

REVIEW ARTICLE

# The embryonic origins and genetic programming of emerging haematopoietic stem cells

Aldo Ciaiu-Uitz and Roger Patient

MRC Molecular Haematology Unit, Weatherall Institute of Molecular Medicine, John Radcliffe Hospital, University of Oxford, UK

## Correspondence

A. Ciaiu-Uitz, MRC Molecular Haematology Unit, Weatherall Institute of Molecular Medicine, John Radcliffe Hospital, University of Oxford, Oxford OX3 9DS, UK  
Fax: +44 1865 222501  
Tel: +44 1865 222470  
E-mail: aldo.ciaiu@imm.ox.ac.uk

R. Patient, MRC Molecular Haematology Unit, Weatherall Institute of Molecular Medicine, John Radcliffe Hospital, University of Oxford, Oxford OX3 9DS, UK  
Fax: +44 1865 222501  
Tel: +44 1865 222613  
E-mail: roger.patient@imm.ox.ac.uk

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**Haematopoietic stem cells (HSCs) emerge from the haemogenic endothelium (HE) localised in the ventral wall of the embryonic dorsal aorta (DA). The HE generates HSCs through a process known as the endothelial to haematopoietic transition (EHT), which has been visualised in live embryos and is currently under intense study. However, EHT is the culmination of multiple programming events, which are as yet poorly understood, that take place before the specification of HE. A number of haematopoietic precursor cells have been described before the emergence of definitive HSCs, but only one haematovascular progenitor, the definitive haemangioblast (DH), gives rise to the DA, HE and HSCs. DHs emerge in the lateral plate mesoderm (LPM) and have a distinct origin and genetic programme compared to other, previously described haematovascular progenitors. Although DHs have so far only been established in *Xenopus* embryos, evidence for their existence in the LPM of mouse and chicken embryos is discussed here. We also review the current knowledge of the origins, lineage relationships, genetic programming and differentiation of the DHs that leads to the generation of HSCs. Importantly, we discuss the significance of the gene regulatory network (GRN) that controls the programming of DHs, a better understanding of which may aid in the establishment of protocols for the *de novo* generation of HSCs *in vitro*.**

**Keywords:** definitive haemangioblast; gene regulatory network; haematopoietic stem cells; lateral plate mesoderm; mouse; quail-chick chimera; Wolffian duct; *Xenopus*; zebrafish

The use of haematopoietic stem cells (HSCs) in the clinic has been a great success for the treatment of blood diseases. HSCs residing in the bone marrow produce all blood cell lineages throughout the lifespan of an organism and are the only cells with the capacity to replenish the whole haematopoietic system upon transplantation into an irradiated adult recipient [1]. However, limited availability has hampered their use and driven efforts for their generation *in vitro* which,

so far, have been unsuccessful. HSCs are generated during embryogenesis and their unsuccessful generation *in vitro* reflects our sparse knowledge of their embryonic ontogeny. The study of the ontogeny of HSCs has a rich history involving the use of chick, amphibian and mouse embryos, and, more recently, the zebrafish. Each model organism provides unique advantages, and a unification of the knowledge generated from them might be required for a complete

## Abbreviations

AGM, aorta-gonads-mesonephros; AP, antero-posterior; DA, dorsal aorta; DH, definitive haemangioblast; DLP, dorsal lateral plate; DMZ, dorsal marginal zone; EHT, endothelial to haematopoietic transition; GRN, gene regulatory network; HE, haemogenic endothelium; Hh, hedgehog; HSCs, haematopoietic stem cells; LPM, lateral plate mesoderm; PCV, posterior cardinal vein; PN, pronephros; TFs, transcription factors; VBI, ventral blood island; VMZ, ventral marginal zone.

understanding of how HSCs are generated during embryogenesis.

The yolk sac is the first haematopoietic organ during embryogenesis. Hence, it was thought that HSCs were generated there and later migrated to intraembryonic organs such as the liver, thymus, spleen and bone marrow [2]. However, the intraembryonic origin of HSCs was clearly demonstrated in chick-quail chimeras [3]. Furthermore, tissue transplantation performed in the frog, *Xenopus*, also demonstrated their intraembryonic origin [4,5]. Subsequently, this was confirmed in the mouse as the aorta-gonads-mesonephros (AGM) region was demonstrated to autonomously generate HSCs [6,7]. Early anatomical analyses in vertebrate embryos described haematopoietic clusters associated with the dorsal aorta (DA) [8–10]. Later these clusters were also described in the chick [11], human [12], mouse [13] and *Xenopus* [14] embryos but it was in the chick where evidence of their endothelial origin was first provided [15,16]. In the last few years, conclusive evidence of the existence of haemogenic endothelium (HE) in the DA has been provided [17–19], triggering intense research on the mechanism by which HSCs are generated from it. However, a complete understanding of the genetic programming of HSCs requires knowledge of the origin of the DA/HE. Lineage labelling in *Xenopus* and mouse has demonstrated that the DA and adult blood derive from the lateral plate mesoderm (LPM) [20,21]. In agreement, definitive haemangioblasts (DHs) have been described in the LPM of *Xenopus* [22,23]. Although DHs have not yet been specifically located in other vertebrates, including the zebrafish, as discussed below, evidence suggests their existence in the LPM of all vertebrates.

