory TNF receptor family member CD27 (*Tnfrsf7*) is mostly observed on the IAHCs with the fewest number of cells (1-2) (Figure 3D) and importantly on all functional AGM HSCs, suggestive of a relation between IAHC size and HSC function (Li et al., 2017; Vink et al., 2020).

High levels of *Gpr56* (*Adgrg1*) expression, one of the most highly upregulated receptor genes during EHT and downstream target of the hematopoietic "heptad" transcription factors (TFs), is seen in all mouse IAHCs and some adjacent ECs (Rao et al., 2015; Solaimani Kartalaei et al., 2015). The hyaluronan receptor CD44 (*Cd44*)

was shown recently to be a functional cell surface marker of mouse HECs at the very moment when they change morphology and express both an endothelial and hematopoietic program (Oatley et al., 2020). Onset of expression is slightly earlier than CD41 and cKit, and continues on most IAHC cells (Oatley et al., 2020), as seen in human (Watt et al., 2000). Others reveal wider and in fact pan-endothelial and -IAHC expression for CD44 (Hou et al., 2020; Wheatley et al., 1993).

The pan-IAHC marker cKit

Exclusive to all IAHC cells is the expression of stem cell factor receptor and well-known embryonic/adult HSC marker cKit (Okada et al., 1991; Sanchez et al., 1996; Yokomizo and Dzierzak, 2010) (Figure 3A,C). Based on its expression, the first IAHCs are observed from E9.5 of murine development and both the total number of cKit$^+$ cells within the aorta and the maximum size of individual clusters peaks at E10.5 ($\pm$ 700 cells, up to 19 cells/IAHC), after which both gradually decrease (Yokomizo and Dzierzak, 2010) (Figure 2A,B). IAHCs are present between E9.5-14.5 and found to be of variable size, containing 1-19 and 1-11 cells per IAHC at E10.5 (Figure 2B) and E11.5 respectively. A significant portion of those ($\pm$ 100 out of 500 total cKit$^+$ cells) are single-cell "clusters" at E11. Based on their shape, IAHCs can generally be classed as either spheroidal, mushroom-like, stacked or single-cell (Boisset et al., 2015) (Figure 1D,E). The fact that slightly more functional HSCs are detected at E11.5 versus E10.5, in combination with an increase of the number of small clusters (1-2 cells) at E11.5, might indicate an inverse correlation between IAHC size and HSC functionality, and/or the way HSCs are *de novo* generated (Kumaravelu et al., 2002; Muller et al., 1994; Yokomizo and Dzierzak, 2010). This is supported by CD27 expression on all functional AGM HSCs and predominantly the smallest IAHCs (Figure 3D) (Vink et al., 2020).

Most IAHCs are found in the middle segment of the aorta at the junction with the VA (2 somites cranial and 5 caudal of junction) at E10.5 and 11.5, with 63% and 37% localized on the E10.5 ventral and dorsal wall, respectively (Yokomizo and Dzierzak, 2010) (Figure 2B,3A). The high density of cKit$^+$ cells in the ventral middle segment of the aorta (Figure 3A) therefore matches the location of the earliest *in vivo*-repopulating HSCs (Mascarenhas et al., 2009; Taoudi and Medvinsky, 2007;

Yokomizo and Dzierzak, 2010). The dorsoventral polarization of HSC induction is in accordance with exposure of ventral ECs to Jag1-Notch1-driven low levels of Notch signaling required for HSC and IAHC formation, whereas the dorsal aspect maintains an arterial program due to Dll4-Notch1-driven high Notch (Bigas and Porcheri, 2018). Despite the almost exclusive generation of HSCs in ventral IAHCs, E10 and E11 ventral IAHCs are more transcriptionally different than dorsal and ventral IAHCs at either of these stages (Baron et al., 2018; Souilhol et al., 2016; Taoudi and Medvinsky, 2007). This could suggest HSC specification is more likely to occur in HE or during EHT, rather than by gradual maturation in IAHCs.

Clusters observed in the UA (Figure 3) and VA are phenotypically similar to those within the aorta and also peak in number and size at E10.5, after which both decrease (Yokomizo and Dzierzak, 2010). It is unknown why, compared to aortic clusters, their size is generally much larger. Those in the VA are localized on all circumferential aspects of the vessel, whilst the UA ones are predominantly on the dorsal side (Yokomizo and Dzierzak, 2010). Intra-arterial clusters are found along the full length of the VA already at E9.5, when the UA and aorta still only contain very few clusters (Garcia-Porrero et al., 1995; Yzaguirre and Speck, 2016b).

Unique intracellular markers

While good antibodies exist for intracellular molecules (e.g. Runx1 (Rybtsov et al., 2014; Sa da Bandeira et al., 2022; Yzaguirre and Speck, 2016a; Yzaguirre and Speck, 2016b)), the isolation and characterization of live (non-fixed/non-permeabilized) cells is more easily performed with fluorescent reporter genes. The intracellular *Ly6A-GFP* (*Sca1-GFP*) reporter (JAX stock no. 012643) is expressed in a select number of (H)ECs, IAHC cells and all long-term adult-repopulating HSCs (de Bruijn et al., 2002; Solaimani Kartalaei et al., 2015; Vink et al., 2020) (Figure 3C,D). Its ability to greatly enrich for the earliest *de novo*-generated HSCs ties in with the importance of pro-inflammatory cytokine IFN-γ for HSC generation (Li et al., 2014), given that two hypersensitive sites in the transgene's transcriptional regulatory fragment are responsible for high-level IFN-γ-inducible (*Ly6A-*)*GFP* expression (Ma et al., 2001; Sinclair et al., 1996). BMP pathway activation, as reported in *BRE-GFP* mouse embryos (Monteiro et al., 2008), is also observed in

some aortic endothelial and IAHC cells, and in all functional AGM HSCs (Crisan et al., 2015).

The pivotal hematopoietic TFs Gata2 and Runx1 also serve as useful HSC markers (Figure 3B). Reporter expression driven from *Runx1*'s hematopoietic-specific *+23* enhancer (*23GFP*) (Nottingham et al., 2007) is observed in restricted numbers of aortic ECs (HECs) and most IAHCs (Swiers et al., 2013). When analyzed by *in situ* hybridization (ISH), *Runx1* expression in the AGM (from any of its enhancers) is seen in all lateral and ventral ECs, IAHCs and several layers subjacent ventral mesenchyme (Bee et al., 2009). The transcriptional repressors *Gfi1* and *Gfib* are downstream of Runx1 and expressed similarly to *23GFP*, with *Gfi1* expression initiated in rare HECs and sustained in basal IAHC cells, and *Gfib* expressed in nearly all IAHC cells (Thambyrajah et al., 2016).

The *Gata2Venus* reporter mouse (JAX stock no. 037700) facilitated the first characterization of live *Gata2*-expressing cells with normal levels and function of Gata2 (Kaimakis et al., 2016). Between E8 and late E11, Gata2 is expressed in a steadily increasing number of (H)ECs and later also in most IAHC cells (Eich et al., 2018; Kaimakis et al., 2016; Minegishi et al., 1999; Minegishi et al., 2003; Robert-Moreno et al., 2005; Vink et al., 2020) (Figure 3B,D). Ninety eight percent of E10.5 IAHC cells also express the TF *Hlf*, a gene not expressed in ECs (Yokomizo et al., 2022; Yokomizo et al., 2019; Zhou et al., 2016). Most recently, an Evi1 (*Mecom*) reporter has been shown to mark HSPCs in the IAHCs, and is an especially potent marker of functional HSPCs (Yokomizo et al., 2022).

Ly6A-GFP, (*Runx1*)*23GFP*, *Gata2Venus* and *Gfi1* are all HEC markers expressed similarly in aortic HECs, with the *Ly6A* transgene highly enriching for "HSC-generating" HE *in vivo* (Chen et al., 2011; Eich et al., 2018; Swiers et al., 2013; Thambyrajah et al., 2016). As a result, *Ly6A-GFP* reveals the most restricted expression pattern in IAHCs. More recently, aortic (CD201⁺cKit⁺CD44⁺) *Neurl3*-expressing (Hou et al., 2020) and (VE-Cadherin⁺CD201⁺CD61⁺) CXCR4-marked (Dignum et al., 2021) cells were termed "HSC-primed" and "HSC-competent" HE, respectively. Based on phenotypic signatures, these cells are most likely basal, bulging and/or 1-2 cell cluster IAHC cells rather than purely HECs.

Unique functional HSC markers

Long-term *in vivo* transplantation assays show that HSCs express many of the aforementioned cell markers. The first functional adult-repopulating HSCs in the AGM are phenotypically characterized (and isolated based on): Ly6A-GFP$^+$ (Chen et al., 2011; de Bruijn et al., 2002; Solaimani Kartalaei et al., 2015), CD27$^{+/high}$ (Li et al., 2017; Vink et al., 2020), CD34$^+$ (Sanchez et al., 1996), cKit$^{+/high}$ (Eich et al., 2018; Sanchez et al., 1996; Solaimani Kartalaei et al., 2015; Vink et al., 2020), Gata2$^+$ (Eich et al., 2018; Kaimakis et al., 2016; Minegishi et al., 1999; Minegishi et al., 2003; Vink et al., 2020), CD31$^{+/high}$ (Eich et al., 2018; North et al., 2002; Solaimani Kartalaei et al., 2015; Vink et al., 2020), VE-Cadherin$^+$, Runx1$^+$ (North et al., 2002), Endoglin$^+$ (Roques et al., 2012), Tie2$^+$ (Liakhovitskaia et al., 2009; Takakura et al., 1998) and CD41^{intermediate} (Robin et al., 2011) expression and are BMP-activated (BRE-GFP$^+$) (Crisan et al., 2015) (Figure 4).

Some marker proteins like CD11b (Mac-1, *Itgam*) (Sanchez et al., 1996), CD45 (Bertrand et al., 2005; North et al., 2002), Sca-1 (de Bruijn et al., 2002; North et al., 2002) and Flk1 (North et al., 2002) are found only on some of the first HSCs, with HSC activity detected in both the expressing and non-expressing populations. Given the transitional and heterogeneous nature of HECs and IAHCs, and the non-synchronous expression onset of most markers, the list is non-exhaustive, and several other endothelial/hematopoietic surface markers and TF reporter lines can be used for purification and/or visualization of the first HSCs. Our lab has found the use of one pan-endothelial-IAHC marker (like CD31), in combination with cKit (pan-IAHC), and Ly6A-GFP or a pivotal hematopoietic TF reporter (like *Gata2Venus*) to yield the best HSC enrichment. The necessity for these combinations of embryonic markers is underlined by the fact most adult BM HSC enrichment strategies (e.g. LSK SLAM) are not suitable for isolation of the earliest adult-repopulating HSCs. As an example, while Ly6a-GFP is expressed in all AGM HSCs, the Sca-1 (Ly6A/E) surface protein is on only 50% of AGM HSCs (de Bruijn et al., 2002), and the SLAM marker CD150 is on very few AGM cells, none of which have HSC potential (McKinney-Freeman et al., 2009).

Lesser-known and/or appreciated IAHC components

A wide range of phenotypic, functional, and transcriptomic heterogeneity of IAHCs has been revealed (Baron et al., 2018; Crisan and Dzierzak, 2016; Crisan et al., 2015; Eich et al., 2018; Kaimakis et al., 2016; Rybtsov et al., 2014; Rybtsov et al., 2011; Solaimani Kartalaei et al., 2015; Taoudi et al., 2008; Vink et al., 2020; Zhou et al., 2016). Functionally, IAHCs have been shown to contain various types of lymphoid and myeloid progenitors, embryonic multipotent progenitors, precursors to HSCs (pre-HSCs, unable to directly reconstitute adult-irradiated recipients), pre-HPSCs, rare long-term multilineage adult-repopulating HSCs and erythro-myeloid progenitors (EMPs), the latter most likely of yolk sac origin (Baron et al., 2018; Boisset et al., 2015; Eich et al., 2018; Li et al., 2014; Patel et al., 2022; Rybtsov et al., 2014; Rybtsov et al., 2011; Taoudi et al., 2008; Vink et al., 2020; Yokomizo et al., 2022). Still, functional (hematopoietic) assays fail to quantitatively match the roughly 500-700 (CD31$^+$cKit$^+$) IAHC cells observed *in vivo*, leaving the question as to what these remaining cells are and might do?

The (unknown) role of Runx1 in IAHCs

IAHC heterogeneity and lack of unique (combinations/ levels of) markers have long impeded isolation, precise localization, and transcriptomic capture of a pure population of the first adult-repopulating HSCs. Recently, SmartSeq2 single-cell RNA sequencing (scRNA-seq) of a near-pure population of AGM HSCs was achieved through reiterations of single-cell functional and transcriptomic analyses, together with index-sorting (Vink et al., 2020) (Figure 4). Despite high-level HSC enrichment and the fact that >50% of (CD31$^+$cKit^{high}Gata2^{medium}) cells exhibit an immature or mature hematopoietic transcriptome, one major subset consisted of *Runx1*-negative/low cells with an endothelial-like transcriptomic signature (Vink et al., 2020). Several other AGM scRNA-seq studies also found E11 *Runx1$^-$* (CD31$^+$cKit$^+$) IAHC cells (Baron et al., 2018; Sa da Bandeira et al., 2022). Rare Runx1$^-$ (CD44$^+$) bulging/IAHC cells were observed in the E10.5 aorta (Hou et al., 2020), and earlier studies using E11.5 (haploinsufficient) *Runx1-LacZ* reporter embryos also revealed *Runx1$^-$* IAHC (CD34$^+$cKit$^+$) cells (North et al., 1999; North et al., 2002). CD41$^+$VE-

Cadherin$^+$ IAHC cells, said to be enriched for HSC precursors, were both Runx1$^-$ and Runx1$^+$ (by intracellular staining) at the time when IAHCs begin to be formed (Rybtsov et al., 2014). Furthermore, the E11 (CD31$^+$cKit$^+$) IAHC fraction of *Runx1-IRES-GFP* reporter embryos (Lorsbach et al., 2004), contains ±55% of *Runx1-GFP^negative* cells (Gonzalez, Crisan, *under revision*).

Importantly, the *Runx1*-negative fraction of IAHCs did not contain any directly-measurable HPC or adult-repopulating HSC function and thus appeared "non-hematopoietic" (Vink et al., 2020). No other studies have so far attempted to characterize Runx1$^-$ IAHC cells, leaving their role undetermined. *Runx1* plays an important role in hematopoietic specification prior to EHT (Swiers et al., 2013; Zhu et al., 2020), is crucial for cell emergence during EHT and is no longer required in *Vav1*-expressing hematopoietic cells/HSPCs from the fetal liver (FL) stage onwards (Chen et al., 2009). *Cdh5/VE-Cadherin*-specific deletion of *Runx1* revealed that it is required for *de novo* generation of (pre-)HSCs until E11.5, after which some become *Runx1*-independent (Tober et al., 2013). However, with *Cdh5* mRNA expression in IAHCs roughly 80% lower than VE-Cadherin surface protein (retained on these cells until shortly after FL colonization), it is unknown whether *Runx1* was deleted in all cells (Yzaguirre et al., 2017). Another study using *CD41-Cre* mediated deletion found *Runx1* only essential during, but not prior to, the transition of CD41$^+$CD43$^-$ IAHC cells ("pro-HSCs") into HSCs (Liakhovitskaia et al., 2014). Still, low-level CD41 expression in early endothelium indicates that Runx1 might be necessary from the earliest HEC specification, during EHT and up until the pro-HSC stage (Swiers et al., 2013; Yzaguirre et al., 2017).

The ambiguity concerning the absolute requirement for Runx1 within IAHCs, raises several possibilities. The Runx1$^-$ IAHC cells may have previously expressed *Runx1* long enough to emerge into IAHCs, but then downregulated its expression to result in an abortive EHT or a functionless cell. Abortive EHT events have been revealed by live-imaging of *runx1*-knockdown zebrafish embryos (Kissa and Herbomel, 2010). In these embryos EHT initiation is rare and when HECs begin bulging out of the endothelium, they immediately burst, thus establishing a crucial role for *runx1* in aortic cell emergence. Some abortive EHT events could be the recently described "doomed" zebrafish HSPCs that did not pass macrophage "quality control" (Wattrus et al., 2022). EHT in fish differs in several aspects from mouse, with

HEC-derived HSPCs extravasating directly into the sub-aortic space instead of forming IAHCs (Boisset et al., 2010; Kissa and Herbomel, 2010). In contrast to zebrafish, abortive EHT events in mice could result in accumulation of such IAHC cells lacking (directly-testable) hematopoietic function. Whether these cells have any potential function (*in vivo* or *in vitro*) remains to be seen.

The IAHC as a micro-niche within the wider embryonic aortic microenvironment

Differently sized IAHCs and/or specific locations within the major embryonic arteries could result in micro-niches promoting generation/maturation of different hematopoietic fates. Some of the uncharacterized IAHC cells may have a supportive role or be in a "neutral"/plastic state awaiting establishment of a hematopoietic identity. Given that *Runx1*- cells express medium/high *CD41* (Figure 4) and/or low *CD43* (and medium levels of cKit) (Vink et al., 2020), they could also represent precursors to HSCs ("pro-/pre-HSCs") (Rybtsov et al., 2014; Rybtsov et al., 2011; Taoudi et al., 2008) unable to directly reveal hematopoietic output. Such cells would only display HSC function upon *in vitro* culturing (Ganuza et al., 2017a; Inlay et al., 2014; Rybtsov et al., 2016) or direct transplantation into neonates, but not adult mice (Boisset et al., 2015; Yoder et al., 1997). These precursors are thought to mature via a multi-step process, transitioning from a "pro-HSC" phenotype (CD41$^+$CD43$^-$CD45$^-$) to "T1 pre-HSC" (CD41^{low}CD43$^+$CD45$^-$) and "T2 pre-HSC" (CD41^{low}CD43^{high}CD45$^+$) (Rybtsov et al., 2014; Rybtsov et al., 2011; Taoudi et al., 2008). Quantitative studies reveal around 1-2 of these pro-HSCs at E9.5, an increase from 5 to 50 pre-HSCs at E10, and a peak of 65 pre-HSCs in the E11 aorta (Rybtsov et al., 2016). By E12.5, approximately 10 pre-HSCs remain (Rybtsov et al., 2016), in line with a decrease in IAHCs and the end of *de novo* HSPC generation in the aorta (Yokomizo and Dzierzak, 2010). Slightly different phenotypic criteria reveal roughly 29 pre-HSCs per E11 AGM (Zhou et al., 2016).

Pre-HSCs are thought to mature to definitive LT-HSCs in the FL. However, one subset supposedly serves as a second origin of innate-like B-1a cells (Hadland et al., 2017). These "pre-HSCs" do not mature into LT-HSCs, but rather developmentally-restricted (B-1a-producing) FL HSCs (Ghosn et al., 2019; Godin et

al., 1993; Hadland et al., 2017). In fact, recent work shows most FL HSCs are biased towards differentiation rather than capable of long-term self-renewal (Ganuza et al., 2022b). Another subset of "pre-HSCs" might give rise to "embryonic multipotent progenitors" (eMPPs) with a predominant role in supporting lymphoid output (Patel et al., 2022), again suggesting not all phenotypic "pre-HSCs" mature into *bona fide* definitive HSCs. The low-efficiency labeling systems and (non-exclusive) phenotypic IAHC markers used in this study do not rule out contribution of AGM HSCs to the adult lymphoid compartment. (e)MPPs are said to play an important role in maintaining unperturbed (non-transplant) hematopoiesis (Busch et al., 2015; Patel et al., 2022; Pei et al., 2020; Rodriguez-Fraticelli et al., 2018; Sun et al., 2014). This is however controversial, with others showing HSCs are the major contributors to steady-state hematopoiesis (Chapple et al., 2018; Sawai et al., 2016; Sawen et al., 2018). Since maturation (and proliferation) of HSC precursors into transplantable HSCs requires *in vitro* culture steps, and phenotypic "pre-HSCs" are clearly heterogeneous, it is uncertain what proportion matures into *bona fide* HSCs and whether all "pre-HSCs" that can attain HSC function *in vitro* will achieve HSC function and identity *in vivo*. In line with this, a recent publication coined the more fitting term "pre-HSPC," for this phenotypically similar, yet functionally heterogeneous IAHC population (Yokomizo et al., 2022). Altogether these precursors are responsible for a fraction of IAHC cells and fail to add up to the 500-700 IAHC cells observed in the E10.5-11.5 aorta (Yokomizo and Dzierzak, 2010).