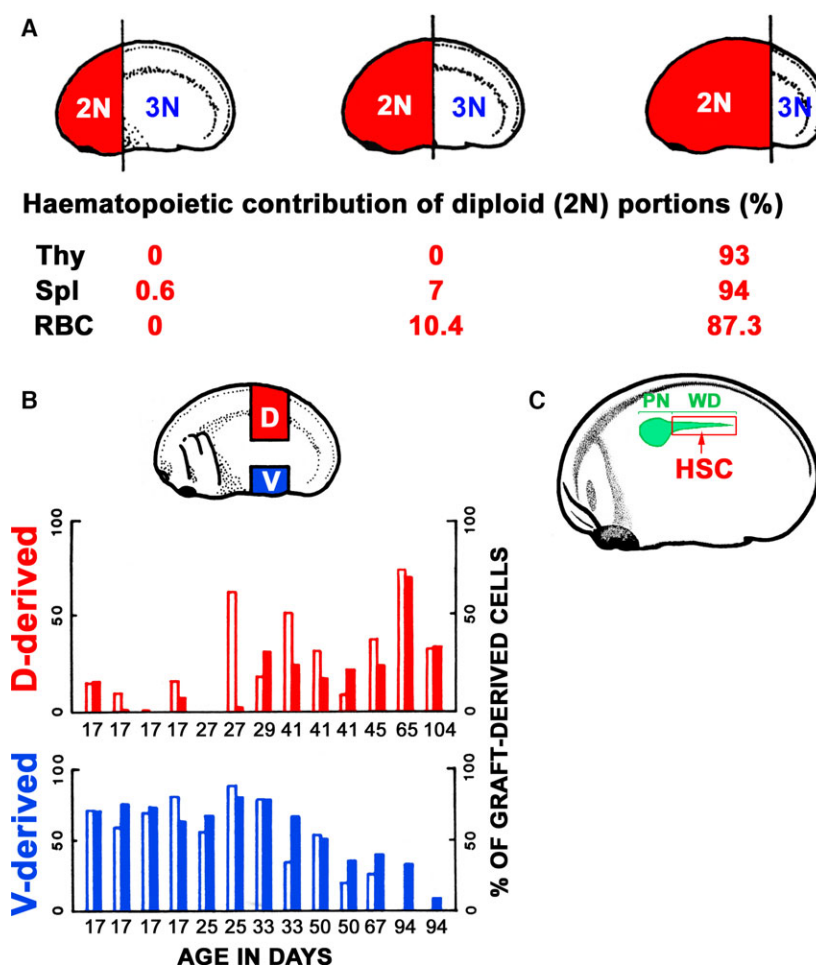
Haemangioblasts are bipotential mesodermal cells with the capacity to give rise to endothelial and blood cells [24]. The specification of DHs in the LPM is the first step in the programming of mesodermal cells towards the HSC lineage. Although the expression of some key haematopoietic transcription factors (TFs) are shared between DHs and other haematovascular progenitors [24], the distinct ontogeny of DHs and the environment where they develop, particularly because of signalling from the somites, make their genetic programming unique. Additionally, endothelial cells give rise to HSCs but endothelial cells do not have a common origin or programming, rather they are patterned along the antero-posterior embryonic axis [25]. Accordingly, lineage labelling experiments in *Xenopus* demonstrate that only cells originating from the anterior half of the posterior LPM are capable of giving rise to HSCs [4,5,26]. This has implications for the *in vitro* generation of HSCs from endothelial cells, as

correctly patterned endothelial precursors would be required. Studies in model organisms, particularly *Xenopus*, have greatly contributed to our understanding of the ontogeny of the DA and HSCs as well as of their precursors, the DHs and other haematovascular tissues, which could inform protocols for the *de novo* generation of HSCs. Here, we briefly summarise these achievements.

## The lateral plate mesoderm gives rise to haematovascular progenitors

The experimental amenability of the *Xenopus* embryo to tissue grafting and fate mapping has allowed the determination of the blood-forming potential of regions and tissues of the early embryo (Fig. 1). The generation of *Xenopus borealis*–*Xenopus laevis* interspecific chimeras as well as 3N–2N *Xenopus laevis* chimeras demonstrated that all adult blood derives from the trunk of the embryo (Fig. 1A) [27]. It has recently been reported that presumptive HSCs are localised in the head of the mid-gestation mouse embryo and that they give rise to postnatal haematopoiesis [28,29]. In *Xenopus*, chimeric embryos have shown that the head does not contribute to adult blood (Fig. 1A) [27]. In agreement, in interspecific chick-quail chimeras, the head does not contribute to adult blood [30]. The generation of the amphibian and avian chimeras was performed long before the onset of blood circulation, whereas the head contribution in the mouse was recorded after the circulation had started, opening the possibility that HSCs from other tissues colonise the head through the circulation; a view supported by the lack of endothelial to haematopoietic transition (EHT) events in the head [31]. Further research in the mouse is required to clarify whether HSCs emerge independently in the head or are contributed by other tissues such as the AGM.

The trunk of the *Xenopus* embryo contains the earliest blood-forming organ, the ventral blood island (VBI), which is equivalent to the amniote yolk sac as it produces primitive erythrocytes and macrophages [32]; and, similarly, it was initially believed to be the only source of blood cells, including HSCs. However, orthotopic transplantation of 2N tissue into 3N host embryos demonstrated that a second, independent, haematopoietic tissue contributing to adult blood was located dorsally in the LPM adjacent to the somites, the dorsal lateral plate (DLP; Fig. 1B) [4,5,27]. Previous experiments in *Rana* showed that the LPM localised just posterior to the pronephros, and alongside the Wolffian duct, gives rise first to the DA, including haematopoietic cells, and later to liver, thymic and



**Fig. 1.** Haematopoietic stem cells derive from the lateral plate mesoderm. (A) *Xenopus* diploid–triploid chimeras showing that adult blood derives from the trunk. Very few, if any, cells derive from the head. Chimeras were generated at stage 22 of development. Thy, thymic blood; Spl, splenic blood; RBC, red blood cells. Data after Maeno *et al.* [27]. (B) Dorsal, not ventral, tissues within the trunk give rise to adult blood. Diploid tissues were grafted into triploid recipients and their contribution to thymocytes (open bars) and red blood cells (closed bars) were analysed throughout development. Dorsal-derived blood increased as the ventral-derived contribution decreased. Grafting experiments were performed at stage 22 of development. D, dorsal; V, ventral. Figure after Maeno *et al.* [4]. (C) HSCs derive from the LPM immediately posterior to the pronephros (PN) and alongside the Wolffian duct (WD).

splenic haematopoiesis [33,34]. Subsequently, this region of the LPM, just posterior to the pronephros, was confirmed by tissue transplantation in *Xenopus* embryos to be the source of haematopoietic cells associated with the DA, the liver, thymus, spleen and, importantly, adult haematopoiesis (Fig. 1C) [4,5,21,26,27]. More recently, direct labelling of the DLP confirmed its contribution to the DA [35]. Furthermore, the DA is absent when the DLP is physically removed [35]. In agreement, lineage labelling in *Xenopus* has demonstrated that a single blastomere of the 32-cell stage embryo, blastomere C3, gives rise to the DLP, the DA and intra-aortic haematopoietic clusters without contributing to the VBI [14,23]; thus, establishing the dorsal LPM, not the VBI, as the tissue from which the progenitors of the DA and HSCs derive.

With the generation of a monoclonal antibody, QH1, that specifically labels endothelial and haematopoietic cells, analysing the development of the first blood vessels in the quail embryo was made possible [36,37]. In amniotes, the DA forms from the fusion

of the paired DA which have migrated from the LPM, one from each side of the embryo [38]. Using the QH1 antibody, it was shown that each of the paired DA forms from single endothelial cells which segregate from the LPM and aggregate to form a cord ventral to the somites; meanwhile, the posterior cardinal vein (PCV) also segregates from the LPM, and both vessels form in association with the Wolffian ducts [37]. Furthermore, transplantation of quail LPM into chick embryos resulted in the contribution of quail endothelial cells to the DA of the host as well to intersomitic vessels [36]. More recently, time-lapse imaging analysis of Cy3-QH1 pulse-labelled quail embryos demonstrated that LPM endothelial cells directly contribute to the primary DA [39]. Interestingly, QH1 cells are positive for an antibody against the haematopoietic determinant, Tal1/Scf [40], indicating that the DA derives from cells coexpressing blood and endothelial genes.

The advent of RNA *in situ* hybridisation further improved the analysis of the haematopoietic

progenitors emerging in the LPM. Hierarchically, *Tall1/Scl* confers a haematopoietic fate on mesodermal cells and its deficiency results in a total absence of blood cells in the mouse [41]. Detailed analysis of *Tall1/Scl* expression during embryogenesis was first performed in zebrafish embryos and revealed the presence of haemangioblasts localised in the LPM [42]. In agreement with its LPM origin, *Tall1/Scl* loss of function results in the absence of the zebrafish DA [43]. Intriguingly, these haemangioblasts give rise to both primitive erythrocytes and macrophages as well as to the precursors of the DA/HSC [32]. However, DA precursors migrate to the midline before the erythrocyte precursors, suggesting that their generation could be temporally distinct [42]. Whether single haemangioblasts in the zebrafish LPM can give rise to both primitive and definitive lineages is not known; either way, zebrafish DHs cannot so far be distinguished from other precursors localised in the LPM. Nevertheless, it is clear that the zebrafish DA derives from the *Tall1/Scl*-expressing LPM.

As discussed above, transplantation experiments defined the location of HSC precursors in the *Xenopus* dorsal LPM. Extensive gene expression analysis has revealed the presence of cells coexpressing endothelial and haematopoietic genes in the *Xenopus* dorsal LPM [22,23], which are very likely to be DHs. However, these DHs cannot anatomically be distinguished from the surrounding mesodermal cells (Fig. 2A). Nevertheless, we have established that they localise immediately ventral to the somites and dorsal to the Wolffian duct anlagen (Fig. 2B,C). The zebrafish LPM haemangioblasts also localise between the somites and the Wolffian duct [42]. This appears to be a common theme in the development of the HSC lineage as the precursors of the DA in the mouse and avian embryos also develop in association with the somites and the Wolffian duct (Fig. 2D).

A genetic hierarchy in the *Xenopus* LPM has been established through the temporal analysis of the expression of blood and endothelial genes [22]. Importantly, the gene regulatory network (GRN) controlling the programming of DHs, as indicated by *Tall1/Scl* expression, has been established through gene perturbation in *Xenopus* [22,44,45]. This GRN shows that, in addition to familiar regulators of haematopoiesis and vascular development, such as *Gata2*, *Kdr/Flk1*, *VEGFA* and the ETS TFs, *Fli1* and *Etv2*, less known regulators such as the ETS TF, *Etv6*, play essential roles in the programming of DHs. *Etv6* has emerged as a critical regulator of *VEGFA* expression in both the somites and the developing DHs themselves [22,46]. Although haemangioblasts are known to

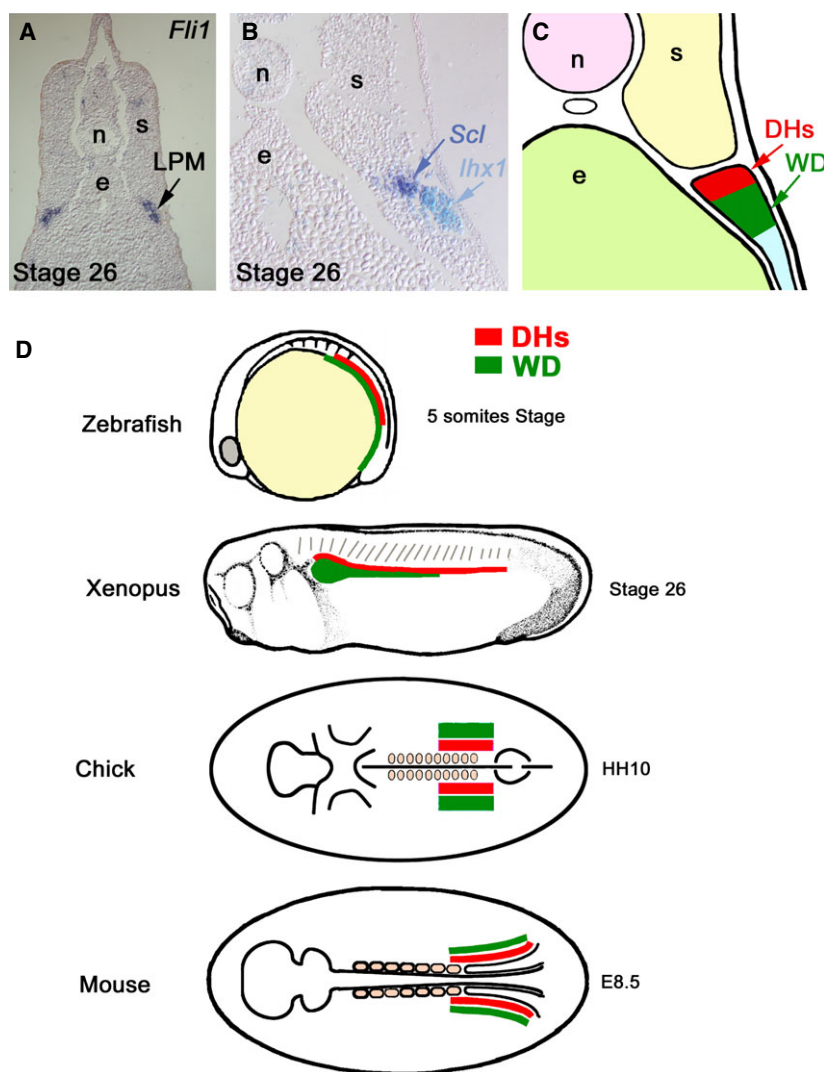
express *Kdr/Flk1*, the receptor for *VEGFA*, the source of *VEGFA* in the LPM was unknown. The DH GRN establishes that paracrine *VEGFA* from the somites is indispensable for the initiation of the DH programme and does so by activating its own expression in *Flk1*-expressing cells through *Etv6* [22]. Expression of *VEGFA* in the somites is also controlled by *Etv6* [46]. This has established that the somites act as a critical signalling centre from the earliest stages of programming of the HSC lineage. Another critical observation is that the establishment of the HSC lineage requires not only TFs but also the intervention of microRNA. The existence of repressive signalling detrimental to DH specification has been revealed by the depletion of *miR-142-3p* [45]. MicroRNAs are known to act as repressors, therefore the failure to establish DH in *miR-142-3p*-depleted embryos suggested that this results from the upregulation of a repressive factor. *miR-142-3p* target analysis suggested *TGF $\beta$*  receptor as a potential target. Indeed, *TGF $\beta$*  appears to be the repressive signal repressed by *miR-142-3p* as its inhibition rescues the DH programme in *miR-142-3p*-depleted embryos [45]. How *miR-142-3p*'s expression is controlled is yet to be determined.