The stratified heterogeneity that could result in micro-niches might also be due to the clonality of IAHCs. Whereas two- and three-cell IAHCs are mostly monoclonal, those with four or more cells are polyclonal (Ganuza et al., 2017b; Porcheri et al., 2020), suggesting that IAHCs commence as monoclonal "units" and then recruit additional cells (migratory or from adjacent clusters). These cells would either already express markers like CD31 and cKit, or initiate expression in the IAHC. On the transcriptional level, rather than being enriched for a particular cell type, the majority of larger IAHCs are heterogeneous, though some were shown to contain mainly erythroid progenitors (Baron et al., 2018). In contrast to direct transplantations revealing 1-2 long-term multilineage-repopulating HSCs per E10.5-11.5 AGM, unperturbed hematopoiesis appears to be sustained by several hundred EC/IAHC precursor clones in the mouse (Ganuza et al., 2017b) and roughly 30 in the

zebrafish AGM (Henninger et al., 2017). Transplanted HSCs are capable of withstanding stress hematopoiesis and functioning in the adult bone marrow niche, thus implicating a microenvironmental role (in pre-HSPC maturation) for some cells in the larger IAHCs. Recent mouse embryo studies suggest that the smallest, one- to two-cell-containing and Dll4-expressing, IAHCs are likely to contain the very first functional HSCs (Porcheri et al., 2020; Vink et al., 2020).

Surprisingly, *Runx1⁻* IAHC cells express *Bmp4* and *Kitl* (Kit ligand or Stem Cell Factor; SCF) (Figure 4; https://gottgens-lab.stemcells.cam.ac.uk/DZIERZAK/ (Vink et al., 2020)). SCF supports AGM HSC generation/maturation and survival, and is expressed/produced by roughly 16% of aortic ECs, 9% mesenchymal cells and some IAHC cells, the latter most likely with HSC potential (Azzoni et al., 2018; Souilhol et al., 2016). These precursors are responsive to SCF (Rybtsov et al., 2014). Cytoplasmic Bmp4 is most highly expressed in ventral subaortic mesenchyme and to a lesser extent in IAHCs, with "foci" of Bmp4 (indicative of receptor activation) predominantly seen on the surface of IAHC cells (Durand et al., 2007; Souilhol et al., 2016). Like Kit ligand, Bmp4 increases HSC maturation in the AGM (Crisan et al., 2016; Durand et al., 2007), however there is a requirement for the timely downregulation of Bmp signaling (Souilhol et al., 2016). The same cells also express high levels of Apelin receptor (*Aplnr* or *APJ*) (Figure 4), which has been implicated in development and maintenance of the hematopoietic system (Jackson et al., 2021). Altogether, these uncharacterized cells might have supportive roles within IAHCs, creating unique hematopoietic (micro-)niches.

Localization of the first HSCs – Does IAHC size correlate with HSC function?

Live-imaging in mouse and zebrafish embryos demonstrates direct emergence of single round cells displaying all phenotypic (and functional) HSC criteria (Bertrand et al., 2010; Boisset et al., 2010; Kissa and Herbomel, 2010). During one rapid transdifferentiation event, these cells are capable of initiating expression of HSC markers cKit, CD41 and Ly6A-GFP (Boisset et al., 2010). Whereas the E11.5 mouse aorta contains around 500 cKit⁺ IAHC cells of which ±100 are present as single-cell "clusters" (Yokomizo and Dzierzak, 2010), zebrafish HSPCs formed by EHT, immediately extravasate without the formation of intra-aortic

clusters. With no formal proof of zebrafish generating multipotent and lineage-restricted progenitors (Stachura and Traver, 2016), one might propose zebrafish have a simpler blood hierarchy than mice, which allows for direct emergence of HSPCs from HECs without the need for any maturation. In mice, IAHCs might function to support the many different types of HPCs, pre-HSPCs and developmentally-restricted HSCs before expansion and differentiation in the FL microenvironment. The same could be true for human embryos that also generate ventral IAHCs. These arise between CS13 (27 dpc) and CS17 (39-42 dpc) and contain functional HSCs between CS14-17 (Ivanovs et al., 2017). Though much less is known about *de novo* HSC generation in human, recent scRNA-seq studies have started to carefully dissect the process to reveal a mix of overlapping and distinct players and markers as compared with mouse aortic HSCs (Calvanese et al., 2022; Crosse et al., 2020; Rix et al., 2022; Zeng et al., 2019).

Recent correlations of CD27 with Ly6A-GFP and Gata2 expression showed HSCs localize to small one- to two-cell clusters in the E10.5-11.5 mouse AGM (Vink et al., 2020) (Figure 3D), in support of a direct HEC-to-HSC transition. Some macrophages interact with these single emerged CD27^{+} cells in mouse (Vink et al., 2020). Macrophages were recently found to be crucial for HSPC generation in mouse (Mariani et al., 2019) and HSPC expansion/quality control in zebrafish (Wattrus et al., 2022) embryos. Interestingly, AGM-associated macrophages showed upregulated expression of *Bmp2* which binds the same type II receptor subunit (Bmpr2; expressed on AGM HSCs (Durand et al., 2007)) as Bmp4 and providing another possible source of Bmp for the generation of the first HSCs (Mariani et al., 2019; Sadlon et al., 2004). Furthermore, *Bmp2k* (Bmp2-inducible kinase), a downstream target of Bmp2, was found upregulated in the first HSCs (Vink et al., 2020). Though not much is known about this protein, it might provide a further link between HSCs and macrophages.

High *Dll4* expression made HSCs stand out from other cells with the same phenotype (CD31^{high}SideScatter^{low}cKit^{high}Gata2[medium]CD27[medium]), in line with others showing that Dll4 maintains smaller IAHCs (Porcheri et al., 2020). Dll4 could play a role in maintenance of HSC quiescence, providing further evidence for the direct HEC-to-HSC transition possibly facilitated by AGM-associated macrophages. The loss of MyD88-dependent toll-like receptor (TLR) signaling, an adaptor protein in one

of two main intracellular TLR pathways, was found to lead to a decrease in IAHC size/cell number and an increase in functional HSCs, whilst the number of HECs remains the same (Bennett et al., 2022). This suggests an uncoupling of IAHC growth and HSC generation and a direct HEC-to-HSC transition, however this will need further investigation.

Altogether, there is increasing evidence in favor of existence of several types of EHT; a rare and direct HEC-to-HSC transition into 1-2 cell-containing IAHCs, and a more frequent EHT resulting in HPCs and immature pre-HSCs in larger IAHCs that mature in the FL to provide for the hematopoietic needs during ontogeny.

Summary and Future Outlook

Intra-aortic clusters were first observed in many early-stage vertebrate embryos more than a century ago. Single-cell approaches have now revealed vast transcriptomic and functional heterogeneity of IAHC cells. Rare adult-repopulating HSCs reside amongst a phenotypically heterogeneous mix of (lymphoid, myeloid, and embryonic multipotent) progenitors and pre-HSPCs, the latter requiring additional maturation before revealing hematopoietic function. The similar developmental landscapes and endothelial origin of these different but phenotypically overlapping IAHC cells, render them challenging to isolate and characterize. Nonetheless, the right combinations of unique embryonic intra- and extracellular markers, and advanced isolation techniques, now allow high-level enrichment. Single-cell transcriptome profiles of IAHC cells, together with the revelation that the mouse embryo contains more IAHC cells than can be accounted for with quantitative functional assays, has raised several questions. What role(s), if any, do these remaining cells play? Do they support maturation, proliferation, or generation of certain hematopoietic cell types/states, or are they abortive EHT events that lead to non-functional cells waiting for clearance? The current state of knowledge suggests that the rare and first adult-repopulating HSCs emerge directly from aortic HECs into small IAHCs, and that *de novo* HSC identity acquisition can occur during EHT rather than by gradual maturation. Many more EHT events lead to an accumulation of "immature" (pre-)HSCs and various types of HPCs in larger IAHCs. The maturation of these precursors and restricted-/biased-HSCs could be

influenced/regulated by transcriptionally-distinct IAHC cells, that lack direct hematopoietic function. Together this suggests an uncoupling of IAHC growth and HSC generation, and a direct HEC-to-HSC transition. A full understanding of the wider aortic microenvironment, and potential IAHC micro-niches, should provide essential clues on how to generate, mature and/or maintain hematopoietic stem and progenitor cells. The vertebrate embryo holds the key to understanding these processes vital for healthy hematopoiesis and regeneration.

Author contributions

CSV and ED wrote the review. CSV made figures.

17

Figure legends

Fig 1. From early observations to high-resolution electron microscopy of EHT and IAHCs. A) Schematic of a midgestation mouse embryo with aorta (and branching at vitelline and umbilical artery) highlighted in red. Blue window indicates transversal cross-section through the aorta **Bi)** Early 20th century drawing of a transverse section through the aorta of a 12mm pig embryo. An intra-aortic haematopoietic cluster (IAHC) is attached to the ventral endothelial lining of the aorta. Ac=aortic cluster; vw=ventral wall; dw=dorsal wall; la=lateral aortic branch; s=mesenchyme. **Bii)** Early drawing of a sagittal section of vascular endothelium at the entrance of an aortic branch/ramus (ar) of a 9mm pig embryo. Morphological changes in (hemogenic) endothelial cells (HECs) are suggestive of IAHC formation (going from endothelial cell e1 to e2 to e3). Right arrow indicates blood flow from aorta into branch. Left arrow indicates direction of circulation along the long axis of the aorta. s=mesenchyme. Both **Bi)** and **Bii)** are effectively some of the first drawings of the endothelial-to-haematopoietic transition (EHT). Reprinted (with minor changes) from (Emmel, 1916), with permission from John Wiley and Sons. **C)** Transmission electron microscopy images of part of the wall of an embryonic day (E)10.5 murine dorsal aorta. Original magnifications 4000x. **Ci)** Endothelial cells (EC) budding out of the ventral aortic wall into the aortic lumen (L) as they undergo EHT. Many of the cells contain large cystic formations (*). Short arrow indicates a tight junction connecting ECs to each other, but not to the underlying mesenchyme. Long arrows point at the basal lamina of extracellular matrix between endothelium and mesenchyme. **Cii)** IAHC of four round haematopoietic cells (h) attached to the endothelial lining. The four cells in the IAHC are interconnected via tight junctions (short arrows). Reprinted (with minor changes) from (Marshall and Thrasher, 2001), with permission from John Wiley and Sons. **D,E)** Scanning electron microscopy (SEM) image of **Di)** thick transverse section through the E10 AGM region (A=inside aorta; top=dorsal; bottom=ventral), and zoom-in of an E10 **Dii)** sphere-shaped, **Ei)** stacked and **Eii)** "mushroom-like" IAHC. **Dii)** IAHC artificially-colored in yellow, endothelium in pink and sub-aortic mesenchyme in blue. **D,E)** Reprinted (with minor changes) from (Boisset et al., 2015), with permission from Elsevier.

Fig 2. Quantifications of IAHC number and size and marker changes during IAHC formation. A) Quantification of number of cKit$^+$ cells within the murine embryonic dorsal aorta (DA) at different times of development (E=embryonic day; sp=somite pairs). **B)** Number of cKit$^+$ cells per intra-aortic hematopoietic cluster (IAHC) in the E10.5 mouse. Each magenta dot indicates a cKit$^+$ cell, with numbers representing cKit$^+$ cells per cluster. One- and two-cell clusters were not drawn/included. Red numbers mark IAHCs with 10 or more cKit$^+$ cells/IAHC. VA=vitelline artery, UA=umbilical artery, FL=fetal liver. **A,B)** Reprinted from (Yokomizo and Dzierzak, 2010), with permission from The Company of Biologists Ltd. **C)** Representation of morphological and cell-specific marker changes during the transdifferentiation of endothelial cells (HECs) into IAHCs and haematopoietic stem and progenitor cells (HSPCs) occurring in the midgestation mouse aorta *in vivo*. Vascular endothelial cells express a wide array of pan-endothelial cell surface markers. Through the expression of certain pivotal haematopoietic genes, a sub-fraction of the endothelium will become hemogenic (HECs; light red) and start undergoing endothelial-to-hematopoietic transition (EHT), leading to the formation of IAHCs that gain hematopoietic characteristics (dark red) as they advance away from the endothelium. Once required/fully matured, the HSPCs will detach from IAHCs and enter circulation. Onset of expression of specific genes and markers is indicated above the schematic of the different EHT stages. Question mark (?) inside an IAHC cell refers to some of the lesser-known and/or appreciated IAHC components discussed in this review.

Fig 3. Immunohistochemistry for a selection of relevant mouse intra-aortic hematopoietic cluster markers. A) Maximum intensity projection of the middle segment of a wild-type (WT) embryonic day (E)10.5 (35 somite pairs; sp) unfixed whole-mount dorsal aorta stained for endothelial and IAHC marker CD31 (red) and intra-aortic hematopoietic cluster (IAHC) marker cKit (cyan). The highest number of IAHCs is observed on the ventral side (left) near the mouths of several aortic branches. Image taken with a Zeiss light-sheet microscope (scale bar=100μm) **B)** Transversal sections through a 35sp Gata2-Venus (top) and 38sp WT (bottom) aorta-gonad-mesonephros (AGM) region. Nuclei are in dark blue (DAPI; top), endothelial (EC) and IAHC cells in red (CD34), and Gata2 (G2V) and Runx1

expression in green and cyan, respectively. White dashed boxes indicate magnified areas **C)** Zoom-in on small IAHCs attached to the ventral aortic wall (top) and large intra-arterial hematopoietic clusters inside the umbilical artery (UA; middle+bottom) of a whole-mount stained Ly6A-GFP embryo (top+middle=38sp; bottom=37sp). All ECs express CD31 (magenta) and IAHC cells express cKit (red) and CD31. Some ECs and IAHC cells also express Ly6A-GFP (green). **D)** Detection of CD27 expression (white) on ventral one- to two-cell clusters present in transversal sections through the aorta of 43sp Gata2-Venus (top+bottom left), 42-43sp Ly6A-GFP (middle), and 36sp MacGreen (bottom right; MacGreen/GFP not shown) embryos. All ECs express CD34 (red). Some ECs and IAHC cells also express Gata2 (G2V; green; top+bottom left) or Ly6A-GFP (green; middle). Three-dimensional image (bottom left) showing a ventral two-cell cluster with one Gata2^{medium}CD27$^+$ cell and one G2^{low}CD27$^-$ cell. See also Video S1 (Vink et al., 2020). Scale bars: 30μm, unless stated otherwise. Tissue sections: 10μm thick.

Fig 4. Transcriptional profiles of select panel of genes in embryonic day 10.5-11.5 intra-aortic hematopoietic cluster cells enriched for functional HSCs.
SPRING visualization corresponding to 1087 single CD31$^+$cKit^{high}Gata2^{medium} intra-aortic hematopoietic cluster (IAHC) cells (including 119 CD31^{high}SideScatter^{low}cKit^{high}Gata2^{medium}CD27^{medium} cells, highly enriched for HSCs and localized to the HSC-containing [circled] transcriptomic sub-cluster; see CD31 plot). Cells are colored according to expression of relevant endothelial-related, hematopoietic-related and transcription factor genes, and genes expressed in the *Runx1* negative/low IAHC fraction (circled; see Runx1 plot). Transcript levels verify enrichment strategies based on expression levels of corresponding intra- and extracellular protein markers, as described in text. Data from (Vink et al., 2020). See also: https://gottgens-lab.stemcells.cam.ac.uk/DZIERZAK/

References

Azzoni, E., V. Frontera, K.E. McGrath, J. Harman, J. Carrelha, C. Nerlov, J. Palis, S.E.W. Jacobsen, and M.F. de Bruijn. 2018. Kit ligand has a critical role in mouse yolk sac and aorta-gonad-mesonephros hematopoiesis. *EMBO Rep* 19:

Baron, C.S., L. Kester, A. Klaus, J.C. Boisset, R. Thambyrajah, L. Yvernogeau, V. Kouskoff, G. Lacaud, A. van Oudenaarden, and C. Robin. 2018. Single-cell transcriptomics reveal the dynamic of haematopoietic stem cell production in the aorta. *Nat Commun* 9:2517.

Bee, T., K. Liddiard, G. Swiers, S.R. Bickley, C.S. Vink, A. Jarratt, J.R. Hughes, A. Medvinsky, and M.F. de Bruijn. 2009. Alternative Runx1 promoter usage in mouse developmental hematopoiesis. *Blood Cells Mol Dis* 43:35-42.

Bennett, L.F., M.D. Mumau, Y. Li, and N.A. Speck. 2022. MyD88-dependent TLR signaling oppositely regulates hematopoietic progenitor and stem cell formation in the embryo. *Development* 149:

Bertrand, J.Y., N.C. Chi, B. Santoso, S. Teng, D.Y. Stainier, and D. Traver. 2010. Haematopoietic stem cells derive directly from aortic endothelium during development. *Nature* 464:108-111.

Bertrand, J.Y., S. Giroux, R. Golub, M. Klaine, A. Jalil, L. Boucontet, I. Godin, and A. Cumano. 2005. Characterization of purified intraembryonic hematopoietic stem cells as a tool to define their site of origin. *Proc Natl Acad Sci U S A* 102:134-139.

Bigas, A., and C. Porcheri. 2018. Notch and Stem Cells. *Adv Exp Med Biol* 1066:235-263.

Boisset, J.C., T. Clapes, A. Klaus, N. Papazian, J. Onderwater, M. Mommaas-Kienhuis, T. Cupedo, and C. Robin. 2015. Progressive maturation toward hematopoietic stem cells in the mouse embryo aorta. *Blood* 125:465-469.

Boisset, J.C., W. van Cappellen, C. Andrieu-Soler, N. Galjart, E. Dzierzak, and C. Robin. 2010. In vivo imaging of haematopoietic cells emerging from the mouse aortic endothelium. *Nature* 464:116-120.

Busch, K., K. Klapproth, M. Barile, M. Flossdorf, T. Holland-Letz, S.M. Schlenner, M. Reth, T. Hofer, and H.R. Rodewald. 2015. Fundamental properties of unperturbed haematopoiesis from stem cells in vivo. *Nature* 518:542-546.

Calvanese, V., S. Capellera-Garcia, F. Ma, I. Fares, S. Liebscher, E.S. Ng, S. Ekstrand, J. Aguade-Gorgorio, A. Vavilina, D. Lefaudeux, B. Nadel, J.Y. Li, Y. Wang, L.K. Lee, R. Ardehali, M.L. Iruela-Arispe, M. Pellegrini, E.G. Stanley, A.G. Elefanty, K. Schenke-Layland, and H.K.A. Mikkola. 2022. Mapping human haematopoietic stem cells from haemogenic endothelium to birth. *Nature* 604:534-540.

Chapple, R.H., Y.J. Tseng, T. Hu, A. Kitano, M. Takeichi, K.A. Hoegenauer, and D. Nakada. 2018. Lineage tracing of murine adult hematopoietic stem cells reveals active contribution to steady-state hematopoiesis. *Blood Adv* 2:1220-1228.

Chen, M.J., Y. Li, M.E. De Obaldia, Q. Yang, A.D. Yzaguirre, T. Yamada-Inagawa, C.S. Vink, A. Bhandoola, E. Dzierzak, and N.A. Speck. 2011. Erythroid/myeloid progenitors and hematopoietic stem cells originate from distinct populations of endothelial cells. *Cell Stem Cell* 9:541-552.

Chen, M.J., T. Yokomizo, B.M. Zeigler, E. Dzierzak, and N.A. Speck. 2009. Runx1 is required for the endothelial to haematopoietic cell transition but not thereafter. *Nature* 457:887-891.

Ciau-Uitz, A., M. Walmsley, and R. Patient. 2000. Distinct origins of adult and embryonic blood in Xenopus. *Cell* 102:787-796.

Crisan, M., and E. Dzierzak. 2016. The many faces of hematopoietic stem cell heterogeneity. *Development* 143:4571-4581.

Crisan, M., P.S. Kartalaei, C.S. Vink, T. Yamada-Inagawa, K. Bollerot, I.W. van, R. van der Linden, S.M.C. de Sousa Lopes, R. Monteiro, C. Mummery, and E. Dzierzak. 2015. Corrigendum: BMP signalling differentially regulates distinct haematopoietic stem cell types. *Nat Commun* 6:8793.