Although the expression of endothelial genes has been analysed in detail in the mouse LPM [47,48], very little is known about the expression of haematopoietic genes. Nevertheless, the expression of *Tall1/Scl* at the stages when endothelial cells segregate from the LPM to form the DA, suggests that DHs also exist in the mouse LPM [47,49]. Importantly, although using a transgenic line that also drives expression in extraembryonic tissues, lineage labelling strongly suggests that LPM not only gives rise to the DA but also to all blood cells in the bone marrow of adult mice [20,50]. Taken together, the LPM origin of HSCs appears to be conserved throughout vertebrate evolution (Fig. 2D).

### Not all endothelial cells are related to haematopoietic progenitors

Global gene expression analysis of human endothelial cells from different body sites, cultured under the same conditions, as well as of established laboratory cell lines, revealed an extraordinary diversity in their molecular programming which appears to reflect their origin [51]. Endothelial cells have been considered as a relatively homogeneous cell type and their differential response to physiological challenges was thought to be due to extrinsic factors; however, the Chi *et al.* findings suggest that these differences might be largely due to intrinsic regulatory mechanisms that appear to reflect regional programming and, perhaps, their





**Fig. 2.** The lateral plate mesoderm contains definitive haemangioblasts (DHs). (A) Expression of *Fli1* in a transverse section of a stage 26 *Xenopus* embryo, showing the localisation of DHs within the LPM. No morphological differences are noticeable with surrounding cells. Dorsal is to the top. s, somites; n, notochord; e, endoderm. (B) *In situ* hybridisation showing that *Tal1/Scf*-expressing DHs localise dorsal to the Wolffian duct anlagen, indicated by *Lhx1* expression. Dorsal is to the top. (C) Diagram showing that DHs localise ventral to the somites and dorsal to the Wolffian duct (WD). (D) The emergence of DHs in association with the somites and WD is conserved through evolution. Although the exact location of DHs is only known in *Xenopus* embryos, *Tal1/Scf* expression in the LPM (shown in red) of other systems is associated with the somites and WD (shown in green). Red bars in the chick and mouse embryos show the localisation of emerging precursors of the DA, which have been shown to express *Tal1/Scf* [40,47].

distinct embryonic origin. Indeed, evidence indicates that embryonic endothelial cells have multiple origins, and that they are patterned and distinctly programmed during early embryogenesis.

In amniotes, the first endothelial cells emerge in extraembryonic tissues and it was initially proposed that the blood vessels within the embryo proper develop from extraembryonic precursors [52]. This view was challenged by the detection of isolated endothelial vesicles in intraembryonic tissues and by the emergence of blood vessels in cultured tissue portions explanted from prevascularised chick embryos (reviewed in [52]). The intraembryonic origin of endothelial cells was confirmed by tissue transplantation, in particular by the contribution of mesodermal cells of quail origin to the blood vessels of recipient chick embryos [52]. In the quail-chick chimeras, mesodermal cell transplantations were performed before

there was any evidence of intraembryonic endothelial development, either in the donor or recipient embryo, and endothelial cells of quail origin were detected with antibodies. These experiments revealed that most mesodermal cells have the capacity to give rise to endothelial cells and that the emergence of endothelial cells was rarely associated with blood cells [52]. The quail-chick chimeras also indicated that the origin of blood vessels is regionalised. For example, specific regions of the cephalic paraxial mesoderm give rise to particular cranial and head blood vessels, with little or no mixing between body segments [53], strongly suggesting an antero-posterior (AP) patterning of the emerging vasculature.

Lineage labelling in early zebrafish embryos also suggested that most mesoderm-forming regions give rise to endothelial cells [54]. In these experiments, caged fluorescein dextran of ubiquitous distribution

was activated with a laser in single cells located within a specific mesodermal quadrant and their descendants, coexpressing either a *Flk1* or *Gata1* transgene, were scored; this was performed both at the blastula and gastrula stages with similar results: cells from most quadrants were found later to express the *Flk1* transgene [54]. A caveat of these experiments is that only cells of the ventral marginal zone were labelled. Also, whether quadrants contribute to specific regions of the vasculature was not examined.

A unique advantage of the *Xenopus* embryo as a model resides in the regularity of the first cell divisions which leads to the generation of embryos with symmetric blastomeres. This allows the generation of reproducible fate maps and has been used to determine the origin of the early vasculature [25]. Labelling single blastomeres with rhodamine dextran at the 16- and 32-cell stage and analysing their contribution to the early embryonic vasculature by colocalisation with the expression of an endothelial gene, apelin receptor (*Aplnr*, also known as *Msr*), determined that all blastomeres fated to the mesodermal lineage give rise to endothelial cells [25]. However, this fate mapping clearly demonstrated an AP organisation/patterning of the *Xenopus* vasculature. Before gastrulation, mesodermal progenitors localise in an equatorial ring (Fig. 3A), where the dorsal marginal zone (DMZ) gives rise to the head region, while the ventral marginal zone (VMZ) gives rise to more posterior mesoderm. Accordingly, the head vasculature derives from the most DMZ blastomeres, while the tail vasculature derives from the most VMZ blastomeres (Fig. 3B) [25]. Importantly, a more medial blastomere, blastomere C3, gives rise to trunk vasculature, including the DA which is fated to give rise to HE. Thus, this fate map agrees with the fate map demonstrating that DH and HE derive from C3 (Fig. 3C) [14].

Analysis in ES cells also indicates that endothelial cells are heterogeneous. For instance, when the expression of a *HoxB6* reporter, which drives expression in posterior LPM, was analysed in mouse ES cells differentiated on the OP9 stromal cell line or during the differentiation of embryoid bodies, it was found to be expressed in only 3% of *Flk1*+ cells [55]. Importantly, the expression of genes associated with arterial specification and critical for definitive haematopoiesis, such as *Notch1*, *Notch4* and *Sox17*, were enriched in this cell population [55], indicating that only a small fraction of *Flk1*+ cells are programmed to the definitive blood lineage.

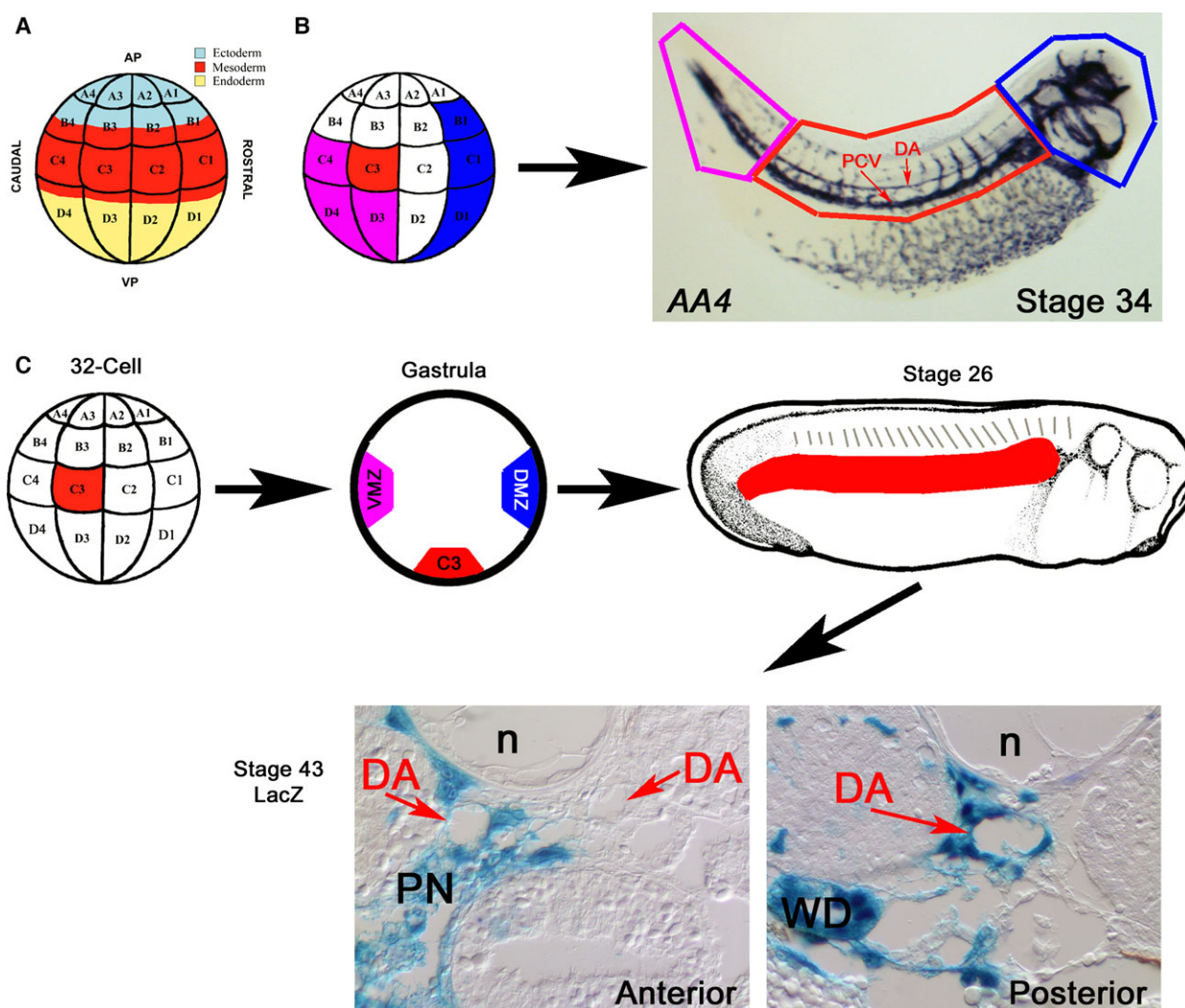
Taken together, the emergence of endothelial cells is not restricted to a single location in the mesodermal layer, instead they appear to emerge from all types of

nascent mesodermal cells. Importantly, the vast majority of endothelial cells emerge independently of haematopoietic cells; in other words, they emerge directly from mesodermal cells rather than through a common blood-endothelial precursor or haemangioblast. In contrast, haematopoietic cells emerge in a few restricted areas of the embryo, for example only three regions of the *Xenopus* embryo give rise to blood: two giving rise to primitive/embryonic blood and only one to definitive blood/HSCs [14]. Also, all haematopoietic cells appear to emerge in association with cells expressing endothelial genes and not directly from mesodermal cells; in other words, through a common haematovascular progenitor, either from haemangioblasts or HE.

### Antero-posterior patterning of endothelial cells

The vertebrate body plan is established early in embryogenesis, so by the end of gastrulation, the body axes (anterior–posterior, dorsal–ventral, left–right) have already been established [56]. This body plan establishes molecular coordinates which dictate the development of particular tissues and organs in specific locations within the body. As a result, body regions become committed to particular fates which cannot be changed beyond certain developmental stages. Regarding the haematopoietic fate, it has been demonstrated in *Xenopus* that ventral tissue, which is fated to give rise to embryonic blood can be reprogrammed to give rise to adult blood when grafted into the dorsal LPM [57]. Conversely, dorsal LPM fated to give rise to adult blood loses this potential when grafted into ventral mesoderm and is reprogrammed to produce embryonic blood [57]. However, this reprogramming cannot be achieved when these tissues are grafted after stage 15 of development which is just a couple of hours after gastrulation [57]. These experiments demonstrate that the embryonic and adult blood lineages are already distinct soon after gastrulation.