Crisan, M., P. Solaimani Kartalaei, A. Neagu, S. Karkanpouna, T. Yamada-Inagawa, C. Purini, C.S. Vink, R. van der Linden, W. van Ijcken, S.M. Chuva de Sousa Lopes, R. Monteiro, C. Mummery, and E. Dzierzak. 2016. BMP and Hedgehog Regulate Distinct AGM Hematopoietic Stem Cells Ex Vivo. *Stem Cell Reports* 6:383-395.

Crosse, E.I., S. Gordon-Keylock, S. Rybtsov, A. Binagui-Casas, H. Felchle, N.C. Nnadi, K. Kirschner, T. Chandra, S. Tamagno, D.J. Webb, F. Rossi, R.A. Anderson, and A. Medvinsky. 2020. Multi-layered Spatial Transcriptomics Identify Secretory Factors Promoting Human Hematopoietic Stem Cell Development. *Cell Stem Cell* 27:822-839 e828.

Daane, J.M., and K.M. Downs. 2011. Hedgehog signaling in the posterior region of the mouse gastrula suggests manifold roles in the fetal-umbilical connection and posterior morphogenesis. *Dev Dyn* 240:2175-2193.

Dantschakoff, V. 1907. Uber das erate auftreten der blutelemente in huhnerembryo. *Folia Haematol* 4:159-166.

de Bruijn, M.F., X. Ma, C. Robin, K. Ottersbach, M.J. Sanchez, and E. Dzierzak. 2002. Hematopoietic stem cells localize to the endothelial cell layer in the midgestation mouse aorta. *Immunity* 16:673-683.

de Bruijn, M.F., N.A. Speck, M.C. Peeters, and E. Dzierzak. 2000. Definitive hematopoietic stem cells first develop within the major arterial regions of the mouse embryo. *EMBO J* 19:2465-2474.

Dieterlen-Lievre, F. 1975. On the origin of haemopoietic stem cells in the avian embryo: an experimental approach. *J Embryol Exp Morphol* 33:607-619.

Dignum, T., B. Varnum-Finney, S.R. Srivatsan, S. Dozono, O. Waltner, A.M. Heck, T. Ishida, C. Nourigat-McKay, D.L. Jackson, S. Rafii, C. Trapnell, I.D. Bernstein, and B. Hadland. 2021. Multipotent progenitors and hematopoietic stem cells arise independently from hemogenic endothelium in the mouse embryo. *Cell Rep* 36:109675.

Durand, C., C. Robin, K. Bollerot, M.H. Baron, K. Ottersbach, and E. Dzierzak. 2007. Embryonic stromal clones reveal developmental regulators of definitive hematopoietic stem cells. *Proc Natl Acad Sci U S A* 104:20838-20843.

Eich, C., J. Arlt, C.S. Vink, P. Solaimani Kartalaei, P. Kaimakis, S.A. Mariani, R. van der Linden, W.A. van Cappellen, and E. Dzierzak. 2018. In vivo single cell analysis reveals Gata2 dynamics in cells transitioning to hematopoietic fate. *J Exp Med* 215:233-248.

Eilken, H.M., S. Nishikawa, and T. Schroeder. 2009. Continuous single-cell imaging of blood generation from haemogenic endothelium. *Nature* 457:896-900.

Emmel, V.E. 1916. The cell clusters in the dorsal aorta of mammalian embryos. *American Journal of Anatomy* 19:401-421.

Ferkowicz, M.J., M. Starr, X. Xie, W. Li, S.A. Johnson, W.C. Shelley, P.R. Morrison, and M.C. Yoder. 2003. CD41 expression defines the onset of primitive and definitive hematopoiesis in the murine embryo. *Development* 130:4393-4403.

Ganuza, M., W. Clements, and S. McKinney-Freeman. 2022a. Specification of hematopoietic stem cells in mammalian embryos: a rare or frequent event? *Blood* 140:309-320.

Ganuza, M., B. Hadland, A. Chabot, C. Li, G. Kang, I. Bernstein, and S. McKinney-Freeman. 2017a. Murine hemogenic endothelial precursors display heterogeneous hematopoietic potential ex vivo. *Exp Hematol* 51:25-35 e26.

Ganuza, M., T. Hall, D. Finkelstein, A. Chabot, G. Kang, and S. McKinney-Freeman. 2017b. Lifelong haematopoiesis is established by hundreds of precursors throughout mammalian ontogeny. *Nat Cell Biol* 19:1153-1163.

Ganuza, M., T. Hall, J. Myers, C. Nevitt, R. Sanchez-Lanzas, A. Chabot, J. Ding, E. Kooienga, C. Caprio, D. Finkelstein, G. Kang, E. Obeng, and S. McKinney-Freeman. 2022b. Murine foetal liver supports limited detectable expansion of life-long haematopoietic progenitors. *Nat Cell Biol*

Garcia-Porrero, J.A., I.E. Godin, and F. Dieterlen-Lievre. 1995. Potential intraembryonic hemogenic sites at pre-liver stages in the mouse. *Anat Embryol (Berl)* 192:425-435.

Garcia-Porrero, J.A., A. Manaia, J. Jimeno, L.L. Lasky, F. Dieterlen-Lievre, and I.E. Godin. 1998. Antigenic profiles of endothelial and hemopoietic lineages in murine intraembryonic hemogenic sites. *Dev Comp Immunol* 22:303-319.

Ghosn, E., M. Yoshimoto, H. Nakauchi, I.L. Weissman, and L.A. Herzenberg. 2019. Hematopoietic stem cell-independent hematopoiesis and the origins of innate-like B lymphocytes. *Development* 146:

Godin, I.E., J.A. Garcia-Porrero, A. Coutinho, F. Dieterlen-Lievre, and M.A. Marcos. 1993. Para-aortic splanchnopleura from early mouse embryos contains B1a cell progenitors. *Nature* 364:67-70.

Graf, T., and T. Enver. 2009. Forcing cells to change lineages. *Nature* 462:587-594.

Haas, S., A. Trumpp, and M.D. Milsom. 2018. Causes and Consequences of Hematopoietic Stem Cell Heterogeneity. *Cell Stem Cell* 22:627-638.

Hadland, B.K., B. Varnum-Finney, P.K. Mandal, D.J. Rossi, M.G. Poulos, J.M. Butler, S. Rafii, M.C. Yoder, M. Yoshimoto, and I.D. Bernstein. 2017. A Common Origin for B-1a and B-2 Lymphocytes in Clonal Pre- Hematopoietic Stem Cells. *Stem Cell Reports* 8:1563-1572.

Henninger, J., B. Santoso, S. Hans, E. Durand, J. Moore, C. Mosimann, M. Brand, D. Traver, and L. Zon. 2017. Corrigendum: Clonal fate mapping quantifies the number of haematopoietic stem cells that arise during development. *Nat Cell Biol* 19:142.

Horton, P.D., S.P. Dumbali, K.R. Bhanu, M.F. Diaz, and P.L. Wenzel. 2021. Biomechanical Regulation of Hematopoietic Stem Cells in the Developing Embryo. *Curr Tissue Microenviron Rep* 2:1-15.

Hou, S., Z. Li, X. Zheng, Y. Gao, J. Dong, Y. Ni, X. Wang, Y. Li, X. Ding, Z. Chang, S. Li, Y. Hu, X. Fan, Y. Hou, L. Wen, B. Liu, F. Tang, and Y. Lan. 2020. Embryonic endothelial evolution towards first hematopoietic stem cells revealed by single-cell transcriptomic and functional analyses. *Cell Res* 30:376-392.

Inlay, M.A., T. Serwold, A. Mosley, J.W. Fathman, I.K. Dimov, J. Seita, and I.L. Weissman. 2014. Identification of multipotent progenitors that emerge prior to

hematopoietic stem cells in embryonic development. *Stem Cell Reports* 2:457-472.

Ivanovs, A., S. Rybtsov, E.S. Ng, E.G. Stanley, A.G. Elefanty, and A. Medvinsky. 2017. Human haematopoietic stem cell development: from the embryo to the dish. *Development* 144:2323-2337.

Jackson, M., A. Fidanza, A.H. Taylor, S. Rybtsov, R. Axton, M. Kydonaki, S. Meek, T. Burdon, A. Medvinsky, and L.M. Forrester. 2021. Modulation of APLNR Signaling Is Required during the Development and Maintenance of the Hematopoietic System. *Stem Cell Reports* 16:727-740.

Jacobsen, S.E.W., and C. Nerlov. 2019. Haematopoiesis in the era of advanced single-cell technologies. *Nat Cell Biol* 21:2-8.

Jaffredo, T., R. Gautier, A. Eichmann, and F. Dieterlen-Lievre. 1998. Intraaortic hemopoietic cells are derived from endothelial cells during ontogeny. *Development* 125:4575-4583.

Jordan, H.E. 1916. Evidence of hemogenic capacity of endothelium. *The Anatomical Record* 10:417-420.

Jordan, H.E. 1917. Aortic Cell Clusters in Vertebrate Embryos. *Proc Natl Acad Sci U S A* 3:149-156.

Kaimakis, P., E. de Pater, C. Eich, P. Solaimani Kartalaei, M.L. Kauts, C.S. Vink, R. van der Linden, M. Jaegle, T. Yokomizo, D. Meijer, and E. Dzierzak. 2016. Functional and molecular characterization of mouse Gata2-independent hematopoietic progenitors. *Blood* 127:1426-1437.

Kissa, K., and P. Herbomel. 2010. Blood stem cells emerge from aortic endothelium by a novel type of cell transition. *Nature* 464:112-115.

Kumano, K., S. Chiba, A. Kunisato, M. Sata, T. Saito, E. Nakagami-Yamaguchi, T. Yamaguchi, S. Masuda, K. Shimizu, T. Takahashi, S. Ogawa, Y. Hamada, and H. Hirai. 2003. Notch1 but not Notch2 is essential for generating hematopoietic stem cells from endothelial cells. *Immunity* 18:699-711.

Kumaravelu, P., L. Hook, A.M. Morrison, J. Ure, S. Zhao, S. Zuyev, J. Ansell, and A. Medvinsky. 2002. Quantitative developmental anatomy of definitive haematopoietic stem cells/long-term repopulating units (HSC/RUs): role of the aorta-gonad-mesonephros (AGM) region and the yolk sac in colonisation of the mouse embryonic liver. *Development* 129:4891-4899.

Lancrin, C., P. Sroczynska, C. Stephenson, T. Allen, V. Kouskoff, and G. Lacaud. 2009. The haemangioblast generates haematopoietic cells through a haemogenic endothelium stage. *Nature* 457:892-895.

Lassila, O., J. Eskola, P. Toivanen, C. Martin, and F. Dieterlen-Lievre. 1978. The origin of lymphoid stem cells studied in chick yold sac-embryo chimaeras. *Nature* 272:353-354.

Li, Y., V. Esain, L. Teng, J. Xu, W. Kwan, I.M. Frost, A.D. Yzaguirre, X. Cai, M. Cortes, M.W. Maijenburg, J. Tober, E. Dzierzak, S.H. Orkin, K. Tan, T.E. North, and N.A. Speck. 2014. Inflammatory signaling regulates embryonic hematopoietic stem and progenitor cell production. *Genes Dev* 28:2597-2612.

Li, Y., L. Gao, B. Hadland, K. Tan, and N.A. Speck. 2017. CD27 marks murine embryonic hematopoietic stem cells and type II prehematopoietic stem cells. *Blood* 130:372-376.

Liakhovitskaia, A., R. Gribi, E. Stamateris, G. Villain, T. Jaffredo, R. Wilkie, D. Gilchrist, J. Yang, J. Ure, and A. Medvinsky. 2009. Restoration of Runx1 expression in the Tie2 cell compartment rescues definitive hematopoietic

stem cells and extends life of Runx1 knockout animals until birth. *Stem Cells* 27:1616-1624.

Liakhovitskaia, A., S. Rybtsov, T. Smith, A. Batsivari, N. Rybtsova, C. Rode, M. de Bruijn, F. Buchholz, S. Gordon-Keylock, S. Zhao, and A. Medvinsky. 2014. Runx1 is required for progression of CD41+ embryonic precursors into HSCs but not prior to this. *Development* 141:3319-3323.

Lorsbach, R.B., J. Moore, S.O. Ang, W. Sun, N. Lenny, and J.R. Downing. 2004. Role of RUNX1 in adult hematopoiesis: analysis of RUNX1-IRES-GFP knock-in mice reveals differential lineage expression. *Blood* 103:2522-2529.

Ma, X., K.W. Ling, and E. Dzierzak. 2001. Cloning of the Ly-6A (Sca-1) gene locus and identification of a 3' distal fragment responsible for high-level gamma-interferon-induced expression in vitro. *Br J Haematol* 114:724-730.

Mariani, S.A., Z. Li, S. Rice, C. Krieg, S. Fragkogianni, M. Robinson, C.S. Vink, J.W. Pollard, and E. Dzierzak. 2019. Pro-inflammatory Aorta-Associated Macrophages Are Involved in Embryonic Development of Hematopoietic Stem Cells. *Immunity* 50:1439-1452 e1435.

Marshall, C.J., and A.J. Thrasher. 2001. The embryonic origins of human haematopoiesis. *Br J Haematol* 112:838-850.

Mascarenhas, M.I., A. Parker, E. Dzierzak, and K. Ottersbach. 2009. Identification of novel regulators of hematopoietic stem cell development through refinement of stem cell localization and expression profiling. *Blood* 114:4645-4653.

Maximow, A.A. 1909. Untersuchungen uber blut und bindegewebe 1. Die fruhesten entwicklungsstadien der blut- und binde-gewebszellan bein saugetierembryo, bis zum anfang der blutbilding unden leber. *Arch Mikroskop Anat* 73:444-561.

McKinney-Freeman, S.L., O. Naveiras, F. Yates, S. Loewer, M. Philitas, M. Curran, P.J. Park, and G.Q. Daley. 2009. Surface antigen phenotypes of hematopoietic stem cells from embryos and murine embryonic stem cells. *Blood* 114:268-278.

Medvinsky, A., and E. Dzierzak. 1996. Definitive hematopoiesis is autonomously initiated by the AGM region. *Cell* 86:897-906.

Mikkola, H.K., Y. Fujiwara, T.M. Schlaeger, D. Traver, and S.H. Orkin. 2003. Expression of CD41 marks the initiation of definitive hematopoiesis in the mouse embryo. *Blood* 101:508-516.

Minegishi, N., J. Ohta, H. Yamagiwa, N. Suzuki, S. Kawauchi, Y. Zhou, S. Takahashi, N. Hayashi, J.D. Engel, and M. Yamamoto. 1999. The mouse GATA-2 gene is expressed in the para-aortic splanchnopleura and aorta-gonads and mesonephros region. *Blood* 93:4196-4207.

Minegishi, N., N. Suzuki, T. Yokomizo, X. Pan, T. Fujimoto, S. Takahashi, T. Hara, A. Miyajima, S. Nishikawa, and M. Yamamoto. 2003. Expression and domain-specific function of GATA-2 during differentiation of the hematopoietic precursor cells in midgestation mouse embryos. *Blood* 102:896-905.

Minot, C., H. Evans, J. Tandler, and F. Sabin. 1912. Development of the blood, the vascular system and the spleen. *Manual of human embryology* 2:498-751.

Monteiro, R.M., S.M. de Sousa Lopes, M. Bialecka, S. de Boer, A. Zwijsen, and C.L. Mummery. 2008. Real time monitoring of BMP Smads transcriptional activity during mouse development. *Genesis* 46:335-346.

Muller, A.M., A. Medvinsky, J. Strouboulis, F. Grosveld, and E. Dzierzak. 1994. Development of hematopoietic stem cell activity in the mouse embryo. *Immunity* 1:291-301.

North, T., T.L. Gu, T. Stacy, Q. Wang, L. Howard, M. Binder, M. Marin-Padilla, and N.A. Speck. 1999. Cbfa2 is required for the formation of intra-aortic hematopoietic clusters. *Development* 126:2563-2575.

North, T.E., M.F. de Bruijn, T. Stacy, L. Talebian, E. Lind, C. Robin, M. Binder, E. Dzierzak, and N.A. Speck. 2002. Runx1 expression marks long-term repopulating hematopoietic stem cells in the midgestation mouse embryo. *Immunity* 16:661-672.

Nottingham, W.T., A. Jarratt, M. Burgess, C.L. Speck, J.F. Cheng, S. Prabhakar, E.M. Rubin, P.S. Li, J. Sloane-Stanley, A.S.J. Kong, and M.F. de Bruijn. 2007. Runx1-mediated hematopoietic stem-cell emergence is controlled by a Gata/Ets/SCL-regulated enhancer. *Blood* 110:4188-4197.

Oatley, M., O.V. Bolukbasi, V. Svensson, M. Shvartsman, K. Ganter, K. Zirngibl, P.V. Pavlovich, V. Milchevskaya, V. Foteva, K.N. Natarajan, B. Baying, V. Benes, K.R. Patil, S.A. Teichmann, and C. Lancrin. 2020. Single-cell transcriptomics identifies CD44 as a marker and regulator of endothelial to haematopoietic transition. *Nat Commun* 11:586.

Okada, S., H. Nakauchi, K. Nagayoshi, S. Nishikawa, S. Nishikawa, Y. Miura, and T. Suda. 1991. Enrichment and characterization of murine hematopoietic stem cells that express c-kit molecule. *Blood* 78:1706-1712.

Olsson, A., M. Venkatasubramanian, V.K. Chaudhri, B.J. Aronow, N. Salomonis, H. Singh, and H.L. Grimes. 2016. Single-cell analysis of mixed-lineage states leading to a binary cell fate choice. *Nature* 537:698-702.

Patel, S.H., C. Christodoulou, C. Weinreb, Q. Yu, E.L. da Rocha, B.J. Pepe-Mooney, S. Bowling, L. Li, F.G. Osorio, G.Q. Daley, and F.D. Camargo. 2022. Lifelong multilineage contribution by embryonic-born blood progenitors. *Nature* 606:747-753.

Pei, W., F. Shang, X. Wang, A.K. Fanti, A. Greco, K. Busch, K. Klapproth, Q. Zhang, C. Quedenau, S. Sauer, T.B. Feyerabend, T. Hofer, and H.R. Rodewald. 2020. Resolving Fates and Single-Cell Transcriptomes of Hematopoietic Stem Cell Clones by PolyloxExpress Barcoding. *Cell Stem Cell* 27:383-395 e388.

Porcheri, C., O. Golan, F.J. Calero-Nieto, R. Thambyrajah, C. Ruiz-Herguido, X. Wang, F. Catto, Y. Guillen, R. Sinha, J. Gonzalez, S.J. Kinston, S.A. Mariani, A. Maglitto, C.S. Vink, E. Dzierzak, P. Charbord, B. Gottgens, L. Espinosa, D. Sprinzak, and A. Bigas. 2020. Notch ligand Dll4 impairs cell recruitment to aortic clusters and limits blood stem cell generation. *EMBO J* 39:e104270.

Rao, T.N., J. Marks-Bluth, J. Sullivan, M.K. Gupta, V. Chandrakanthan, S.R. Fitch, K. Ottersbach, Y.C. Jang, X. Piao, R.N. Kulkarni, T. Serwold, J.E. Pimanda, and A.J. Wagers. 2015. High-level Gpr56 expression is dispensable for the maintenance and function of hematopoietic stem and progenitor cells in mice. *Stem Cell Res* 14:307-322.

Rix, B., A.H. Maduro, K.S. Bridge, and W. Grey. 2022. Markers for human haematopoietic stem cells: The disconnect between an identification marker and its function. *Front Physiol* 13:1009160.

Robert-Moreno, A., L. Espinosa, J.L. de la Pompa, and A. Bigas. 2005. RBPjkappa-dependent Notch function regulates Gata2 and is essential for the formation of intra-embryonic hematopoietic cells. *Development* 132:1117-1126.

Robert-Moreno, A., J. Guiu, C. Ruiz-Herguido, M.E. Lopez, J. Ingles-Esteve, L. Riera, A. Tipping, T. Enver, E. Dzierzak, T. Gridley, L. Espinosa, and A. Bigas. 2008. Impaired embryonic haematopoiesis yet normal arterial

development in the absence of the Notch ligand Jagged1. *EMBO J* 27:1886-1895.