The *Xenopus* fate map and tissue transplantation in avian embryos demonstrate that the emerging embryonic vasculature is patterned along the AP axis. Recent evidence indicates that this patterning is conserved in the mouse. Regional deletion of the ETS TF, *Etv2*, which is essential for endothelial development, demonstrated a regionalisation of the endothelium as avascular areas resulting from the deletion of *Etv2* are not revascularised by endothelial cells from neighbouring regions [55]. For instance, *Etv2* deletion in *Mesp1*-expressing cells results in the loss of vasculature anterior to the forelimb and posterior to the hindlimb,



**Fig. 3.** Fate map in *Xenopus* embryos indicates AP patterning of the endothelium. (A) Localisation of the germ layers in the 32-cell stage embryo. Haematopoietic and endothelial cells emerge from the mesodermal layer. AP, animal pole; VP, vegetal pole. (B) The 32-cell stage fate map of the dorsal aorta (DA) and other axial vessels shows an AP regionalisation of the vasculature. The contribution of blastomeres of the 32-cell stage embryo to the vasculature are outlined on an embryo stained for expression of the vascular gene, *AA4*. Anterior to the right and dorsal to the top. PCV, posterior cardinal vein. (C) Time course of blastomere C3 contribution to haematopoietic tissues. Blastomere C3 gives rise to the dorsal LPM at stage 26 and to the DA and HSCs at stage 43 [23]. During gastrulation, C3 derivatives are distinctly localised from the progenitors of embryonic blood in the DMZ and VMZ. Sections show LacZ staining from one of the two C3 blastomeres at two axial levels: at the level of the bifurcated DA (Anterior) and at the level of the medial DA in the trunk (Posterior). C3 contributes to the entire DA of the trunk and, anteriorly, to the bifurcated DA proximal to the trunk. PN, pronephros; n, notochord; WD, Wolffian duct.

with the trunk vasculature, encompassing the AGM region, much less affected [55]. Unless *Etv2* deletion in *Mesp1* cells indirectly affects the migratory behaviour of normal endothelial cells, this indicates that trunk endothelium does not contribute to, and cannot compensate for, the loss of more anterior or posterior endothelial cells. Furthermore, although somite-derived endothelium has been shown to be highly migratory [58], it appears to be unable to colonise the

avascular regions generated by *Etv2* deletion. Thus, the persistence of avascular regions in *Etv2*-deficient embryos is more likely due to regional programming rather than defects in the migratory behaviour of normal endothelial cells. Interestingly, this reduces the possibility that a migratory endothelial precursor is responsible for HSCs in the head, and suggests that a more mature precursor cell, with haematopoietic potential, is a more likely contributor.

It is noteworthy that, in birds, endothelial cells derived from the somites contribute to the embryonic DA [59]. Although the DA is initially formed by endothelial cells derived from the LPM [36,37,39,40], by the time intra-aortic haematopoietic clusters sprout from the ventral wall, the dorsal wall consists of somite-derived endothelium [60]. Haematopoietic potential, however, is restricted to the splanchnic mesoderm-derived ventral wall. Later, after HSCs have sprouted, even the ventral wall is replaced by somite-derived endothelium [61]. In the mouse, however, somite contribution to the DA endothelium is rare [58,62]. Furthermore, lineage labelling strongly indicates that all endothelium of the embryonic and adult DA derives from the LPM [20,50], ruling out a potential contribution of the somites to HSCs. In the mouse, a later contribution of the somites to the HSC lineage is also very unlikely as only endothelial cells labelled during a narrow window of development give rise to adult blood [63].

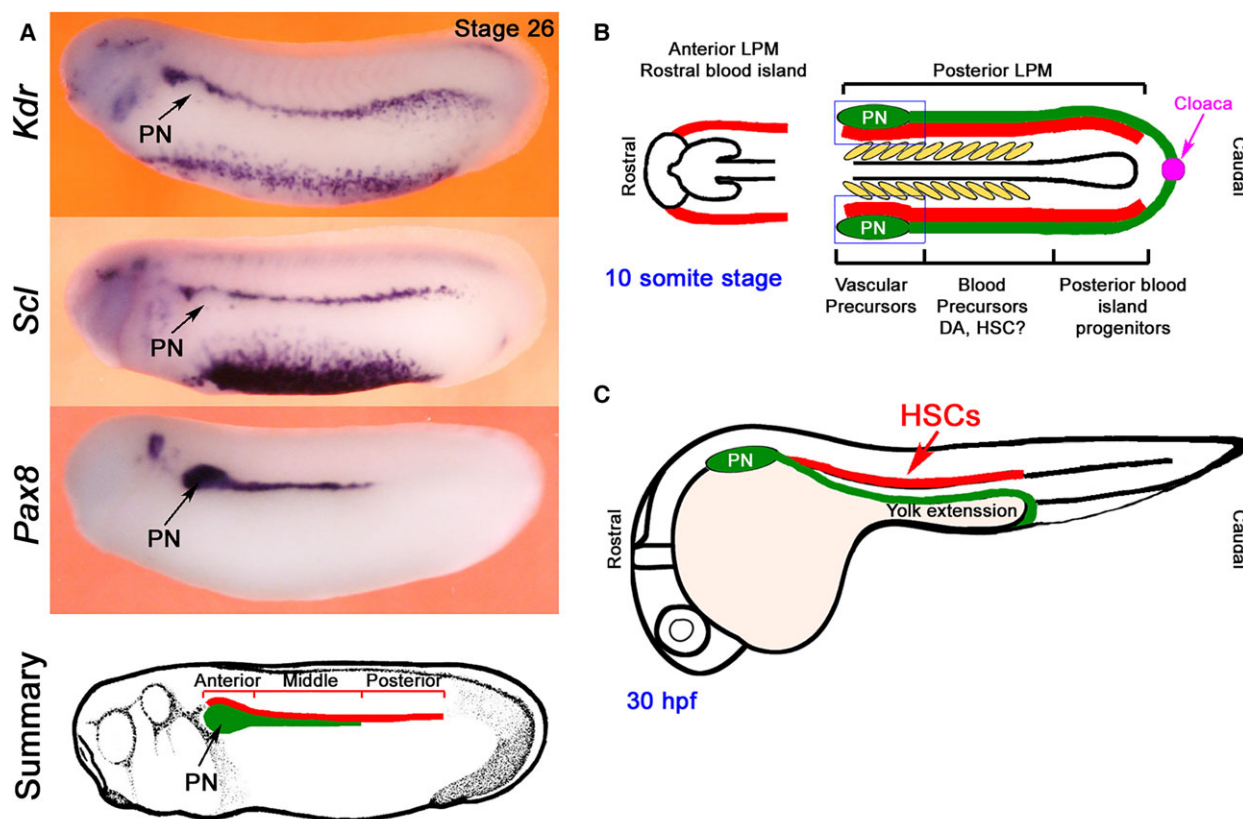
In *Xenopus*, HSCs originate from a restricted region of the LPM (Fig. 1) [4,5,21,26], suggesting that the LPM is patterned. Importantly, the *Xenopus* Wolffian duct, a structure developing in close association with DHs, has been shown to be segmented in an AP manner [64]. The zebrafish Wolffian duct also develops in close association with haemangioblasts and has also been shown to be segmented [65,66]. Although segmentation of the amniote Wolffian duct has not yet been described, its function evolves with time, reflecting its sequential connection with the transient pronephros, the mesonephros and, finally, the metanephros and the reproductive organs [67]. In amniotes, the Wolffian duct also develops alongside the DA precursors (Fig. 2D) and its development may reflect regional patterning cues that could also influence the emergence of DHs.

In *Xenopus* embryos, the expression of *Tall/Scl* and *Kdr/Flk1* in the LPM starts at the level of the pronephros (PN) and extends posteriorly to the tail bud (Fig. 4A). Their expression can be subdivided into three regions relative to the developing pronephros and Wolffian duct: an anterior region associated with the pronephric head, a middle region running alongside the Wolffian duct, and a posterior region without any association with pronephric tissue (Fig. 4A). Transplantation experiments have demonstrated that HSCs derive from the region localised immediately posterior to the pronephric head (Fig. 1); therefore, it is likely that DHs giving rise to HSCs reside in the middle portion of the PLM and that they develop in association with the Wolffian duct. However, the precise location of the DHs requires further investigation.

At the 10-somite stage, the LPM of the zebrafish embryo can be subdivided into anterior and posterior regions according to *Tall/Scl* expression (Fig. 4B). The anterior region is located in the head and gives rise to the rostral blood island. This region produces myeloid and endothelial cells and also contains cardiac precursors [42,68–70]. The posterior LPM localises in between the somites and the pronephric anlagen; however, in contrast to the Wolffian duct, *Scl* does not reach the cloaca (Fig. 4B). The posterior LPM can be further subdivided into two regions according to their potential to give rise only to vascular precursors or to both blood and vascular precursors. *Gata1* expression in the posterior LPM localises from somite 5 and extends posteriorly to cover the *Tall/Scl* expression domain. Therefore, it is thought that haematopoietic potential is contained within the *Gata1*-expressing cells, whereas the *Gata1*-negative domain localised anterior to somite 5 and running alongside the pronephros (Fig. 4B, blue-boxed region) only generates vascular precursors. The *Gata1*-expressing domain can further be subdivided, as its most posterior region is believed to later give rise to the posterior blood island which has erythromyeloid potential [71]. The region between the vascular precursors and the posterior blood island progenitors is where embryonic erythrocyte precursors reside and, additionally, it is thought to give rise to HSCs, as endothelial cells derived from this region form the trunk DA [32,72], where EHT has been observed [17,18]. However, the boundaries between the regions of the posterior LPM have not been clearly delineated and lineage labelling experiments will be required to define where in the LPM of the 10-somite embryo HSC potential is located. At the 10-somite stage, HSC potential has been proposed to be located posterior to the pronephros (Fig. 4B); this could be the case as *Runx1* expression in the DA of the 30-h postfertilisation (hpf) embryo is also restricted posterior to the PN and extends posteriorly as far as the yolk extension (Fig. 4C). Taken together, these observations indicate AP patterning of the HSC potential in zebrafish.

The functional significance of endothelial patterning is exemplified by the role of hedgehog (Hh) in arterial specification [73]. In the zebrafish, Hh signalling has been implicated in the regulation of *VEGFA* expression in the somites. Subsequent VEGFA secretion from the somites activates notch signalling in the DA and this establishes the arterial programme [74]. In parallel, this Hh-VEGFA-notch signalling cascade initiates the HE programme in the zebrafish DA [75]. In the mouse, expression in developing arteries of Dll4, a notch ligand that is essential for arterial identity [76],



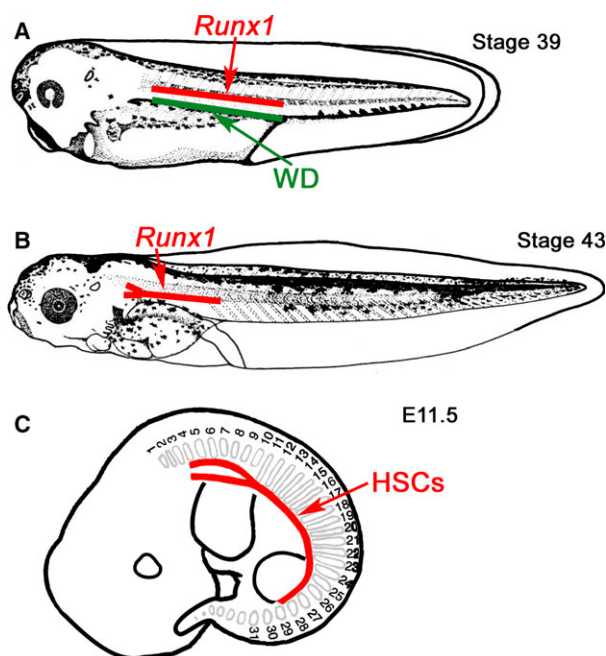


**Fig. 4.** Haematovascular progenitors within the LPM are regionalised. (A) Comparison of *Kdr/Flk1*, *Tal1/Scl* and *Pax8* expression in the dorsal LPM of stage 26 *Xenopus* embryos showing three regions according to their association with the developing pronephric system: an anterior region associated with the pronephros (PN), a middle region associated with the Wolffian duct, and a posterior region without any association with the pronephric system. Transplantation experiments have shown that HSCs derive from the middle region of the LPM. However, further analysis is required to establish whether the anterior and posterior LPM give rise to any HSCs. (B) Regionalisation of the LPM in 10-somite zebrafish embryos. *Scl* expression, shown in red, subdivides the LPM into anterior and posterior. The posterior LPM localises alongside the Wolffian duct (shown in green) and can be further subdivided into vascular precursors which do not express Gata1, a region proximal to the cloaca which gives rise to the posterior blood island and a middle region which gives rise to embryonic blood. This middle region is also thought to contain HSC precursors. (C) *Runx1* expression (red) in the DA of the 30hpf zebrafish embryo localises in the trunk. This region has been shown to undergo EHT to give rise to HSCs. The area expressing *Runx1* spans the Wolffian duct (green), is posterior to the pronephros (PN) and does not extend posterior to the yolk extension. Thus, emergence of HSCs appears to be restricted to the trunk.

is completely dependent on VEGFA signalling [73]. However, in Hh-deficient embryos, *Dll4* expression is only lost in the head [73]. This suggests that the Hh-VEGFA-Dll4 cascade is only functional in the head region and not in the DA which gives rise to HSCs. In support of this, although *Dll4* expression in *Xenopus* embryos is completely dependent on VEGFA, inhibition of Hh signalling has no effect on *Dll4* expression in the DA, and *VEGFA* expression in the somites is normal [77], thus confirming that *Dll4* expression in the trunk DA is not dependent on Hh signalling. The differential requirement of Hh for the expression of *Dll4* in the head and the trunk indicates that

regionalisation greatly influences the response of endothelial cells to signalling pathways.