Robin, C., K. Ottersbach, J.C. Boisset, A. Oziemlak, and E. Dzierzak. 2011. CD41 is developmentally regulated and differentially expressed on mouse hematopoietic stem cells. *Blood* 117:5088-5091.

Rodriguez-Fraticelli, A.E., S.L. Wolock, C.S. Weinreb, R. Panero, S.H. Patel, M. Jankovic, J. Sun, R.A. Calogero, A.M. Klein, and F.D. Camargo. 2018. Clonal analysis of lineage fate in native haematopoiesis. *Nature* 553:212-216.

Roques, M., C. Durand, R. Gautier, P.Y. Canto, L. Petit-Cocault, L. Yvernogeau, D. Dunon, M. Souyri, and T. Jaffredo. 2012. Endoglin expression level discriminates long-term hematopoietic from short-term clonogenic progenitor cells in the aorta. *Haematologica* 97:975-979.

Rybtsov, S., A. Batsivari, K. Bilotkach, D. Paruzina, J. Senserrich, O. Nerushev, and A. Medvinsky. 2014. Tracing the origin of the HSC hierarchy reveals an SCF-dependent, IL-3-independent CD43(-) embryonic precursor. *Stem Cell Reports* 3:489-501.

Rybtsov, S., A. Ivanovs, S. Zhao, and A. Medvinsky. 2016. Concealed expansion of immature precursors underpins acute burst of adult HSC activity in foetal liver. *Development* 143:1284-1289.

Rybtsov, S., M. Sobiesiak, S. Taoudi, C. Souilhol, J. Senserrich, A. Liakhovitskaia, A. Ivanovs, J. Frampton, S. Zhao, and A. Medvinsky. 2011. Hierarchical organization and early hematopoietic specification of the developing HSC lineage in the AGM region. *J Exp Med* 208:1305-1315.

Sa da Bandeira, D., A.M. Kilpatrick, M. Marques, M. Gomez-Salazar, T. Ventura, Z.N. Gonzalez, D. Stefancova, F. Rossi, M. Vermeren, C.S. Vink, M. Beltran, N.C. Henderson, B. Jung, R. van der Linden, H.J.G. van de Werken, W.F.J. van Ijcken, C. Betsholtz, S.J. Forbes, H. Cuervo, and M. Crisan. 2022. PDGFRbeta(+) cells play a dual role as hematopoietic precursors and niche cells during mouse ontogeny. *Cell Rep* 40:111114.

Sabin, F.R. 1920. Studies on the origin of blood-vessels and of red blood-corpuscles as seen in the living blastoderm of chicks during the second day of incubation. *Contributions to embryology.* IX:213-262.

Sadlon, T.J., I.D. Lewis, and R.J. D'Andrea. 2004. BMP4: its role in development of the hematopoietic system and potential as a hematopoietic growth factor. *Stem Cells* 22:457-474.

Sanchez, M.J., A. Holmes, C. Miles, and E. Dzierzak. 1996. Characterization of the first definitive hematopoietic stem cells in the AGM and liver of the mouse embryo. *Immunity* 5:513-525.

Sawai, C.M., S. Babovic, S. Upadhaya, D. Knapp, Y. Lavin, C.M. Lau, A. Goloborodko, J. Feng, J. Fujisaki, L. Ding, L.A. Mirny, M. Merad, C.J. Eaves, and B. Reizis. 2016. Hematopoietic Stem Cells Are the Major Source of Multilineage Hematopoiesis in Adult Animals. *Immunity* 45:597-609.

Sawen, P., M. Eldeeb, E. Erlandsson, T.A. Kristiansen, C. Laterza, Z. Kokaia, G. Karlsson, J. Yuan, S. Soneji, P.K. Mandal, D.J. Rossi, and D. Bryder. 2018. Murine HSCs contribute actively to native hematopoiesis but with reduced differentiation capacity upon aging. *Elife* 7:

Sinclair, A., B. Daly, and E. Dzierzak. 1996. The Ly-6E.1 (Sca-1) gene requires a 3' chromatin-dependent region for high-level gamma-interferon-induced hematopoietic cell expression. *Blood* 87:2750-2761.

Smith, R.A., and C.A. Glomski. 1982. "Hemogenic endothelium" of the embryonic aorta: Does it exist? *Dev Comp Immunol* 6:359-368.

Solaimani Kartalaei, P., T. Yamada-Inagawa, C.S. Vink, E. de Pater, R. van der Linden, J. Marks-Bluth, A. van der Sloot, M. van den Hout, T. Yokomizo, M.L. van Schaick-Solerno, R. Delwel, J.E. Pimanda, I.W.F. van, and E. Dzierzak. 2015. Whole-transcriptome analysis of endothelial to hematopoietic stem cell transition reveals a requirement for Gpr56 in HSC generation. *J Exp Med* 212:93-106.

Souilhol, C., C. Gonneau, J.G. Lendinez, A. Batsivari, S. Rybtsov, H. Wilson, L. Morgado-Palacin, D. Hills, S. Taoudi, J. Antonchuk, S. Zhao, and A. Medvinsky. 2016. Inductive interactions mediated by interplay of asymmetric signalling underlie development of adult haematopoietic stem cells. *Nat Commun* 7:10784.

Stachura, D.L., and D. Traver. 2016. Cellular dissection of zebrafish hematopoiesis. *Methods Cell Biol* 133:11-53.

Stricht, M. 1899. L'origine des premières cellules sanguines et des premiers vaisseaux sanguins dans l'aire vasculaire de chauves-souris'. *Bull. Acad. R. Med. Belg* 13:336-351.

Sun, J., A. Ramos, B. Chapman, J.B. Johnnidis, L. Le, Y.J. Ho, A. Klein, O. Hofmann, and F.D. Camargo. 2014. Clonal dynamics of native haematopoiesis. *Nature* 514:322-327.

Swiers, G., C. Baumann, J. O'Rourke, E. Giannoulatou, S. Taylor, A. Joshi, V. Moignard, C. Pina, T. Bee, K.D. Kokkaliaris, M. Yoshimoto, M.C. Yoder, J. Frampton, T. Schroeder, T. Enver, B. Gottgens, and M. de Bruijn. 2013. Early dynamic fate changes in haemogenic endothelium characterized at the single-cell level. *Nat Commun* 4:2924.

Takakura, N., X.L. Huang, T. Naruse, I. Hamaguchi, D.J. Dumont, G.D. Yancopoulos, and T. Suda. 1998. Critical role of the TIE2 endothelial cell receptor in the development of definitive hematopoiesis. *Immunity* 9:677-686.

Taoudi, S., C. Gonneau, K. Moore, J.M. Sheridan, C.C. Blackburn, E. Taylor, and A. Medvinsky. 2008. Extensive hematopoietic stem cell generation in the AGM region via maturation of VE-cadherin+CD45+ pre-definitive HSCs. *Cell Stem Cell* 3:99-108.

Taoudi, S., and A. Medvinsky. 2007. Functional identification of the hematopoietic stem cell niche in the ventral domain of the embryonic dorsal aorta. *Proc Natl Acad Sci U S A* 104:9399-9403.

Tavian, M., L. Coulombel, D. Luton, H.S. Clemente, F. Dieterlen-Lievre, and B. Peault. 1996. Aorta-associated CD34+ hematopoietic cells in the early human embryo. *Blood* 87:67-72.

Thambyrajah, R., and A. Bigas. 2022. Notch Signaling in HSC Emergence: When, Why and How. *Cells* 11:

Thambyrajah, R., M. Mazan, R. Patel, V. Moignard, M. Stefanska, E. Marinopoulou, Y. Li, C. Lancrin, T. Clapes, T. Moroy, C. Robin, C. Miller, S. Cowley, B. Gottgens, V. Kouskoff, and G. Lacaud. 2016. GFI1 proteins orchestrate the emergence of haematopoietic stem cells through recruitment of LSD1. *Nat Cell Biol* 18:21-32.

Tober, J., A.D. Yzaguirre, E. Piwarzyk, and N.A. Speck. 2013. Distinct temporal requirements for Runx1 in hematopoietic progenitors and stem cells. *Development* 140:3765-3776.

Turpen, J.B., C.M. Knudson, and P.S. Hoefen. 1981. The early ontogeny of hematopoietic cells studied by grafting cytogenetically labeled tissue anlagen: localization of a prospective stem cell compartment. *Dev Biol* 85:99-112.

Vink, C.S., F.J. Calero-Nieto, X. Wang, A. Maglitto, S.A. Mariani, W. Jawaid, B. Gottgens, and E. Dzierzak. 2020. Iterative Single-Cell Analyses Define the Transcriptome of the First Functional Hematopoietic Stem Cells. *Cell Rep* 31:107627.

Watt, S.M., L.H. Butler, M. Tavian, H.J. Buhring, I. Rappold, P.J. Simmons, A.C. Zannettino, D. Buck, A. Fuchs, R. Doyonnas, J.Y. Chan, J.P. Levesque, B. Peault, and I. Roxanis. 2000. Functionally defined CD164 epitopes are expressed on CD34(+) cells throughout ontogeny but display distinct distribution patterns in adult hematopoietic and nonhematopoietic tissues. *Blood* 95:3113-3124.

Wattrus, S.J., M.L. Smith, C.P. Rodrigues, E.J. Hagedorn, J.W. Kim, B. Budnik, and L.I. Zon. 2022. Quality assurance of hematopoietic stem cells by macrophages determines stem cell clonality. *Science* 377:1413-1419.

Wheatley, S.C., C.M. Isacke, and P.H. Crossley. 1993. Restricted expression of the hyaluronan receptor, CD44, during postimplantation mouse embryogenesis suggests key roles in tissue formation and patterning. *Development* 119:295-306.

Yoder, M.C., K. Hiatt, P. Dutt, P. Mukherjee, D.M. Bodine, and D. Orlic. 1997. Characterization of definitive lymphohematopoietic stem cells in the day 9 murine yolk sac. *Immunity* 7:335-344.

Yokomizo, T., and E. Dzierzak. 2010. Three-dimensional cartography of hematopoietic clusters in the vasculature of whole mouse embryos. *Development* 137:3651-3661.

Yokomizo, T., T. Ideue, S. Morino-Koga, C.Y. Tham, T. Sato, N. Takeda, Y. Kubota, M. Kurokawa, N. Komatsu, M. Ogawa, K. Araki, M. Osato, and T. Suda. 2022. Independent origins of fetal liver haematopoietic stem and progenitor cells. *Nature* 609:779-784.

Yokomizo, T., N. Watanabe, T. Umemoto, J. Matsuo, R. Harai, Y. Kihara, E. Nakamura, N. Tada, T. Sato, T. Takaku, A. Shimono, H. Takizawa, N. Nakagata, S. Mori, M. Kurokawa, D.G. Tenen, M. Osato, T. Suda, and N. Komatsu. 2019. Hlf marks the developmental pathway for hematopoietic stem cells but not for erythro-myeloid progenitors. *J Exp Med* 216:1599-1614.

Yzaguirre, A.D., M.F. de Bruijn, and N.A. Speck. 2017. The Role of Runx1 in Embryonic Blood Cell Formation. *Adv Exp Med Biol* 962:47-64.

Yzaguirre, A.D., and N.A. Speck. 2016a. Extravascular endothelial and hematopoietic islands form through multiple pathways in midgestation mouse embryos. *Dev Biol* 415:111-121.

Yzaguirre, A.D., and N.A. Speck. 2016b. Insights into blood cell formation from hemogenic endothelium in lesser-known anatomic sites. *Dev Dyn* 245:1011-1028.

Zeng, Y., J. He, Z. Bai, Z. Li, Y. Gong, C. Liu, Y. Ni, J. Du, C. Ma, L. Bian, Y. Lan, and B. Liu. 2019. Tracing the first hematopoietic stem cell generation in human embryo by single-cell RNA sequencing. *Cell Res* 29:881-894.

Zhou, F., X. Li, W. Wang, P. Zhu, J. Zhou, W. He, M. Ding, F. Xiong, X. Zheng, Z. Li, Y. Ni, X. Mu, L. Wen, T. Cheng, Y. Lan, W. Yuan, F. Tang, and B. Liu. 2016. Tracing haematopoietic stem cell formation at single-cell resolution. *Nature* 533:487-492.

Zhu, Q., P. Gao, J. Tober, L. Bennett, C. Chen, Y. Uzun, Y. Li, E.D. Howell, M. Mumau, W. Yu, B. He, N.A. Speck, and K. Tan. 2020. Developmental trajectory of prehematopoietic stem cell formation from endothelium. *Blood* 136:845-856.

Zovein, A.C., K.A. Turlo, R.M. Ponec, M.R. Lynch, K.C. Chen, J.J. Hofmann, T.C. Cox, J.C. Gasson, and M.L. Iruela-Arispe. 2010. Vascular remodeling of the vitelline artery initiates extravascular emergence of hematopoietic clusters. *Blood* 116:3435-3444.

Abstract

The adult-definitive hematopoietic hierarchy and hematopoietic stem cells (HSCs) residing in the bone marrow are established during embryonic development. In mouse, human and many other mammals, it is the sudden formation of so-called intra-aortic/arterial hematopoietic clusters (IAHCs) that best signifies and visualizes this *de novo* generation of HSCs and hematopoietic progenitor cells (HPCs). Cluster cells arise through an endothelial-to-hematopoietic transition and, for some time, express markers/genes of both tissue types, whilst acquiring more hematopoietic features and losing endothelial ones. Amongst several hundreds of IAHC cells, the midgestation mouse embryo contains only very few *bona fide* adult-repopulating HSCs, suggestive of a challenging cell fate to achieve. Most others are HPCs of various types, some of which have the potential to mature into HSCs *in vitro*. Based on the number of cells that reveal hematopoietic function, a fraction of IAHC cells is uncharacterized. This review aims to explore the current state of knowledge on IAHC cells. We will describe markers useful for isolation and characterization of these fleetingly-produced yet vitally-important cells and for the refined enrichment of the HSCs they contain, and speculate on the role of some IAHC cells that are as-yet functionally uncharacterized.

Early observations of intra-aortic clusters

Understanding *de novo* hematopoietic cell generation, i.e. transition or differentiation of a non-hematopoietic precursor to a hematopoietic cell identity, has long been a goal in the field of developmental hematopoiesis. Over a century ago, before functional assays existed, histological studies on many species of early-stage vertebrate embryos (Figure 1A) revealed the existence of clusters of hematopoietic-like cells tightly associated to the ventral aortic endothelium, frequently near the divergence of vascular branches (Figure 1B) (Dantschakoff, 1907; Emmel, 1916; Jordan, 1916; Jordan, 1917; Maximow, 1909; Minot et al., 1912; Sabin, 1920; Stricht, 1899). Given the cross-species conservation of intra-aortic hematopoietic clusters (IAHCs), it was thought that the biomechanical process(es) giving rise to them would also be conserved. Rather than an incidental accretion and accumulation of IAHCs from the circulating blood, the acquisition of a spheroidal shape and exclusive localization to the vascular wall, implicated an active transition state of endothelial cells (ECs) with a unique hemogenic capacity (Emmel, 1916; Jordan, 1916; Jordan, 1917; Sabin, 1920). Similar intra-arterial masses/clusters were observed in the umbilical (UA) and vitelline arteries (VA), and other temporary ventral aortic branches, leaving several of these studies to postulate a causal relationship between IAHC formation and vascular remodeling/atrophy (Emmel, 1916; Jordan, 1916; Jordan, 1917). Indeed, the remodeling of the VA (first being connected to the aorta via the "vessel of confluence" (Daane and Downs, 2011), then via a plexus of small ventral aortic branches, to forming a singular arterial connection) and the UA was shown to have a strong and direct effect on intra- and even extravascular cluster formation and blood-forming capacity of this artery (Yzaguirre and Speck, 2016a; Zovein et al., 2010). The extravascular clusters of hematopoietic and/or endothelial cells, also referred to as mesenteric blood islands, were derived intravascularly from both the VA and UA (Yzaguirre and Speck, 2016a; Zovein et al., 2010).

The early 20th century observations, combined with later functional proof of the definitive hematopoietic potency of hematopoietic cluster-containing embryonic arteries (Ciau-Uitz et al., 2000; de Bruijn et al., 2000; Dieterlen-Lievre, 1975; Lassila et al., 1978; Medvinsky and Dzierzak, 1996; Turpen et al., 1981), strongly suggested a correlation between cluster formation and hematopoietic stem and progenitor cell

(HSPC) generation. Additionally, these studies shed light on the unique spatiotemporal relationship between the hematopoietic and (cardio)vascular systems.

The first experimental evidence that endothelial cells could adopt blood cell morphology and form IAHCs came from the avian model (Jaffredo et al., 1998). Specific dye-labelling of endothelial cells in chick embryos prior to the onset of hematopoiesis and IAHC formation, resulted in the later appearance of dye-positive IAHCs expressing hematopoietic surface markers (Jaffredo et al., 1998) indicating a cell transition or transdifferentiation. The ECs endowed with hemogenic capacity were called "hemogenic endothelium" (HE). This term was proposed in the early 80s when electron microscopy surprisingly revealed ultrastructural similarities and tight junctions between IAHCs and the underlying endothelial cells (Smith and Glomski, 1982) (Figure 1C). Disjointedness of the endothelial layer and basal lamina was revealed where IAHCs form/emerge, with the most lumenal IAHC cells lacking a basal lamina (Garcia-Porrero et al., 1995; Jaffredo et al., 1998; Tavian et al., 1996). Real-time *in vitro* and *in vivo* imaging ultimately demonstrated that IAHCs arise via an endothelial-to-hematopoietic transition (EHT) event (Bertrand et al., 2010; Boisset et al., 2010; Eilken et al., 2009; Kissa and Herbomel, 2010; Lancrin et al., 2009).

These events and resulting hematopoietic clusters are found predominantly (if not exclusively) in the arterial (as opposed to venous) vasculature. This is likely due to the requirement for Notch signaling in both the acquisition of arterial fate and generation of hematopoietic clusters (Kumano et al., 2003; Robert-Moreno et al., 2005; Robert-Moreno et al., 2008; Thambyrajah and Bigas, 2022), and differences in blood-flow associated hemodynamic forces (Horton et al., 2021).

Isolation and characterization of IAHC cells and the first adult-repopulating HSCs

While the developmental landscapes of HECs, IAHCs and HSPCs are highly similar, these individual populations are phenotypically overlapping and functionally heterogeneous, and therefore challenging to isolate and characterize. Cells undergoing EHT still express varying levels of many endothelial genes and surface

markers for some period, while already starting to display hematopoietic features (Swiers et al., 2013) (Figure 2C).

Despite their transitional and potentially "metastable" state, as indicated by co-expression of counteracting/cross-antagonistic (endothelial and hematopoietic) transcriptional programs (Graf and Enver, 2009; Olsson et al., 2016), useful markers to enrich for HECs, HSCs and IAHCs exist (Figure 2C). Mouse embryos contain around 500-700 phenotypic IAHC cells at embryonic day (E)10.5-11.5 (Figure 2A), with only 1-2 of these being *bona fide* HSCs at this time of development, as determined by long-term serial multilineage repopulation of lethally-irradiated adults (Kumaravelu et al., 2002; Muller et al., 1994; Yokomizo and Dzierzak, 2010). As the "gold standard" to test HSC functionality, such transplantations trigger an HSC towards expansion and multilineage differentiation to ultimately reveal its true potential at the time of isolation. When examined in their biological undisturbed (non-transplant) environment, the actual *in vivo* cell fate of these *de novo*-generated HSCs might not necessarily be the same (Ganuza et al., 2022a; Haas et al., 2018; Jacobsen and Nerlov, 2019). The same might hold true for cells revealing progenitor function during *in vitro* hematopoietic assays. Similarly, other cells that are non-transplantable and do not reveal HSC potential at the time of isolation, might be fated to mature into HSCs (Ganuza et al., 2022a). Understanding *de novo* generation of a cell that can directly display HSC potential versus a cell that might be fated to become an HSC, will be of great importance to produce HSCs for clinical (transplantation) application. Though fate-mapping/lineage-tracing (genetic-barcoding) studies have revealed valuable information on contribution of various HSPCs to steady-state hematopoiesis (Ganuza et al., 2022a), the maturation of these HSC-fated cells into *bona fide* HSCs under homeostatic conditions will rely on variable factors unlikely to be easily translatable for regenerative purposes.

The rarity of functional HSCs within the heterogeneous mix of IAHC cells, together with the lack of an exclusive HSC phenotypic signature, are amongst some of the challenges that must be overcome in attempts to uncover how *de novo* acquisition of HSC identity is achieved.

Unique extracellular markers

All mouse IAHCs and the entire aortic endothelial lining can be phenotypically characterized and isolated (by FACS) based on expression of endothelial surface markers like CD31 (*Pecam1*) and CD34 (*Cd34*) (Garcia-Porrero et al., 1998; Yokomizo and Dzierzak, 2010) (Figure 3). Additionally, all ECs and IAHC cells express VE-Cadherin (CD144, *Cdh5*), Tie2 (CD202B, *Tek*), Endoglin (CD105, *Eng*), and Esam (*Esam*) (Chen et al., 2011; Liakhovitskaia et al., 2009; Roques et al., 2012; Takakura et al., 1998; Yokomizo and Dzierzak, 2010). Flk1 (VEGFR-2, *Kdr*) and mature pan-hematopoietic marker CD45 (*Ptprc*) show mutually exclusive expression, with Flk1 expressed on all ECs and the most basal IAHC cells, and CD45 mainly on the outer/lumenal cells of IAHCs (Bertrand et al., 2005; Yokomizo and Dzierzak, 2010). This expression pattern is like that observed in chick ECs and IAHCs (Jaffredo et al., 1998). In contrast to the more broadly expressed surface markers, CD41 (*Itga2b*) is observed on some IAHC cells (presumably those transitioning from Flk1$^+$ to CD45$^+$) but not on ECs (Bertrand et al., 2005; Ferkowicz et al., 2003; Mikkola et al., 2003; Robin et al., 2011; Yokomizo and Dzierzak, 2010) and thus is generally considered the earliest marker of definitive hematopoietic fate commitment. CD43 (*Spn*) is another marker expressed only on some IAHC cells, and specifically on those with potential to mature into definitive HSCs upon *in vitro* culture ("T[ype]1/2 pre-HSCs") (Rybtsov et al., 2014; Rybtsov et al., 2016). During IAHC maturation, its expression comes on slightly later than CD41 and increases before the onset of CD45 expression. CD201 (*Procr* or *Epcr*) expression likely initiates slightly before pan-IAHC marker cKit (CD117, *Kit*) during/before EHT, with expression sustained on HSCs (Baron et al., 2018; Hou et al., 2020; Zhou et al., 2016). Expression of pro-inflammatory TNF receptor family member CD27 (*Tnfrsf7*) is mostly observed on the IAHCs with the fewest number of cells (1-2) (Figure 3D) and importantly on all functional AGM HSCs, suggestive of a relation between IAHC size and HSC function (Li et al., 2017; Vink et al., 2020).

High levels of *Gpr56* (*Adgrg1*) expression, one of the most highly upregulated receptor genes during EHT and downstream target of the hematopoietic "heptad" transcription factors (TFs), is seen in all mouse IAHCs and some adjacent ECs (Rao et al., 2015; Solaimani Kartalaei et al., 2015). The hyaluronan receptor CD44 (*Cd44*)

5

was shown recently to be a functional cell surface marker of mouse HECs at the very moment when they change morphology and express both an endothelial and hematopoietic program (Oatley et al., 2020). Onset of expression is slightly earlier than CD41 and cKit, and continues on most IAHC cells (Oatley et al., 2020), as seen in human (Watt et al., 2000). Others reveal wider and in fact pan-endothelial and -IAHC expression for CD44 (Hou et al., 2020; Wheatley et al., 1993).

The pan-IAHC marker cKit

Exclusive to all IAHC cells is the expression of stem cell factor receptor and well-known embryonic/adult HSC marker cKit (Okada et al., 1991; Sanchez et al., 1996; Yokomizo and Dzierzak, 2010) (Figure 3A,C). Based on its expression, the first IAHCs are observed from E9.5 of murine development and both the total number of cKit$^+$ cells within the aorta and the maximum size of individual clusters peaks at E10.5 ($\pm$ 700 cells, up to 19 cells/IAHC), after which both gradually decrease (Yokomizo and Dzierzak, 2010) (Figure 2A,B). IAHCs are present between E9.5-14.5 and found to be of variable size, containing 1-19 and 1-11 cells per IAHC at E10.5 (Figure 2B) and E11.5 respectively. A significant portion of those ($\pm$ 100 out of 500 total cKit$^+$ cells) are single-cell "clusters" at E11. Based on their shape, IAHCs can generally be classed as either spheroidal, mushroom-like, stacked or single-cell (Boisset et al., 2015) (Figure 1D,E). The fact that slightly more functional HSCs are detected at E11.5 versus E10.5, in combination with an increase of the number of small clusters (1-2 cells) at E11.5, might indicate an inverse correlation between IAHC size and HSC functionality, and/or the way HSCs are *de novo* generated (Kumaravelu et al., 2002; Muller et al., 1994; Yokomizo and Dzierzak, 2010). This is supported by CD27 expression on all functional AGM HSCs and predominantly the smallest IAHCs (Figure 3D) (Vink et al., 2020).

Most IAHCs are found in the middle segment of the aorta at the junction with the VA (2 somites cranial and 5 caudal of junction) at E10.5 and 11.5, with 63% and 37% localized on the E10.5 ventral and dorsal wall, respectively (Yokomizo and Dzierzak, 2010) (Figure 2B,3A). The high density of cKit$^+$ cells in the ventral middle segment of the aorta (Figure 3A) therefore matches the location of the earliest *in vivo*-repopulating HSCs (Mascarenhas et al., 2009; Taoudi and Medvinsky, 2007;

Yokomizo and Dzierzak, 2010). The dorsoventral polarization of HSC induction is in accordance with exposure of ventral ECs to Jag1-Notch1-driven low levels of Notch signaling required for HSC and IAHC formation, whereas the dorsal aspect maintains an arterial program due to Dll4-Notch1-driven high Notch (Bigas and Porcheri, 2018). Despite the almost exclusive generation of HSCs in ventral IAHCs, E10 and E11 ventral IAHCs are more transcriptionally different than dorsal and ventral IAHCs at either of these stages (Baron et al., 2018; Souilhol et al., 2016; Taoudi and Medvinsky, 2007). This could suggest HSC specification is more likely to occur in HE or during EHT, rather than by gradual maturation in IAHCs.

Clusters observed in the UA (Figure 3) and VA are phenotypically similar to those within the aorta and also peak in number and size at E10.5, after which both decrease (Yokomizo and Dzierzak, 2010). It is unknown why, compared to aortic clusters, their size is generally much larger. Those in the VA are localized on all circumferential aspects of the vessel, whilst the UA ones are predominantly on the dorsal side (Yokomizo and Dzierzak, 2010). Intra-arterial clusters are found along the full length of the VA already at E9.5, when the UA and aorta still only contain very few clusters (Garcia-Porrero et al., 1995; Yzaguirre and Speck, 2016b).

Unique intracellular markers

While good antibodies exist for intracellular molecules (e.g. Runx1 (Rybtsov et al., 2014; Sa da Bandeira et al., 2022; Yzaguirre and Speck, 2016a; Yzaguirre and Speck, 2016b)), the isolation and characterization of live (non-fixed/non-permeabilized) cells is more easily performed with fluorescent reporter genes. The intracellular *Ly6A-GFP* (*Sca1-GFP*) reporter (JAX stock no. 012643) is expressed in a select number of (H)ECs, IAHC cells and all long-term adult-repopulating HSCs (de Bruijn et al., 2002; Solaimani Kartalaei et al., 2015; Vink et al., 2020) (Figure 3C,D). Its ability to greatly enrich for the earliest *de novo*-generated HSCs ties in with the importance of pro-inflammatory cytokine IFN-γ for HSC generation (Li et al., 2014), given that two hypersensitive sites in the transgene's transcriptional regulatory fragment are responsible for high-level IFN-γ-inducible (*Ly6A-*)*GFP* expression (Ma et al., 2001; Sinclair et al., 1996). BMP pathway activation, as reported in *BRE-GFP* mouse embryos (Monteiro et al., 2008), is also observed in

some aortic endothelial and IAHC cells, and in all functional AGM HSCs (Crisan et al., 2015).

The pivotal hematopoietic TFs Gata2 and Runx1 also serve as useful HSC markers (Figure 3B). Reporter expression driven from *Runx1*'s hematopoietic-specific *+23* enhancer (*23GFP*) (Nottingham et al., 2007) is observed in restricted numbers of aortic ECs (HECs) and most IAHCs (Swiers et al., 2013). When analyzed by *in situ* hybridization (ISH), *Runx1* expression in the AGM (from any of its enhancers) is seen in all lateral and ventral ECs, IAHCs and several layers subjacent ventral mesenchyme (Bee et al., 2009). The transcriptional repressors *Gfi1* and *Gfib* are downstream of Runx1 and expressed similarly to *23GFP*, with *Gfi1* expression initiated in rare HECs and sustained in basal IAHC cells, and *Gfib* expressed in nearly all IAHC cells (Thambyrajah et al., 2016).

The *Gata2Venus* reporter mouse (JAX stock no. 037700) facilitated the first characterization of live *Gata2*-expressing cells with normal levels and function of Gata2 (Kaimakis et al., 2016). Between E8 and late E11, Gata2 is expressed in a steadily increasing number of (H)ECs and later also in most IAHC cells (Eich et al., 2018; Kaimakis et al., 2016; Minegishi et al., 1999; Minegishi et al., 2003; Robert-Moreno et al., 2005; Vink et al., 2020) (Figure 3B,D). Ninety eight percent of E10.5 IAHC cells also express the TF *Hlf*, a gene not expressed in ECs (Yokomizo et al., 2022; Yokomizo et al., 2019; Zhou et al., 2016). Most recently, an Evi1 (*Mecom*) reporter has been shown to mark HSPCs in the IAHCs, and is an especially potent marker of functional HSPCs (Yokomizo et al., 2022).

Ly6A-GFP, (*Runx1*)*23GFP*, *Gata2Venus* and *Gfi1* are all HEC markers expressed similarly in aortic HECs, with the *Ly6A* transgene highly enriching for "HSC-generating" HE *in vivo* (Chen et al., 2011; Eich et al., 2018; Swiers et al., 2013; Thambyrajah et al., 2016). As a result, *Ly6A-GFP* reveals the most restricted expression pattern in IAHCs. More recently, aortic (CD201$^+$cKit$^+$CD44$^+$) *Neurl3*-expressing (Hou et al., 2020) and (VE-Cadherin$^+$CD201$^+$CD61$^+$) CXCR4-marked (Dignum et al., 2021) cells were termed "HSC-primed" and "HSC-competent" HE, respectively. Based on phenotypic signatures, these cells are most likely basal, bulging and/or 1-2 cell cluster IAHC cells rather than purely HECs.

Unique functional HSC markers

Long-term *in vivo* transplantation assays show that HSCs express many of the aforementioned cell markers. The first functional adult-repopulating HSCs in the AGM are phenotypically characterized (and isolated based on): Ly6A-GFP$^+$ (Chen et al., 2011; de Bruijn et al., 2002; Solaimani Kartalaei et al., 2015), CD27$^{+/high}$ (Li et al., 2017; Vink et al., 2020), CD34$^+$ (Sanchez et al., 1996), cKit$^{+/high}$ (Eich et al., 2018; Sanchez et al., 1996; Solaimani Kartalaei et al., 2015; Vink et al., 2020), Gata2$^+$ (Eich et al., 2018; Kaimakis et al., 2016; Minegishi et al., 1999; Minegishi et al., 2003; Vink et al., 2020), CD31$^{+/high}$ (Eich et al., 2018; North et al., 2002; Solaimani Kartalaei et al., 2015; Vink et al., 2020), VE-Cadherin$^+$, Runx1$^+$ (North et al., 2002), Endoglin$^+$ (Roques et al., 2012), Tie2$^+$ (Liakhovitskaia et al., 2009; Takakura et al., 1998) and CD41^{intermediate} (Robin et al., 2011) expression and are BMP-activated (BRE-GFP$^+$) (Crisan et al., 2015) (Figure 4).

Some marker proteins like CD11b (Mac-1, *Itgam*) (Sanchez et al., 1996), CD45 (Bertrand et al., 2005; North et al., 2002), Sca-1 (de Bruijn et al., 2002; North et al., 2002) and Flk1 (North et al., 2002) are found only on some of the first HSCs, with HSC activity detected in both the expressing and non-expressing populations. Given the transitional and heterogeneous nature of HECs and IAHCs, and the non-synchronous expression onset of most markers, the list is non-exhaustive, and several other endothelial/hematopoietic surface markers and TF reporter lines can be used for purification and/or visualization of the first HSCs. Our lab has found the use of one pan-endothelial-IAHC marker (like CD31), in combination with cKit (pan-IAHC), and Ly6A-GFP or a pivotal hematopoietic TF reporter (like *Gata2Venus*) to yield the best HSC enrichment. The necessity for these combinations of embryonic markers is underlined by the fact most adult BM HSC enrichment strategies (e.g. LSK SLAM) are not suitable for isolation of the earliest adult-repopulating HSCs. As an example, while Ly6a-GFP is expressed in all AGM HSCs, the Sca-1 (Ly6A/E) surface protein is on only 50% of AGM HSCs (de Bruijn et al., 2002), and the SLAM marker CD150 is on very few AGM cells, none of which have HSC potential (McKinney-Freeman et al., 2009).

Lesser-known and/or appreciated IAHC components

A wide range of phenotypic, functional, and transcriptomic heterogeneity of IAHCs has been revealed (Baron et al., 2018; Crisan and Dzierzak, 2016; Crisan et al., 2015; Eich et al., 2018; Kaimakis et al., 2016; Rybtsov et al., 2014; Rybtsov et al., 2011; Solaimani Kartalaei et al., 2015; Taoudi et al., 2008; Vink et al., 2020; Zhou et al., 2016). Functionally, IAHCs have been shown to contain various types of lymphoid and myeloid progenitors, embryonic multipotent progenitors, precursors to HSCs (pre-HSCs, unable to directly reconstitute adult-irradiated recipients), pre-HPSCs, rare long-term multilineage adult-repopulating HSCs and erythro-myeloid progenitors (EMPs), the latter most likely of yolk sac origin (Baron et al., 2018; Boisset et al., 2015; Eich et al., 2018; Li et al., 2014; Patel et al., 2022; Rybtsov et al., 2014; Rybtsov et al., 2011; Taoudi et al., 2008; Vink et al., 2020; Yokomizo et al., 2022). Still, functional (hematopoietic) assays fail to quantitatively match the roughly 500-700 (CD31$^+$cKit$^+$) IAHC cells observed *in vivo*, leaving the question as to what these remaining cells are and might do?

The (unknown) role of Runx1 in IAHCs

IAHC heterogeneity and lack of unique (combinations/ levels of) markers have long impeded isolation, precise localization, and transcriptomic capture of a pure population of the first adult-repopulating HSCs. Recently, SmartSeq2 single-cell RNA sequencing (scRNA-seq) of a near-pure population of AGM HSCs was achieved through reiterations of single-cell functional and transcriptomic analyses, together with index-sorting (Vink et al., 2020) (Figure 4). Despite high-level HSC enrichment and the fact that >50% of (CD31$^+$cKit^{high}Gata2^{medium}) cells exhibit an immature or mature hematopoietic transcriptome, one major subset consisted of *Runx1*-negative/low cells with an endothelial-like transcriptomic signature (Vink et al., 2020). Several other AGM scRNA-seq studies also found E11 *Runx1$^-$* (CD31$^+$cKit$^+$) IAHC cells (Baron et al., 2018; Sa da Bandeira et al., 2022). Rare Runx1$^-$ (CD44$^+$) bulging/IAHC cells were observed in the E10.5 aorta (Hou et al., 2020), and earlier studies using E11.5 (haploinsufficient) *Runx1-LacZ* reporter embryos also revealed *Runx1$^-$* IAHC (CD34$^+$cKit$^+$) cells (North et al., 1999; North et al., 2002). CD41$^+$VE-

Cadherin⁺ IAHC cells, said to be enriched for HSC precursors, were both Runx1⁻ and Runx1⁺ (by intracellular staining) at the time when IAHCs begin to be formed (Rybtsov et al., 2014). Furthermore, the E11 (CD31⁺cKit⁺) IAHC fraction of *Runx1-IRES-GFP* reporter embryos (Lorsbach et al., 2004), contains ±55% of *Runx1-GFP^negative* cells (Gonzalez, Crisan, *under revision*).

Importantly, the *Runx1*-negative fraction of IAHCs did not contain any directly-measurable HPC or adult-repopulating HSC function and thus appeared "non-hematopoietic" (Vink et al., 2020). No other studies have so far attempted to characterize Runx1⁻ IAHC cells, leaving their role undetermined. *Runx1* plays an important role in hematopoietic specification prior to EHT (Swiers et al., 2013; Zhu et al., 2020), is crucial for cell emergence during EHT and is no longer required in *Vav1*-expressing hematopoietic cells/HSPCs from the fetal liver (FL) stage onwards (Chen et al., 2009). *Cdh5/VE-Cadherin*-specific deletion of *Runx1* revealed that it is required for *de novo* generation of (pre-)HSCs until E11.5, after which some become *Runx1*-independent (Tober et al., 2013). However, with *Cdh5* mRNA expression in IAHCs roughly 80% lower than VE-Cadherin surface protein (retained on these cells until shortly after FL colonization), it is unknown whether *Runx1* was deleted in all cells (Yzaguirre et al., 2017). Another study using *CD41-Cre* mediated deletion found *Runx1* only essential during, but not prior to, the transition of CD41⁺CD43⁻ IAHC cells ("pro-HSCs") into HSCs (Liakhovitskaia et al., 2014). Still, low-level CD41 expression in early endothelium indicates that Runx1 might be necessary from the earliest HEC specification, during EHT and up until the pro-HSC stage (Swiers et al., 2013; Yzaguirre et al., 2017).

The ambiguity concerning the absolute requirement for Runx1 within IAHCs, raises several possibilities. The Runx1⁻ IAHC cells may have previously expressed *Runx1* long enough to emerge into IAHCs, but then downregulated its expression to result in an abortive EHT or a functionless cell. Abortive EHT events have been revealed by live-imaging of *runx1*-knockdown zebrafish embryos (Kissa and Herbomel, 2010). In these embryos EHT initiation is rare and when HECs begin bulging out of the endothelium, they immediately burst, thus establishing a crucial role for *runx1* in aortic cell emergence. Some abortive EHT events could be the recently described "doomed" zebrafish HSPCs that did not pass macrophage "quality control" (Wattrus et al., 2022). EHT in fish differs in several aspects from mouse, with

HEC-derived HSPCs extravasating directly into the sub-aortic space instead of forming IAHCs (Boisset et al., 2010; Kissa and Herbomel, 2010). In contrast to zebrafish, abortive EHT events in mice could result in accumulation of such IAHC cells lacking (directly-testable) hematopoietic function. Whether these cells have any potential function (*in vivo* or *in vitro*) remains to be seen.

The IAHC as a micro-niche within the wider embryonic aortic microenvironment

Differently sized IAHCs and/or specific locations within the major embryonic arteries could result in micro-niches promoting generation/maturation of different hematopoietic fates. Some of the uncharacterized IAHC cells may have a supportive role or be in a "neutral"/plastic state awaiting establishment of a hematopoietic identity. Given that *Runx1*- cells express medium/high *CD41* (Figure 4) and/or low *CD43* (and medium levels of cKit) (Vink et al., 2020), they could also represent precursors to HSCs ("pro-/pre-HSCs") (Rybtsov et al., 2014; Rybtsov et al., 2011; Taoudi et al., 2008) unable to directly reveal hematopoietic output. Such cells would only display HSC function upon *in vitro* culturing (Ganuza et al., 2017a; Inlay et al., 2014; Rybtsov et al., 2016) or direct transplantation into neonates, but not adult mice (Boisset et al., 2015; Yoder et al., 1997). These precursors are thought to mature via a multi-step process, transitioning from a "pro-HSC" phenotype ($CD41^+CD43^-CD45^-$) to "T1 pre-HSC" ($CD41^{low}CD43^+CD45^-$) and "T2 pre-HSC" ($CD41^{low}CD43^{high}CD45^+$) (Rybtsov et al., 2014; Rybtsov et al., 2011; Taoudi et al., 2008). Quantitative studies reveal around 1-2 of these pro-HSCs at E9.5, an increase from 5 to 50 pre-HSCs at E10, and a peak of 65 pre-HSCs in the E11 aorta (Rybtsov et al., 2016). By E12.5, approximately 10 pre-HSCs remain (Rybtsov et al., 2016), in line with a decrease in IAHCs and the end of *de novo* HSPC generation in the aorta (Yokomizo and Dzierzak, 2010). Slightly different phenotypic criteria reveal roughly 29 pre-HSCs per E11 AGM (Zhou et al., 2016).