Haematopoietic stem cells emerge in a restricted AP position within the DA. In *Xenopus* embryos, expression within the DA of *Runx1*, a TF essential for EHT [78], is restricted to the trunk (Fig. 5A). The main axial artery of the embryo spans from the head to the tail and can be subdivided, from anterior to posterior, into the head arteries, the paired or bifurcated DA, the medial DA of the trunk and the tail artery. *Runx1*'s expression spans the Wolffian duct and never extends posteriorly into the tail artery, with its posterior boundary of expression located at the level of the



**Fig. 5.** HSCs emerge in the single medial DA of the trunk and the bifurcated DA proximal to it. (A) *Runx1* expression in the *Xenopus* DA is restricted to the trunk and encompasses the WDs. This suggests that HE only emerges in the trunk. (B) At the time when sprouting of intra-aortic haematopoietic clusters is observed, *Runx1* expression is confined to the trunk and also extends anteriorly to the bifurcated DA, suggesting that HSCs may emerge in these vessels too. (C) Functional assays in the mouse have demonstrated that HSCs emerge in the single medial DA of the trunk as well as in the bifurcated DA proximal to it [80].

cloaca [32]. *Runx1*'s anterior boundary is located in the bifurcated DA, not far from where they fuse to form the medial DA (Fig. 5B) [32]. In the mouse, intra-aortic haematopoietic clusters are also only found in the trunk DA, with an anterior boundary localised at the level of the forelimb and the posterior boundary at the level of the hindlimb [13,79]. More recently, it has been confirmed in the mouse that functional HSCs only emerge in the trunk DA, which extends between the anterior and posterior limb buds and anteriorly includes the most proximal portion of the bifurcated DA (Fig. 5C) [80]. Interestingly, the LPM that gives rise to this region of the DA is already patterned [81]. This patterning positions specification of the forelimb and hindlimb buds and is mediated by a GRN, which includes *Hox* genes [81,82].

*Hox* genes are key determinants of AP identity [83] and have been shown to determine the formation of the kidney morphogenetic field in chick [84] and mouse embryos [85]. Furthermore, *Cdx* genes, which are known to regulate segmental identity through *Hox*

genes, have been shown to regulate the segmentation of the Wolffian duct. *Cdx* deficiency in the zebrafish causes a posterior shift of the entire Wolffian duct with the loss of the most posterior segments [66]. Regarding haematopoiesis, *Cdx* genes have been shown to regulate embryonic erythropoiesis in the zebrafish through *Hox* genes [86,87]; however, whether this is due to changes in AP patterning is not known. As the DA/HSC precursors develop in close proximity to the Wolffian duct and the precursors of embryonic erythrocytes in this organism, these observations suggest a potential involvement of *Hox* genes in the AP restriction of HSC emergence.

Evidence that *Hox* genes can control the AP expression of key HE/HSC genes has been provided by studies on the specification of chick motor neurons [88]. Motor neurons with distinct function are specified as pools of cells at different AP positions in the neural tube. *Runx1* is expressed in, and essential for, the specification of one of these pools. Parologue 5 *Hox* genes (*Hox5*) are expressed by the same pool of motor neurons and are required for *Runx1* expression. *HoxC8* is expressed posterior to the pool of *Runx1*+ motor neurons and gene perturbation has demonstrated that it regulates the posterior boundary of *Runx1* expression by repressing *Hox5* [88]. Within the mouse DA, *HoxA3* has been shown to repress the expression of *Runx1* [89]. At E8.25, expression of *Runx1* is not detected by *in situ* hybridisation in the DA of wild-type embryos; however, when *HoxA3* is deleted, premature expression is observed [89]. This suggests that repression of *HoxA3* is required for the initiation of the expression of *Runx1* and, consequently, for the emergence of HE in the DA. How *HoxA3* is repressed in a timely fashion, though, is not known. Similarly, whether deletion of *HoxA3* is accompanied by AP changes is not known. Nevertheless, these observations indicate that HE fate is established early during development, potentially in the LPM, and that a *Hox* GRN is involved.

## Conclusions and perspectives

Although DHs have so far only been described and characterised in *Xenopus* embryos, indirect evidence strongly indicates their existence in the LPM of all vertebrates. In *Xenopus*, the dorsal LPM of the trunk provides a unique environment for the specification of DHs. Critically, tissues such as the somites, which do not appear to be involved in the specification of other haematovascular progenitors, contribute to the unique signalling regime that initiates the programming of the HSC lineage. Perhaps, one of the main functions of this environment is to prevent emerging DHs from

immediately differentiating into mature blood cells, while retaining their capacity to differentiate into angioblasts, as well as priming them to give rise to HSCs when localised in the correct DA microenvironment. Important to note is that the TFs controlling the DH GRN, such as Gata2, Scl and ETS transcription factors, also form the core of the GRN programming HE in the DA [22,90]. Similarly, key signalling pathways, such as VEGFA and BMP, are equally essential for DHs and HE. Nevertheless, the programming outputs of the interaction between TFs and signalling pathways are significantly different. This suggests that their combinatorial activity, their regulation and/or their mechanism of action is different. Indeed, we have demonstrated in *Xenopus* that the small isoform of VEGFA is sufficient for DH programming but that the medium and large isoforms of VEGFA, generated by alternative splicing, are essential for the emergence of HE [77]. The use of model organisms with externally developing embryos will continue to contribute to our understanding of how HSCs are programmed from nascent mesoderm. These important lessons should be used to inform the establishment of protocols for the *de novo* generation of HSCs *in vitro*.

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