Pre-HSCs are thought to mature to definitive LT-HSCs in the FL. However, one subset supposedly serves as a second origin of innate-like B-1a cells (Hadland et al., 2017). These "pre-HSCs" do not mature into LT-HSCs, but rather developmentally-restricted (B-1a-producing) FL HSCs (Ghosn et al., 2019; Godin et

al., 1993; Hadland et al., 2017). In fact, recent work shows most FL HSCs are biased towards differentiation rather than capable of long-term self-renewal (Ganuza et al., 2022b). Another subset of "pre-HSCs" might give rise to "embryonic multipotent progenitors" (eMPPs) with a predominant role in supporting lymphoid output (Patel et al., 2022), again suggesting not all phenotypic "pre-HSCs" mature into *bona fide* definitive HSCs. The low-efficiency labeling systems and (non-exclusive) phenotypic IAHC markers used in this study do not rule out contribution of AGM HSCs to the adult lymphoid compartment. (e)MPPs are said to play an important role in maintaining unperturbed (non-transplant) hematopoiesis (Busch et al., 2015; Patel et al., 2022; Pei et al., 2020; Rodriguez-Fraticelli et al., 2018; Sun et al., 2014). This is however controversial, with others showing HSCs are the major contributors to steady-state hematopoiesis (Chapple et al., 2018; Sawai et al., 2016; Sawen et al., 2018). Since maturation (and proliferation) of HSC precursors into transplantable HSCs requires *in vitro* culture steps, and phenotypic "pre-HSCs" are clearly heterogeneous, it is uncertain what proportion matures into *bona fide* HSCs and whether all "pre-HSCs" that can attain HSC function *in vitro* will achieve HSC function and identity *in vivo*. In line with this, a recent publication coined the more fitting term "pre-HSPC," for this phenotypically similar, yet functionally heterogeneous IAHC population (Yokomizo et al., 2022). Altogether these precursors are responsible for a fraction of IAHC cells and fail to add up to the 500-700 IAHC cells observed in the E10.5-11.5 aorta (Yokomizo and Dzierzak, 2010).

The stratified heterogeneity that could result in micro-niches might also be due to the clonality of IAHCs. Whereas two- and three-cell IAHCs are mostly monoclonal, those with four or more cells are polyclonal (Ganuza et al., 2017b; Porcheri et al., 2020), suggesting that IAHCs commence as monoclonal "units" and then recruit additional cells (migratory or from adjacent clusters). These cells would either already express markers like CD31 and cKit, or initiate expression in the IAHC. On the transcriptional level, rather than being enriched for a particular cell type, the majority of larger IAHCs are heterogeneous, though some were shown to contain mainly erythroid progenitors (Baron et al., 2018). In contrast to direct transplantations revealing 1-2 long-term multilineage-repopulating HSCs per E10.5-11.5 AGM, unperturbed hematopoiesis appears to be sustained by several hundred EC/IAHC precursor clones in the mouse (Ganuza et al., 2017b) and roughly 30 in the

zebrafish AGM (Henninger et al., 2017). Transplanted HSCs are capable of withstanding stress hematopoiesis and functioning in the adult bone marrow niche, thus implicating a microenvironmental role (in pre-HSPC maturation) for some cells in the larger IAHCs. Recent mouse embryo studies suggest that the smallest, one- to two-cell-containing and Dll4-expressing, IAHCs are likely to contain the very first functional HSCs (Porcheri et al., 2020; Vink et al., 2020).

Surprisingly, *Runx1⁻* IAHC cells express *Bmp4* and *Kitl* (Kit ligand or Stem Cell Factor; SCF) (Figure 4; https://gottgens-lab.stemcells.cam.ac.uk/DZIERZAK/ (Vink et al., 2020)). SCF supports AGM HSC generation/maturation and survival, and is expressed/produced by roughly 16% of aortic ECs, 9% mesenchymal cells and some IAHC cells, the latter most likely with HSC potential (Azzoni et al., 2018; Souilhol et al., 2016). These precursors are responsive to SCF (Rybtsov et al., 2014). Cytoplasmic Bmp4 is most highly expressed in ventral subaortic mesenchyme and to a lesser extent in IAHCs, with "foci" of Bmp4 (indicative of receptor activation) predominantly seen on the surface of IAHC cells (Durand et al., 2007; Souilhol et al., 2016). Like Kit ligand, Bmp4 increases HSC maturation in the AGM (Crisan et al., 2016; Durand et al., 2007), however there is a requirement for the timely downregulation of Bmp signaling (Souilhol et al., 2016). The same cells also express high levels of Apelin receptor (*Aplnr* or *APJ*) (Figure 4), which has been implicated in development and maintenance of the hematopoietic system (Jackson et al., 2021). Altogether, these uncharacterized cells might have supportive roles within IAHCs, creating unique hematopoietic (micro-)niches.

Localization of the first HSCs – Does IAHC size correlate with HSC function?

Live-imaging in mouse and zebrafish embryos demonstrates direct emergence of single round cells displaying all phenotypic (and functional) HSC criteria (Bertrand et al., 2010; Boisset et al., 2010; Kissa and Herbomel, 2010). During one rapid transdifferentiation event, these cells are capable of initiating expression of HSC markers cKit, CD41 and Ly6A-GFP (Boisset et al., 2010). Whereas the E11.5 mouse aorta contains around 500 cKit⁺ IAHC cells of which ±100 are present as single-cell "clusters" (Yokomizo and Dzierzak, 2010), zebrafish HSPCs formed by EHT, immediately extravasate without the formation of intra-aortic

clusters. With no formal proof of zebrafish generating multipotent and lineage-restricted progenitors (Stachura and Traver, 2016), one might propose zebrafish have a simpler blood hierarchy than mice, which allows for direct emergence of HSPCs from HECs without the need for any maturation. In mice, IAHCs might function to support the many different types of HPCs, pre-HSPCs and developmentally-restricted HSCs before expansion and differentiation in the FL microenvironment. The same could be true for human embryos that also generate ventral IAHCs. These arise between CS13 (27 dpc) and CS17 (39-42 dpc) and contain functional HSCs between CS14-17 (Ivanovs et al., 2017). Though much less is known about *de novo* HSC generation in human, recent scRNA-seq studies have started to carefully dissect the process to reveal a mix of overlapping and distinct players and markers as compared with mouse aortic HSCs (Calvanese et al., 2022; Crosse et al., 2020; Rix et al., 2022; Zeng et al., 2019).

Recent correlations of CD27 with Ly6A-GFP and Gata2 expression showed HSCs localize to small one- to two-cell clusters in the E10.5-11.5 mouse AGM (Vink et al., 2020) (Figure 3D), in support of a direct HEC-to-HSC transition. Some macrophages interact with these single emerged CD27^{+} cells in mouse (Vink et al., 2020). Macrophages were recently found to be crucial for HSPC generation in mouse (Mariani et al., 2019) and HSPC expansion/quality control in zebrafish (Wattrus et al., 2022) embryos. Interestingly, AGM-associated macrophages showed upregulated expression of *Bmp2* which binds the same type II receptor subunit (Bmpr2; expressed on AGM HSCs (Durand et al., 2007)) as Bmp4 and providing another possible source of Bmp for the generation of the first HSCs (Mariani et al., 2019; Sadlon et al., 2004). Furthermore, *Bmp2k* (Bmp2-inducible kinase), a downstream target of Bmp2, was found upregulated in the first HSCs (Vink et al., 2020). Though not much is known about this protein, it might provide a further link between HSCs and macrophages.

High *Dll4* expression made HSCs stand out from other cells with the same phenotype (CD31^{high}SideScatter^{low}cKit^{high}Gata2[medium]CD27[medium]), in line with others showing that Dll4 maintains smaller IAHCs (Porcheri et al., 2020). Dll4 could play a role in maintenance of HSC quiescence, providing further evidence for the direct HEC-to-HSC transition possibly facilitated by AGM-associated macrophages. The loss of MyD88-dependent toll-like receptor (TLR) signaling, an adaptor protein in one

of two main intracellular TLR pathways, was found to lead to a decrease in IAHC size/cell number and an increase in functional HSCs, whilst the number of HECs remains the same (Bennett et al., 2022). This suggests an uncoupling of IAHC growth and HSC generation and a direct HEC-to-HSC transition, however this will need further investigation.

Altogether, there is increasing evidence in favor of existence of several types of EHT; a rare and direct HEC-to-HSC transition into 1-2 cell-containing IAHCs, and a more frequent EHT resulting in HPCs and immature pre-HSCs in larger IAHCs that mature in the FL to provide for the hematopoietic needs during ontogeny.

Summary and Future Outlook

Intra-aortic clusters were first observed in many early-stage vertebrate embryos more than a century ago. Single-cell approaches have now revealed vast transcriptomic and functional heterogeneity of IAHC cells. Rare adult-repopulating HSCs reside amongst a phenotypically heterogeneous mix of (lymphoid, myeloid, and embryonic multipotent) progenitors and pre-HSPCs, the latter requiring additional maturation before revealing hematopoietic function. The similar developmental landscapes and endothelial origin of these different but phenotypically overlapping IAHC cells, render them challenging to isolate and characterize. Nonetheless, the right combinations of unique embryonic intra- and extracellular markers, and advanced isolation techniques, now allow high-level enrichment. Single-cell transcriptome profiles of IAHC cells, together with the revelation that the mouse embryo contains more IAHC cells than can be accounted for with quantitative functional assays, has raised several questions. What role(s), if any, do these remaining cells play? Do they support maturation, proliferation, or generation of certain hematopoietic cell types/states, or are they abortive EHT events that lead to non-functional cells waiting for clearance? The current state of knowledge suggests that the rare and first adult-repopulating HSCs emerge directly from aortic HECs into small IAHCs, and that *de novo* HSC identity acquisition can occur during EHT rather than by gradual maturation. Many more EHT events lead to an accumulation of "immature" (pre-)HSCs and various types of HPCs in larger IAHCs. The maturation of these precursors and restricted-/biased-HSCs could be

16

influenced/regulated by transcriptionally-distinct IAHC cells, that lack direct hematopoietic function. Together this suggests an uncoupling of IAHC growth and HSC generation, and a direct HEC-to-HSC transition. A full understanding of the wider aortic microenvironment, and potential IAHC micro-niches, should provide essential clues on how to generate, mature and/or maintain hematopoietic stem and progenitor cells. The vertebrate embryo holds the key to understanding these processes vital for healthy hematopoiesis and regeneration.

Sources of Funding

Author contributions

CSV and ED wrote the review. CSV made figures.

Figure legends

Fig 1. From early observations to high-resolution electron microscopy of EHT and IAHCs. A) Schematic of a midgestation mouse embryo with aorta (and branching at vitelline and umbilical artery) highlighted in red. Blue window indicates transversal cross-section through the aorta **Bi)** Early 20th century drawing of a transverse section through the aorta of a 12mm pig embryo. An intra-aortic haematopoietic cluster (IAHC) is attached to the ventral endothelial lining of the aorta. Ac=aortic cluster; vw=ventral wall; dw=dorsal wall; la=lateral aortic branch; s=mesenchyme. **Bii)** Early drawing of a sagittal section of vascular endothelium at the entrance of an aortic branch/ramus (ar) of a 9mm pig embryo. Morphological changes in (hemogenic) endothelial cells (HECs) are suggestive of IAHC formation (going from endothelial cell e1 to e2 to e3). Right arrow indicates blood flow from aorta into branch. Left arrow indicates direction of circulation along the long axis of the aorta. s=mesenchyme. Both **Bi)** and **Bii)** are effectively some of the first drawings of the endothelial-to-haematopoietic transition (EHT). Reprinted (with minor changes) from (Emmel, 1916), with permission from John Wiley and Sons. **C)** Transmission electron microscopy images of part of the wall of an embryonic day (E)10.5 murine dorsal aorta. Original magnifications 4000x. **Ci)** Endothelial cells (EC) budding out of the ventral aortic wall into the aortic lumen (L) as they undergo EHT. Many of the cells contain large cystic formations (*). Short arrow indicates a tight junction connecting ECs to each other, but not to the underlying mesenchyme. Long arrows point at the basal lamina of extracellular matrix between endothelium and mesenchyme. **Cii)** IAHC of four round haematopoietic cells (h) attached to the endothelial lining. The four cells in the IAHC are interconnected via tight junctions (short arrows). Reprinted (with minor changes) from (Marshall and Thrasher, 2001), with permission from John Wiley and Sons. **D,E)** Scanning electron microscopy (SEM) image of **Di)** thick transverse section through the E10 AGM region (A=inside aorta; top=dorsal; bottom=ventral), and zoom-in of an E10 **Dii)** sphere-shaped, **Ei)** stacked and **Eii)** "mushroom-like" IAHC. **Dii)** IAHC artificially-colored in yellow, endothelium in pink and sub-aortic mesenchyme in blue. **D,E)** Reprinted (with minor changes) from (Boisset et al., 2015), with permission from Elsevier.

Fig 2. Quantifications of IAHC number and size and marker changes during IAHC formation. A) Quantification of number of cKit$^+$ cells within the murine embryonic dorsal aorta (DA) at different times of development (E=embryonic day; sp=somite pairs). **B)** Number of cKit$^+$ cells per intra-aortic hematopoietic cluster (IAHC) in the E10.5 mouse. Each magenta dot indicates a cKit$^+$ cell, with numbers representing cKit$^+$ cells per cluster. One- and two-cell clusters were not drawn/included. Red numbers mark IAHCs with 10 or more cKit$^+$ cells/IAHC. VA=vitelline artery, UA=umbilical artery, FL=fetal liver. **A,B)** Reprinted from (Yokomizo and Dzierzak, 2010), with permission from The Company of Biologists Ltd. **C)** Representation of morphological and cell-specific marker changes during the transdifferentiation of endothelial cells (HECs) into IAHCs and haematopoietic stem and progenitor cells (HSPCs) occurring in the midgestation mouse aorta *in vivo*. Vascular endothelial cells express a wide array of pan-endothelial cell surface markers. Through the expression of certain pivotal haematopoietic genes, a sub-fraction of the endothelium will become hemogenic (HECs; light red) and start undergoing endothelial-to-hematopoietic transition (EHT), leading to the formation of IAHCs that gain hematopoietic characteristics (dark red) as they advance away from the endothelium. Once required/fully matured, the HSPCs will detach from IAHCs and enter circulation. Onset of expression of specific genes and markers is indicated above the schematic of the different EHT stages. Question mark (?) inside an IAHC cell refers to some of the lesser-known and/or appreciated IAHC components discussed in this review.

Fig 3. Immunohistochemistry for a selection of relevant mouse intra-aortic hematopoietic cluster markers. A) Maximum intensity projection of the middle segment of a wild-type (WT) embryonic day (E)10.5 (35 somite pairs; sp) unfixed whole-mount dorsal aorta stained for endothelial and IAHC marker CD31 (red) and intra-aortic hematopoietic cluster (IAHC) marker cKit (cyan). The highest number of IAHCs is observed on the ventral side (left) near the mouths of several aortic branches. Image taken with a Zeiss light-sheet microscope (scale bar=100µm) **B)** Transversal sections through a 35sp Gata2-Venus (top) and 38sp WT (bottom) aorta-gonad-mesonephros (AGM) region. Nuclei are in dark blue (DAPI; top), endothelial (EC) and IAHC cells in red (CD34), and Gata2 (G2V) and Runx1

expression in green and cyan, respectively. White dashed boxes indicate magnified areas **C)** Zoom-in on small IAHCs attached to the ventral aortic wall (top) and large intra-arterial hematopoietic clusters inside the umbilical artery (UA; middle+bottom) of a whole-mount stained Ly6A-GFP embryo (top+middle=38sp; bottom=37sp). All ECs express CD31 (magenta) and IAHC cells express cKit (red) and CD31. Some ECs and IAHC cells also express Ly6A-GFP (green). **D)** Detection of CD27 expression (white) on ventral one- to two-cell clusters present in transversal sections through the aorta of 43sp Gata2-Venus (top+bottom left), 42-43sp Ly6A-GFP (middle), and 36sp MacGreen (bottom right; MacGreen/GFP not shown) embryos. All ECs express CD34 (red). Some ECs and IAHC cells also express Gata2 (G2V; green; top+bottom left) or Ly6A-GFP (green; middle). Three-dimensional image (bottom left) showing a ventral two-cell cluster with one $Gata2^{medium}CD27^+$ cell and one $G2^{low}CD27^-$ cell. See also Video S1 (Vink et al., 2020). Scale bars: 30µm, unless stated otherwise. Tissue sections: 10µm thick.

Fig 4. Transcriptional profiles of select panel of genes in embryonic day 10.5-11.5 intra-aortic hematopoietic cluster cells enriched for functional HSCs.
SPRING visualization corresponding to 1087 single $CD31^+cKit^{high}Gata2^{medium}$ intra-aortic hematopoietic cluster (IAHC) cells (including 119 $CD31^{high}SideScatter^{low}cKit^{high}Gata2^{medium}CD27^{medium}$ cells, highly enriched for HSCs and localized to the HSC-containing [circled] transcriptomic sub-cluster; see CD31 plot). Cells are colored according to expression of relevant endothelial-related, hematopoietic-related and transcription factor genes, and genes expressed in the *Runx1* negative/low IAHC fraction (circled; see Runx1 plot). Transcript levels verify enrichment strategies based on expression levels of corresponding intra- and extracellular protein markers, as described in text. Data from (Vink et al., 2020). See also: https://gottgens-lab.stemcells.cam.ac.uk/DZIERZAK/

References

Azzoni, E., V. Frontera, K.E. McGrath, J. Harman, J. Carrelha, C. Nerlov, J. Palis, S.E.W. Jacobsen, and M.F. de Bruijn. 2018. Kit ligand has a critical role in mouse yolk sac and aorta-gonad-mesonephros hematopoiesis. *EMBO Rep* 19:

Baron, C.S., L. Kester, A. Klaus, J.C. Boisset, R. Thambyrajah, L. Yvernogeau, V. Kouskoff, G. Lacaud, A. van Oudenaarden, and C. Robin. 2018. Single-cell transcriptomics reveal the dynamic of haematopoietic stem cell production in the aorta. *Nat Commun* 9:2517.

Bee, T., K. Liddiard, G. Swiers, S.R. Bickley, C.S. Vink, A. Jarratt, J.R. Hughes, A. Medvinsky, and M.F. de Bruijn. 2009. Alternative Runx1 promoter usage in mouse developmental hematopoiesis. *Blood Cells Mol Dis* 43:35-42.

Bennett, L.F., M.D. Mumau, Y. Li, and N.A. Speck. 2022. MyD88-dependent TLR signaling oppositely regulates hematopoietic progenitor and stem cell formation in the embryo. *Development* 149:

Bertrand, J.Y., N.C. Chi, B. Santoso, S. Teng, D.Y. Stainier, and D. Traver. 2010. Haematopoietic stem cells derive directly from aortic endothelium during development. *Nature* 464:108-111.

Bertrand, J.Y., S. Giroux, R. Golub, M. Klaine, A. Jalil, L. Boucontet, I. Godin, and A. Cumano. 2005. Characterization of purified intraembryonic hematopoietic stem cells as a tool to define their site of origin. *Proc Natl Acad Sci U S A* 102:134-139.

Bigas, A., and C. Porcheri. 2018. Notch and Stem Cells. *Adv Exp Med Biol* 1066:235-263.

Boisset, J.C., T. Clapes, A. Klaus, N. Papazian, J. Onderwater, M. Mommaas-Kienhuis, T. Cupedo, and C. Robin. 2015. Progressive maturation toward hematopoietic stem cells in the mouse embryo aorta. *Blood* 125:465-469.

Boisset, J.C., W. van Cappellen, C. Andrieu-Soler, N. Galjart, E. Dzierzak, and C. Robin. 2010. In vivo imaging of haematopoietic cells emerging from the mouse aortic endothelium. *Nature* 464:116-120.

Busch, K., K. Klapproth, M. Barile, M. Flossdorf, T. Holland-Letz, S.M. Schlenner, M. Reth, T. Hofer, and H.R. Rodewald. 2015. Fundamental properties of unperturbed haematopoiesis from stem cells in vivo. *Nature* 518:542-546.

Calvanese, V., S. Capellera-Garcia, F. Ma, I. Fares, S. Liebscher, E.S. Ng, S. Ekstrand, J. Aguade-Gorgorio, A. Vavilina, D. Lefaudeux, B. Nadel, J.Y. Li, Y. Wang, L.K. Lee, R. Ardehali, M.L. Iruela-Arispe, M. Pellegrini, E.G. Stanley, A.G. Elefanty, K. Schenke-Layland, and H.K.A. Mikkola. 2022. Mapping human haematopoietic stem cells from haemogenic endothelium to birth. *Nature* 604:534-540.

Chapple, R.H., Y.J. Tseng, T. Hu, A. Kitano, M. Takeichi, K.A. Hoegenauer, and D. Nakada. 2018. Lineage tracing of murine adult hematopoietic stem cells reveals active contribution to steady-state hematopoiesis. *Blood Adv* 2:1220-1228.

Chen, M.J., Y. Li, M.E. De Obaldia, Q. Yang, A.D. Yzaguirre, T. Yamada-Inagawa, C.S. Vink, A. Bhandoola, E. Dzierzak, and N.A. Speck. 2011. Erythroid/myeloid progenitors and hematopoietic stem cells originate from distinct populations of endothelial cells. *Cell Stem Cell* 9:541-552.

Chen, M.J., T. Yokomizo, B.M. Zeigler, E. Dzierzak, and N.A. Speck. 2009. Runx1 is required for the endothelial to haematopoietic cell transition but not thereafter. *Nature* 457:887-891.

Ciau-Uitz, A., M. Walmsley, and R. Patient. 2000. Distinct origins of adult and embryonic blood in Xenopus. *Cell* 102:787-796.

Crisan, M., and E. Dzierzak. 2016. The many faces of hematopoietic stem cell heterogeneity. *Development* 143:4571-4581.

Crisan, M., P.S. Kartalaei, C.S. Vink, T. Yamada-Inagawa, K. Bollerot, I.W. van, R. van der Linden, S.M.C. de Sousa Lopes, R. Monteiro, C. Mummery, and E. Dzierzak. 2015. Corrigendum: BMP signalling differentially regulates distinct haematopoietic stem cell types. *Nat Commun* 6:8793.

Crisan, M., P. Solaimani Kartalaei, A. Neagu, S. Karkanpouna, T. Yamada-Inagawa, C. Purini, C.S. Vink, R. van der Linden, W. van Ijcken, S.M. Chuva de Sousa Lopes, R. Monteiro, C. Mummery, and E. Dzierzak. 2016. BMP and Hedgehog Regulate Distinct AGM Hematopoietic Stem Cells Ex Vivo. *Stem Cell Reports* 6:383-395.

Crosse, E.I., S. Gordon-Keylock, S. Rybtsov, A. Binagui-Casas, H. Felchle, N.C. Nnadi, K. Kirschner, T. Chandra, S. Tamagno, D.J. Webb, F. Rossi, R.A. Anderson, and A. Medvinsky. 2020. Multi-layered Spatial Transcriptomics Identify Secretory Factors Promoting Human Hematopoietic Stem Cell Development. *Cell Stem Cell* 27:822-839 e828.

Daane, J.M., and K.M. Downs. 2011. Hedgehog signaling in the posterior region of the mouse gastrula suggests manifold roles in the fetal-umbilical connection and posterior morphogenesis. *Dev Dyn* 240:2175-2193.

Dantschakoff, V. 1907. Uber das erate auftreten der blutelemente in huhnerembryo. *Folia Haematol* 4:159-166.

de Bruijn, M.F., X. Ma, C. Robin, K. Ottersbach, M.J. Sanchez, and E. Dzierzak. 2002. Hematopoietic stem cells localize to the endothelial cell layer in the midgestation mouse aorta. *Immunity* 16:673-683.

de Bruijn, M.F., N.A. Speck, M.C. Peeters, and E. Dzierzak. 2000. Definitive hematopoietic stem cells first develop within the major arterial regions of the mouse embryo. *EMBO J* 19:2465-2474.

Dieterlen-Lievre, F. 1975. On the origin of haemopoietic stem cells in the avian embryo: an experimental approach. *J Embryol Exp Morphol* 33:607-619.

Dignum, T., B. Varnum-Finney, S.R. Srivatsan, S. Dozono, O. Waltner, A.M. Heck, T. Ishida, C. Nourigat-McKay, D.L. Jackson, S. Rafii, C. Trapnell, I.D. Bernstein, and B. Hadland. 2021. Multipotent progenitors and hematopoietic stem cells arise independently from hemogenic endothelium in the mouse embryo. *Cell Rep* 36:109675.

Durand, C., C. Robin, K. Bollerot, M.H. Baron, K. Ottersbach, and E. Dzierzak. 2007. Embryonic stromal clones reveal developmental regulators of definitive hematopoietic stem cells. *Proc Natl Acad Sci U S A* 104:20838-20843.

Eich, C., J. Arlt, C.S. Vink, P. Solaimani Kartalaei, P. Kaimakis, S.A. Mariani, R. van der Linden, W.A. van Cappellen, and E. Dzierzak. 2018. In vivo single cell analysis reveals Gata2 dynamics in cells transitioning to hematopoietic fate. *J Exp Med* 215:233-248.

Eilken, H.M., S. Nishikawa, and T. Schroeder. 2009. Continuous single-cell imaging of blood generation from haemogenic endothelium. *Nature* 457:896-900.

Emmel, V.E. 1916. The cell clusters in the dorsal aorta of mammalian embryos. *American Journal of Anatomy* 19:401-421.

Ferkowicz, M.J., M. Starr, X. Xie, W. Li, S.A. Johnson, W.C. Shelley, P.R. Morrison, and M.C. Yoder. 2003. CD41 expression defines the onset of primitive and definitive hematopoiesis in the murine embryo. *Development* 130:4393-4403.

Ganuza, M., W. Clements, and S. McKinney-Freeman. 2022a. Specification of hematopoietic stem cells in mammalian embryos: a rare or frequent event? *Blood* 140:309-320.

Ganuza, M., B. Hadland, A. Chabot, C. Li, G. Kang, I. Bernstein, and S. McKinney-Freeman. 2017a. Murine hemogenic endothelial precursors display heterogeneous hematopoietic potential ex vivo. *Exp Hematol* 51:25-35 e26.

Ganuza, M., T. Hall, D. Finkelstein, A. Chabot, G. Kang, and S. McKinney-Freeman. 2017b. Lifelong haematopoiesis is established by hundreds of precursors throughout mammalian ontogeny. *Nat Cell Biol* 19:1153-1163.

Ganuza, M., T. Hall, J. Myers, C. Nevitt, R. Sanchez-Lanzas, A. Chabot, J. Ding, E. Kooienga, C. Caprio, D. Finkelstein, G. Kang, E. Obeng, and S. McKinney-Freeman. 2022b. Murine foetal liver supports limited detectable expansion of life-long haematopoietic progenitors. *Nat Cell Biol*

Garcia-Porrero, J.A., I.E. Godin, and F. Dieterlen-Lievre. 1995. Potential intraembryonic hemogenic sites at pre-liver stages in the mouse. *Anat Embryol (Berl)* 192:425-435.

Garcia-Porrero, J.A., A. Manaia, J. Jimeno, L.L. Lasky, F. Dieterlen-Lievre, and I.E. Godin. 1998. Antigenic profiles of endothelial and hemopoietic lineages in murine intraembryonic hemogenic sites. *Dev Comp Immunol* 22:303-319.

Ghosn, E., M. Yoshimoto, H. Nakauchi, I.L. Weissman, and L.A. Herzenberg. 2019. Hematopoietic stem cell-independent hematopoiesis and the origins of innate-like B lymphocytes. *Development* 146:

Godin, I.E., J.A. Garcia-Porrero, A. Coutinho, F. Dieterlen-Lievre, and M.A. Marcos. 1993. Para-aortic splanchnopleura from early mouse embryos contains B1a cell progenitors. *Nature* 364:67-70.

Graf, T., and T. Enver. 2009. Forcing cells to change lineages. *Nature* 462:587-594.

Haas, S., A. Trumpp, and M.D. Milsom. 2018. Causes and Consequences of Hematopoietic Stem Cell Heterogeneity. *Cell Stem Cell* 22:627-638.

Hadland, B.K., B. Varnum-Finney, P.K. Mandal, D.J. Rossi, M.G. Poulos, J.M. Butler, S. Rafii, M.C. Yoder, M. Yoshimoto, and I.D. Bernstein. 2017. A Common Origin for B-1a and B-2 Lymphocytes in Clonal Pre- Hematopoietic Stem Cells. *Stem Cell Reports* 8:1563-1572.

Henninger, J., B. Santoso, S. Hans, E. Durand, J. Moore, C. Mosimann, M. Brand, D. Traver, and L. Zon. 2017. Corrigendum: Clonal fate mapping quantifies the number of haematopoietic stem cells that arise during development. *Nat Cell Biol* 19:142.

Horton, P.D., S.P. Dumbali, K.R. Bhanu, M.F. Diaz, and P.L. Wenzel. 2021. Biomechanical Regulation of Hematopoietic Stem Cells in the Developing Embryo. *Curr Tissue Microenviron Rep* 2:1-15.

Hou, S., Z. Li, X. Zheng, Y. Gao, J. Dong, Y. Ni, X. Wang, Y. Li, X. Ding, Z. Chang, S. Li, Y. Hu, X. Fan, Y. Hou, L. Wen, B. Liu, F. Tang, and Y. Lan. 2020. Embryonic endothelial evolution towards first hematopoietic stem cells revealed by single-cell transcriptomic and functional analyses. *Cell Res* 30:376-392.

Inlay, M.A., T. Serwold, A. Mosley, J.W. Fathman, I.K. Dimov, J. Seita, and I.L. Weissman. 2014. Identification of multipotent progenitors that emerge prior to

hematopoietic stem cells in embryonic development. *Stem Cell Reports* 2:457-472.

Ivanovs, A., S. Rybtsov, E.S. Ng, E.G. Stanley, A.G. Elefanty, and A. Medvinsky. 2017. Human haematopoietic stem cell development: from the embryo to the dish. *Development* 144:2323-2337.

Jackson, M., A. Fidanza, A.H. Taylor, S. Rybtsov, R. Axton, M. Kydonaki, S. Meek, T. Burdon, A. Medvinsky, and L.M. Forrester. 2021. Modulation of APLNR Signaling Is Required during the Development and Maintenance of the Hematopoietic System. *Stem Cell Reports* 16:727-740.

Jacobsen, S.E.W., and C. Nerlov. 2019. Haematopoiesis in the era of advanced single-cell technologies. *Nat Cell Biol* 21:2-8.

Jaffredo, T., R. Gautier, A. Eichmann, and F. Dieterlen-Lievre. 1998. Intraaortic hemopoietic cells are derived from endothelial cells during ontogeny. *Development* 125:4575-4583.

Jordan, H.E. 1916. Evidence of hemogenic capacity of endothelium. *The Anatomical Record* 10:417-420.

Jordan, H.E. 1917. Aortic Cell Clusters in Vertebrate Embryos. *Proc Natl Acad Sci U S A* 3:149-156.

Kaimakis, P., E. de Pater, C. Eich, P. Solaimani Kartalaei, M.L. Kauts, C.S. Vink, R. van der Linden, M. Jaegle, T. Yokomizo, D. Meijer, and E. Dzierzak. 2016. Functional and molecular characterization of mouse Gata2-independent hematopoietic progenitors. *Blood* 127:1426-1437.

Kissa, K., and P. Herbomel. 2010. Blood stem cells emerge from aortic endothelium by a novel type of cell transition. *Nature* 464:112-115.

Kumano, K., S. Chiba, A. Kunisato, M. Sata, T. Saito, E. Nakagami-Yamaguchi, T. Yamaguchi, S. Masuda, K. Shimizu, T. Takahashi, S. Ogawa, Y. Hamada, and H. Hirai. 2003. Notch1 but not Notch2 is essential for generating hematopoietic stem cells from endothelial cells. *Immunity* 18:699-711.

Kumaravelu, P., L. Hook, A.M. Morrison, J. Ure, S. Zhao, S. Zuyev, J. Ansell, and A. Medvinsky. 2002. Quantitative developmental anatomy of definitive haematopoietic stem cells/long-term repopulating units (HSC/RUs): role of the aorta-gonad-mesonephros (AGM) region and the yolk sac in colonisation of the mouse embryonic liver. *Development* 129:4891-4899.

Lancrin, C., P. Sroczynska, C. Stephenson, T. Allen, V. Kouskoff, and G. Lacaud. 2009. The haemangioblast generates haematopoietic cells through a haemogenic endothelium stage. *Nature* 457:892-895.

Lassila, O., J. Eskola, P. Toivanen, C. Martin, and F. Dieterlen-Lievre. 1978. The origin of lymphoid stem cells studied in chick yold sac-embryo chimaeras. *Nature* 272:353-354.

Li, Y., V. Esain, L. Teng, J. Xu, W. Kwan, I.M. Frost, A.D. Yzaguirre, X. Cai, M. Cortes, M.W. Maijenburg, J. Tober, E. Dzierzak, S.H. Orkin, K. Tan, T.E. North, and N.A. Speck. 2014. Inflammatory signaling regulates embryonic hematopoietic stem and progenitor cell production. *Genes Dev* 28:2597-2612.

Li, Y., L. Gao, B. Hadland, K. Tan, and N.A. Speck. 2017. CD27 marks murine embryonic hematopoietic stem cells and type II prehematopoietic stem cells. *Blood* 130:372-376.

Liakhovitskaia, A., R. Gribi, E. Stamateris, G. Villain, T. Jaffredo, R. Wilkie, D. Gilchrist, J. Yang, J. Ure, and A. Medvinsky. 2009. Restoration of Runx1 expression in the Tie2 cell compartment rescues definitive hematopoietic

stem cells and extends life of Runx1 knockout animals until birth. *Stem Cells* 27:1616-1624.

Liakhovitskaia, A., S. Rybtsov, T. Smith, A. Batsivari, N. Rybtsova, C. Rode, M. de Bruijn, F. Buchholz, S. Gordon-Keylock, S. Zhao, and A. Medvinsky. 2014. Runx1 is required for progression of CD41+ embryonic precursors into HSCs but not prior to this. *Development* 141:3319-3323.

Lorsbach, R.B., J. Moore, S.O. Ang, W. Sun, N. Lenny, and J.R. Downing. 2004. Role of RUNX1 in adult hematopoiesis: analysis of RUNX1-IRES-GFP knock-in mice reveals differential lineage expression. *Blood* 103:2522-2529.

Ma, X., K.W. Ling, and E. Dzierzak. 2001. Cloning of the Ly-6A (Sca-1) gene locus and identification of a 3' distal fragment responsible for high-level gamma-interferon-induced expression in vitro. *Br J Haematol* 114:724-730.

Mariani, S.A., Z. Li, S. Rice, C. Krieg, S. Fragkogianni, M. Robinson, C.S. Vink, J.W. Pollard, and E. Dzierzak. 2019. Pro-inflammatory Aorta-Associated Macrophages Are Involved in Embryonic Development of Hematopoietic Stem Cells. *Immunity* 50:1439-1452 e1435.

Marshall, C.J., and A.J. Thrasher. 2001. The embryonic origins of human haematopoiesis. *Br J Haematol* 112:838-850.

Mascarenhas, M.I., A. Parker, E. Dzierzak, and K. Ottersbach. 2009. Identification of novel regulators of hematopoietic stem cell development through refinement of stem cell localization and expression profiling. *Blood* 114:4645-4653.

Maximow, A.A. 1909. Untersuchungen uber blut und bindegewebe 1. Die fruhesten entwicklungsstadien der blut- und binde-gewebszellan bein saugetierembryo, bis zum anfang der blutbilding unden leber. *Arch Mikroskop Anat* 73:444-561.

McKinney-Freeman, S.L., O. Naveiras, F. Yates, S. Loewer, M. Philitas, M. Curran, P.J. Park, and G.Q. Daley. 2009. Surface antigen phenotypes of hematopoietic stem cells from embryos and murine embryonic stem cells. *Blood* 114:268-278.

Medvinsky, A., and E. Dzierzak. 1996. Definitive hematopoiesis is autonomously initiated by the AGM region. *Cell* 86:897-906.

Mikkola, H.K., Y. Fujiwara, T.M. Schlaeger, D. Traver, and S.H. Orkin. 2003. Expression of CD41 marks the initiation of definitive hematopoiesis in the mouse embryo. *Blood* 101:508-516.

Minegishi, N., J. Ohta, H. Yamagiwa, N. Suzuki, S. Kawauchi, Y. Zhou, S. Takahashi, N. Hayashi, J.D. Engel, and M. Yamamoto. 1999. The mouse GATA-2 gene is expressed in the para-aortic splanchnopleura and aorta-gonads and mesonephros region. *Blood* 93:4196-4207.

Minegishi, N., N. Suzuki, T. Yokomizo, X. Pan, T. Fujimoto, S. Takahashi, T. Hara, A. Miyajima, S. Nishikawa, and M. Yamamoto. 2003. Expression and domain-specific function of GATA-2 during differentiation of the hematopoietic precursor cells in midgestation mouse embryos. *Blood* 102:896-905.

Minot, C., H. Evans, J. Tandler, and F. Sabin. 1912. Development of the blood, the vascular system and the spleen. *Manual of human embryology* 2:498-751.

Monteiro, R.M., S.M. de Sousa Lopes, M. Bialecka, S. de Boer, A. Zwijsen, and C.L. Mummery. 2008. Real time monitoring of BMP Smads transcriptional activity during mouse development. *Genesis* 46:335-346.

Muller, A.M., A. Medvinsky, J. Strouboulis, F. Grosveld, and E. Dzierzak. 1994. Development of hematopoietic stem cell activity in the mouse embryo. *Immunity* 1:291-301.

North, T., T.L. Gu, T. Stacy, Q. Wang, L. Howard, M. Binder, M. Marin-Padilla, and N.A. Speck. 1999. Cbfa2 is required for the formation of intra-aortic hematopoietic clusters. *Development* 126:2563-2575.

North, T.E., M.F. de Bruijn, T. Stacy, L. Talebian, E. Lind, C. Robin, M. Binder, E. Dzierzak, and N.A. Speck. 2002. Runx1 expression marks long-term repopulating hematopoietic stem cells in the midgestation mouse embryo. *Immunity* 16:661-672.

Nottingham, W.T., A. Jarratt, M. Burgess, C.L. Speck, J.F. Cheng, S. Prabhakar, E.M. Rubin, P.S. Li, J. Sloane-Stanley, A.S.J. Kong, and M.F. de Bruijn. 2007. Runx1-mediated hematopoietic stem-cell emergence is controlled by a Gata/Ets/SCL-regulated enhancer. *Blood* 110:4188-4197.

Oatley, M., O.V. Bolukbasi, V. Svensson, M. Shvartsman, K. Ganter, K. Zirngibl, P.V. Pavlovich, V. Milchevskaya, V. Foteva, K.N. Natarajan, B. Baying, V. Benes, K.R. Patil, S.A. Teichmann, and C. Lancrin. 2020. Single-cell transcriptomics identifies CD44 as a marker and regulator of endothelial to haematopoietic transition. *Nat Commun* 11:586.

Okada, S., H. Nakauchi, K. Nagayoshi, S. Nishikawa, S. Nishikawa, Y. Miura, and T. Suda. 1991. Enrichment and characterization of murine hematopoietic stem cells that express c-kit molecule. *Blood* 78:1706-1712.

Olsson, A., M. Venkatasubramanian, V.K. Chaudhri, B.J. Aronow, N. Salomonis, H. Singh, and H.L. Grimes. 2016. Single-cell analysis of mixed-lineage states leading to a binary cell fate choice. *Nature* 537:698-702.

Patel, S.H., C. Christodoulou, C. Weinreb, Q. Yu, E.L. da Rocha, B.J. Pepe-Mooney, S. Bowling, L. Li, F.G. Osorio, G.Q. Daley, and F.D. Camargo. 2022. Lifelong multilineage contribution by embryonic-born blood progenitors. *Nature* 606:747-753.

Pei, W., F. Shang, X. Wang, A.K. Fanti, A. Greco, K. Busch, K. Klapproth, Q. Zhang, C. Quedenau, S. Sauer, T.B. Feyerabend, T. Hofer, and H.R. Rodewald. 2020. Resolving Fates and Single-Cell Transcriptomes of Hematopoietic Stem Cell Clones by PolyloxExpress Barcoding. *Cell Stem Cell* 27:383-395 e388.

Porcheri, C., O. Golan, F.J. Calero-Nieto, R. Thambyrajah, C. Ruiz-Herguido, X. Wang, F. Catto, Y. Guillen, R. Sinha, J. Gonzalez, S.J. Kinston, S.A. Mariani, A. Maglitto, C.S. Vink, E. Dzierzak, P. Charbord, B. Gottgens, L. Espinosa, D. Sprinzak, and A. Bigas. 2020. Notch ligand Dll4 impairs cell recruitment to aortic clusters and limits blood stem cell generation. *EMBO J* 39:e104270.

Rao, T.N., J. Marks-Bluth, J. Sullivan, M.K. Gupta, V. Chandrakanthan, S.R. Fitch, K. Ottersbach, Y.C. Jang, X. Piao, R.N. Kulkarni, T. Serwold, J.E. Pimanda, and A.J. Wagers. 2015. High-level Gpr56 expression is dispensable for the maintenance and function of hematopoietic stem and progenitor cells in mice. *Stem Cell Res* 14:307-322.

Rix, B., A.H. Maduro, K.S. Bridge, and W. Grey. 2022. Markers for human haematopoietic stem cells: The disconnect between an identification marker and its function. *Front Physiol* 13:1009160.

Robert-Moreno, A., L. Espinosa, J.L. de la Pompa, and A. Bigas. 2005. RBPjkappa-dependent Notch function regulates Gata2 and is essential for the formation of intra-embryonic hematopoietic cells. *Development* 132:1117-1126.

Robert-Moreno, A., J. Guiu, C. Ruiz-Herguido, M.E. Lopez, J. Ingles-Esteve, L. Riera, A. Tipping, T. Enver, E. Dzierzak, T. Gridley, L. Espinosa, and A. Bigas. 2008. Impaired embryonic haematopoiesis yet normal arterial

development in the absence of the Notch ligand Jagged1. *EMBO J* 27:1886-1895.

Robin, C., K. Ottersbach, J.C. Boisset, A. Oziemlak, and E. Dzierzak. 2011. CD41 is developmentally regulated and differentially expressed on mouse hematopoietic stem cells. *Blood* 117:5088-5091.

Rodriguez-Fraticelli, A.E., S.L. Wolock, C.S. Weinreb, R. Panero, S.H. Patel, M. Jankovic, J. Sun, R.A. Calogero, A.M. Klein, and F.D. Camargo. 2018. Clonal analysis of lineage fate in native haematopoiesis. *Nature* 553:212-216.

Roques, M., C. Durand, R. Gautier, P.Y. Canto, L. Petit-Cocault, L. Yvernogeau, D. Dunon, M. Souyri, and T. Jaffredo. 2012. Endoglin expression level discriminates long-term hematopoietic from short-term clonogenic progenitor cells in the aorta. *Haematologica* 97:975-979.

Rybtsov, S., A. Batsivari, K. Bilotkach, D. Paruzina, J. Senserrich, O. Nerushev, and A. Medvinsky. 2014. Tracing the origin of the HSC hierarchy reveals an SCF-dependent, IL-3-independent CD43(-) embryonic precursor. *Stem Cell Reports* 3:489-501.

Rybtsov, S., A. Ivanovs, S. Zhao, and A. Medvinsky. 2016. Concealed expansion of immature precursors underpins acute burst of adult HSC activity in foetal liver. *Development* 143:1284-1289.

Rybtsov, S., M. Sobiesiak, S. Taoudi, C. Souilhol, J. Senserrich, A. Liakhovitskaia, A. Ivanovs, J. Frampton, S. Zhao, and A. Medvinsky. 2011. Hierarchical organization and early hematopoietic specification of the developing HSC lineage in the AGM region. *J Exp Med* 208:1305-1315.

Sa da Bandeira, D., A.M. Kilpatrick, M. Marques, M. Gomez-Salazar, T. Ventura, Z.N. Gonzalez, D. Stefancova, F. Rossi, M. Vermeren, C.S. Vink, M. Beltran, N.C. Henderson, B. Jung, R. van der Linden, H.J.G. van de Werken, W.F.J. van Ijcken, C. Betsholtz, S.J. Forbes, H. Cuervo, and M. Crisan. 2022. PDGFRbeta(+) cells play a dual role as hematopoietic precursors and niche cells during mouse ontogeny. *Cell Rep* 40:111114.

Sabin, F.R. 1920. Studies on the origin of blood-vessels and of red blood-corpuscles as seen in the living blastoderm of chicks during the second day of incubation. *Contributions to embryology.* IX:213-262.

Sadlon, T.J., I.D. Lewis, and R.J. D'Andrea. 2004. BMP4: its role in development of the hematopoietic system and potential as a hematopoietic growth factor. *Stem Cells* 22:457-474.

Sanchez, M.J., A. Holmes, C. Miles, and E. Dzierzak. 1996. Characterization of the first definitive hematopoietic stem cells in the AGM and liver of the mouse embryo. *Immunity* 5:513-525.

Sawai, C.M., S. Babovic, S. Upadhaya, D. Knapp, Y. Lavin, C.M. Lau, A. Goloborodko, J. Feng, J. Fujisaki, L. Ding, L.A. Mirny, M. Merad, C.J. Eaves, and B. Reizis. 2016. Hematopoietic Stem Cells Are the Major Source of Multilineage Hematopoiesis in Adult Animals. *Immunity* 45:597-609.

Sawen, P., M. Eldeeb, E. Erlandsson, T.A. Kristiansen, C. Laterza, Z. Kokaia, G. Karlsson, J. Yuan, S. Soneji, P.K. Mandal, D.J. Rossi, and D. Bryder. 2018. Murine HSCs contribute actively to native hematopoiesis but with reduced differentiation capacity upon aging. *Elife* 7:

Sinclair, A., B. Daly, and E. Dzierzak. 1996. The Ly-6E.1 (Sca-1) gene requires a 3' chromatin-dependent region for high-level gamma-interferon-induced hematopoietic cell expression. *Blood* 87:2750-2761.

Smith, R.A., and C.A. Glomski. 1982. "Hemogenic endothelium" of the embryonic aorta: Does it exist? *Dev Comp Immunol* 6:359-368.

Solaimani Kartalaei, P., T. Yamada-Inagawa, C.S. Vink, E. de Pater, R. van der Linden, J. Marks-Bluth, A. van der Sloot, M. van den Hout, T. Yokomizo, M.L. van Schaick-Solerno, R. Delwel, J.E. Pimanda, I.W.F. van, and E. Dzierzak. 2015. Whole-transcriptome analysis of endothelial to hematopoietic stem cell transition reveals a requirement for Gpr56 in HSC generation. *J Exp Med* 212:93-106.

Souilhol, C., C. Gonneau, J.G. Lendinez, A. Batsivari, S. Rybtsov, H. Wilson, L. Morgado-Palacin, D. Hills, S. Taoudi, J. Antonchuk, S. Zhao, and A. Medvinsky. 2016. Inductive interactions mediated by interplay of asymmetric signalling underlie development of adult haematopoietic stem cells. *Nat Commun* 7:10784.

Stachura, D.L., and D. Traver. 2016. Cellular dissection of zebrafish hematopoiesis. *Methods Cell Biol* 133:11-53.

Stricht, M. 1899. L'origine des premières cellules sanguines et des premiers vaisseaux sanguins dans l'aire vasculaire de chauves-souris'. *Bull. Acad. R. Med. Belg* 13:336-351.

Sun, J., A. Ramos, B. Chapman, J.B. Johnnidis, L. Le, Y.J. Ho, A. Klein, O. Hofmann, and F.D. Camargo. 2014. Clonal dynamics of native haematopoiesis. *Nature* 514:322-327.

Swiers, G., C. Baumann, J. O'Rourke, E. Giannoulatou, S. Taylor, A. Joshi, V. Moignard, C. Pina, T. Bee, K.D. Kokkaliaris, M. Yoshimoto, M.C. Yoder, J. Frampton, T. Schroeder, T. Enver, B. Gottgens, and M. de Bruijn. 2013. Early dynamic fate changes in haemogenic endothelium characterized at the single-cell level. *Nat Commun* 4:2924.

Takakura, N., X.L. Huang, T. Naruse, I. Hamaguchi, D.J. Dumont, G.D. Yancopoulos, and T. Suda. 1998. Critical role of the TIE2 endothelial cell receptor in the development of definitive hematopoiesis. *Immunity* 9:677-686.

Taoudi, S., C. Gonneau, K. Moore, J.M. Sheridan, C.C. Blackburn, E. Taylor, and A. Medvinsky. 2008. Extensive hematopoietic stem cell generation in the AGM region via maturation of VE-cadherin+CD45+ pre-definitive HSCs. *Cell Stem Cell* 3:99-108.

Taoudi, S., and A. Medvinsky. 2007. Functional identification of the hematopoietic stem cell niche in the ventral domain of the embryonic dorsal aorta. *Proc Natl Acad Sci U S A* 104:9399-9403.

Tavian, M., L. Coulombel, D. Luton, H.S. Clemente, F. Dieterlen-Lievre, and B. Peault. 1996. Aorta-associated CD34+ hematopoietic cells in the early human embryo. *Blood* 87:67-72.

Thambyrajah, R., and A. Bigas. 2022. Notch Signaling in HSC Emergence: When, Why and How. *Cells* 11:

Thambyrajah, R., M. Mazan, R. Patel, V. Moignard, M. Stefanska, E. Marinopoulou, Y. Li, C. Lancrin, T. Clapes, T. Moroy, C. Robin, C. Miller, S. Cowley, B. Gottgens, V. Kouskoff, and G. Lacaud. 2016. GFI1 proteins orchestrate the emergence of haematopoietic stem cells through recruitment of LSD1. *Nat Cell Biol* 18:21-32.

Tober, J., A.D. Yzaguirre, E. Piwarzyk, and N.A. Speck. 2013. Distinct temporal requirements for Runx1 in hematopoietic progenitors and stem cells. *Development* 140:3765-3776.

Turpen, J.B., C.M. Knudson, and P.S. Hoefen. 1981. The early ontogeny of hematopoietic cells studied by grafting cytogenetically labeled tissue anlagen: localization of a prospective stem cell compartment. *Dev Biol* 85:99-112.

Vink, C.S., F.J. Calero-Nieto, X. Wang, A. Maglitto, S.A. Mariani, W. Jawaid, B. Gottgens, and E. Dzierzak. 2020. Iterative Single-Cell Analyses Define the Transcriptome of the First Functional Hematopoietic Stem Cells. *Cell Rep* 31:107627.

Watt, S.M., L.H. Butler, M. Tavian, H.J. Buhring, I. Rappold, P.J. Simmons, A.C. Zannettino, D. Buck, A. Fuchs, R. Doyonnas, J.Y. Chan, J.P. Levesque, B. Peault, and I. Roxanis. 2000. Functionally defined CD164 epitopes are expressed on CD34(+) cells throughout ontogeny but display distinct distribution patterns in adult hematopoietic and nonhematopoietic tissues. *Blood* 95:3113-3124.

Wattrus, S.J., M.L. Smith, C.P. Rodrigues, E.J. Hagedorn, J.W. Kim, B. Budnik, and L.I. Zon. 2022. Quality assurance of hematopoietic stem cells by macrophages determines stem cell clonality. *Science* 377:1413-1419.

Wheatley, S.C., C.M. Isacke, and P.H. Crossley. 1993. Restricted expression of the hyaluronan receptor, CD44, during postimplantation mouse embryogenesis suggests key roles in tissue formation and patterning. *Development* 119:295-306.

Yoder, M.C., K. Hiatt, P. Dutt, P. Mukherjee, D.M. Bodine, and D. Orlic. 1997. Characterization of definitive lymphohematopoietic stem cells in the day 9 murine yolk sac. *Immunity* 7:335-344.

Yokomizo, T., and E. Dzierzak. 2010. Three-dimensional cartography of hematopoietic clusters in the vasculature of whole mouse embryos. *Development* 137:3651-3661.

Yokomizo, T., T. Ideue, S. Morino-Koga, C.Y. Tham, T. Sato, N. Takeda, Y. Kubota, M. Kurokawa, N. Komatsu, M. Ogawa, K. Araki, M. Osato, and T. Suda. 2022. Independent origins of fetal liver haematopoietic stem and progenitor cells. *Nature* 609:779-784.

Yokomizo, T., N. Watanabe, T. Umemoto, J. Matsuo, R. Harai, Y. Kihara, E. Nakamura, N. Tada, T. Sato, T. Takaku, A. Shimono, H. Takizawa, N. Nakagata, S. Mori, M. Kurokawa, D.G. Tenen, M. Osato, T. Suda, and N. Komatsu. 2019. Hlf marks the developmental pathway for hematopoietic stem cells but not for erythro-myeloid progenitors. *J Exp Med* 216:1599-1614.

Yzaguirre, A.D., M.F. de Bruijn, and N.A. Speck. 2017. The Role of Runx1 in Embryonic Blood Cell Formation. *Adv Exp Med Biol* 962:47-64.

Yzaguirre, A.D., and N.A. Speck. 2016a. Extravascular endothelial and hematopoietic islands form through multiple pathways in midgestation mouse embryos. *Dev Biol* 415:111-121.

Yzaguirre, A.D., and N.A. Speck. 2016b. Insights into blood cell formation from hemogenic endothelium in lesser-known anatomic sites. *Dev Dyn* 245:1011-1028.

Zeng, Y., J. He, Z. Bai, Z. Li, Y. Gong, C. Liu, Y. Ni, J. Du, C. Ma, L. Bian, Y. Lan, and B. Liu. 2019. Tracing the first hematopoietic stem cell generation in human embryo by single-cell RNA sequencing. *Cell Res* 29:881-894.

Zhou, F., X. Li, W. Wang, P. Zhu, J. Zhou, W. He, M. Ding, F. Xiong, X. Zheng, Z. Li, Y. Ni, X. Mu, L. Wen, T. Cheng, Y. Lan, W. Yuan, F. Tang, and B. Liu. 2016. Tracing haematopoietic stem cell formation at single-cell resolution. *Nature* 533:487-492.

Zhu, Q., P. Gao, J. Tober, L. Bennett, C. Chen, Y. Uzun, Y. Li, E.D. Howell, M. Mumau, W. Yu, B. He, N.A. Speck, and K. Tan. 2020. Developmental trajectory of prehematopoietic stem cell formation from endothelium. *Blood* 136:845-856.

Zovein, A.C., K.A. Turlo, R.M. Ponec, M.R. Lynch, K.C. Chen, J.J. Hofmann, T.C. Cox, J.C. Gasson, and M.L. Iruela-Arispe. 2010. Vascular remodeling of the vitelline artery initiates extravascular emergence of hematopoietic clusters. *Blood* 116:3435-3444.
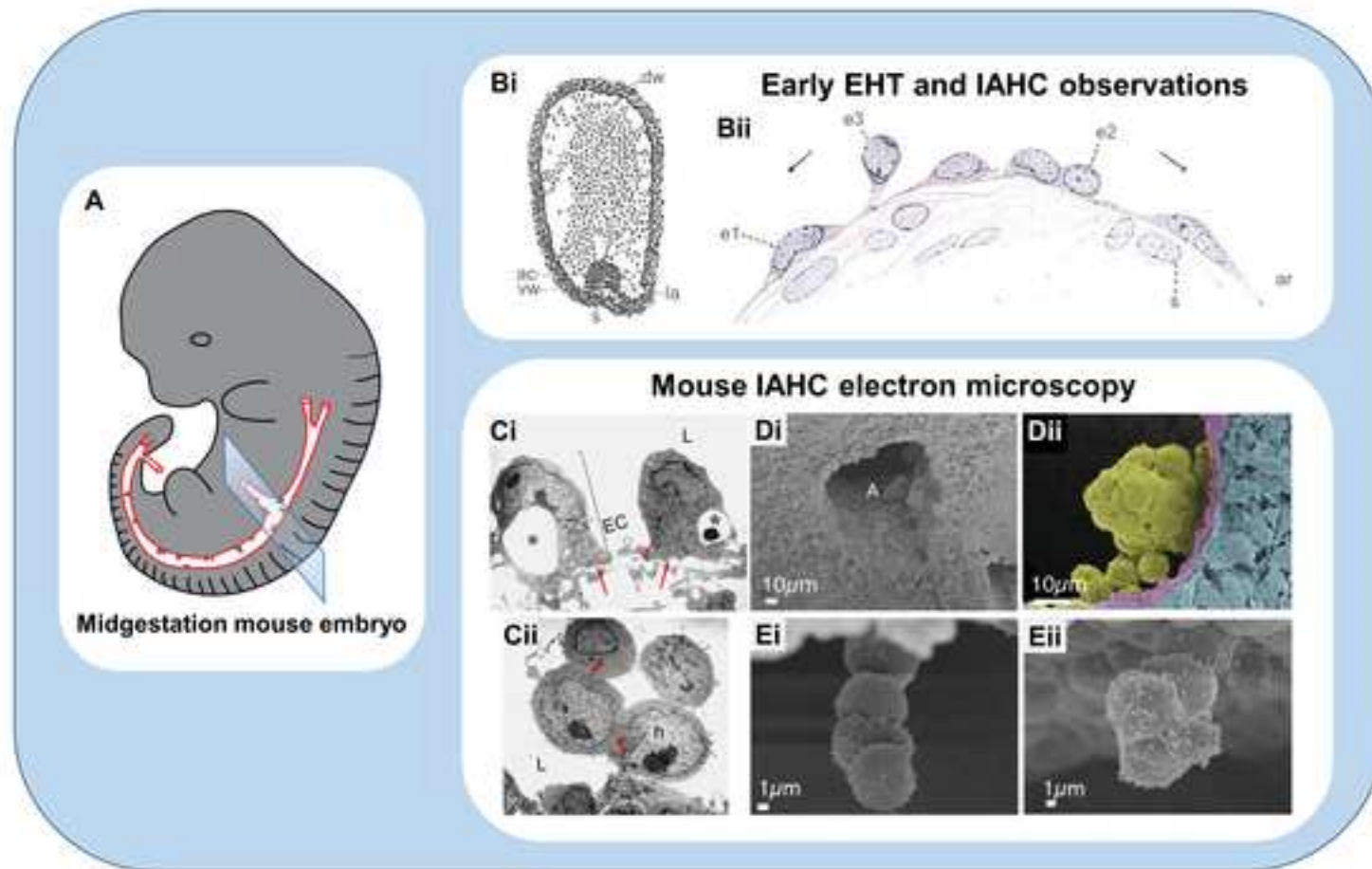
Fig1

Figure 2

Click here to access/download;Figure;Fig2_final.tif ±

A

c-Kit⁺ cells per DA (y-axis, 0–900)

P=0.014

25 sp / E9.5   35 sp / E10.5   45 sp / E11.5   55 sp / E14.5

B

E10.5

DA, FL, VA, UA

C

Aortic endothelium	HEC	EHT	Aortic clusters (IAHC)
VE-Cadherin Endoglin CD31 Esam CD34 Flk1 Tie2 Dll4	Ly6A-GFP CXCR4 Neurl3-GFP Notch1 Evi1-GFP Jag1 Runx1 CD44 Gata2 Gfi1	CD201 Gpr56 CD27 Gfi1b cKit	Hlf-tdTomato CD11b CD41 CD43 CD45

Fig2

Figure 3

Click here to access/download;Figure;Fig3_final.tif

IAHC immunofluorescence

A — cKit, CD31

B — Gata2 and Runx1 — DAPI, G2V, CD34; Runx1, CD34

C — Ly6A-GFP — cKit, CD31, Ly6A-GFP; UA

D — CD27 — G2V, CD34, DAPI, CD27; CD27; CD34

Fig3

Figure 4

Click here to access/download;Figure;Fig4_final.tif ⬇

Fig4