

Signalling pathways that control vertebrate haematopoietic stem cell specification

Wilson K. Clements¹ and David Traver²

Abstract | Haematopoietic stem cells (HSCs) are tissue-specific stem cells that replenish all mature blood lineages during the lifetime of an individual. Clinically, HSCs form the foundation of transplantation-based therapies for leukaemias and congenital blood disorders. Researchers have long been interested in understanding the normal signalling mechanisms that specify HSCs in the embryo, in part because recapitulating these requirements *in vitro* might provide a means to generate immune-compatible HSCs for transplantation. Recent embryological work has demonstrated the existence of previously unknown signalling requirements. Moreover, it is now clear that gene expression in the nearby somite is integrally involved in regulating the transition of the embryonic endothelium to a haemogenic fate. Here, we review current knowledge of the intraembryonic signals required for the specification of HSCs in vertebrates.

Induced pluripotent stem cell

(iPS cell). An adult cell reprogrammed by one of several protocols to 'pluripotency', a state competent to form all embryonic tissues.

¹Department of Hematology, Division of Experimental Hematology, St Jude Children's Research Hospital, 262 Danny Thomas Pl., Memphis, Tennessee 38105, USA.

²Department of Cellular and Molecular Medicine and Section of Cell and Developmental Biology, University of California at San Diego, 9500 Gilman Dr., La Jolla, California 92093-0380, USA. Correspondence to D.T. e-mail: dtraver@ucsd.edu doi:10.1038/nri3443

Haematopoietic stem cells (HSCs) are self-renewing blood and immune cell precursors capable of producing daughter cells that proliferate and mature to provide all adult blood effector cells, including erythroid, myeloid and lymphoid cells¹. Clinically, these cells are the relevant component of bone marrow transplants, which are used to treat patients with leukaemia and congenital blood disorders, but the availability of immune-compatible donors remains a problem². The advent of induced pluripotent stem cell (iPS cell) technology has raised the possibility of making HSCs from a patient's own non-haematopoietic tissues^{3,4}, but it is not possible so far to convert pluripotent cells to HSCs that are capable of long-term self-renewal and generation of normal distributions of the complete set of mature blood cell lineages^{5,6}. This suggests that key specification requirements are unknown. Attempts to generate HSCs *in vitro* could be informed by understanding the normal *in vivo* mechanisms that generate these cells during embryonic development. A clear understanding of the developmental specification of HSCs might moreover provide insight into the causes of congenital diseases, such as aplastic anaemias and congenital neutropenia. Recent work in multiple species has uncovered several previously unknown signalling inputs required for HSC specification; here, we review these advances and place them in a developmental context.

Establishment of adult haematopoiesis

In all vertebrates, the establishment of self-renewing HSCs with the full set of lineage potentials is preceded during development by earlier primitive and definitive 'waves' of haematopoiesis (FIG. 1a), which take place in various anatomical locations (FIG. 1b). Primitive myeloid and erythroid waves (BOX 1) transiently generate limited sets of effector cells. In *Xenopus laevis*, the spatial separation of cells fated to generate primitive and definitive haematopoietic tissues is well defined, with primitive precursors occupying the ventral blood islands (VBIs) and definitive blood arising from the dorsal lateral plate mesoderm (DLP mesoderm)⁷⁻¹⁰; separation occurs as early as the 32-cell stage⁷. Interestingly, these populations retain significant plasticity with respect to their potential to adopt primitive or definitive haematopoietic fates, as transplantation of VBI cells to the DLP mesoderm and vice versa until mid-neurula stages results in cells taking on the identity specified by the site of engraftment¹¹. These results indicate that the signalling environment has a major role in assignment of primitive versus definitive haematopoietic fates until relatively late in development.

Definitive haematopoiesis can be split into two waves. An earlier wave proceeds through a multipotent progenitor — known as the erythromyeloid progenitor (EMP) — with lineage potential limited to erythrocytes, megakaryocytes and myeloid cells¹²⁻¹⁹. This wave is followed

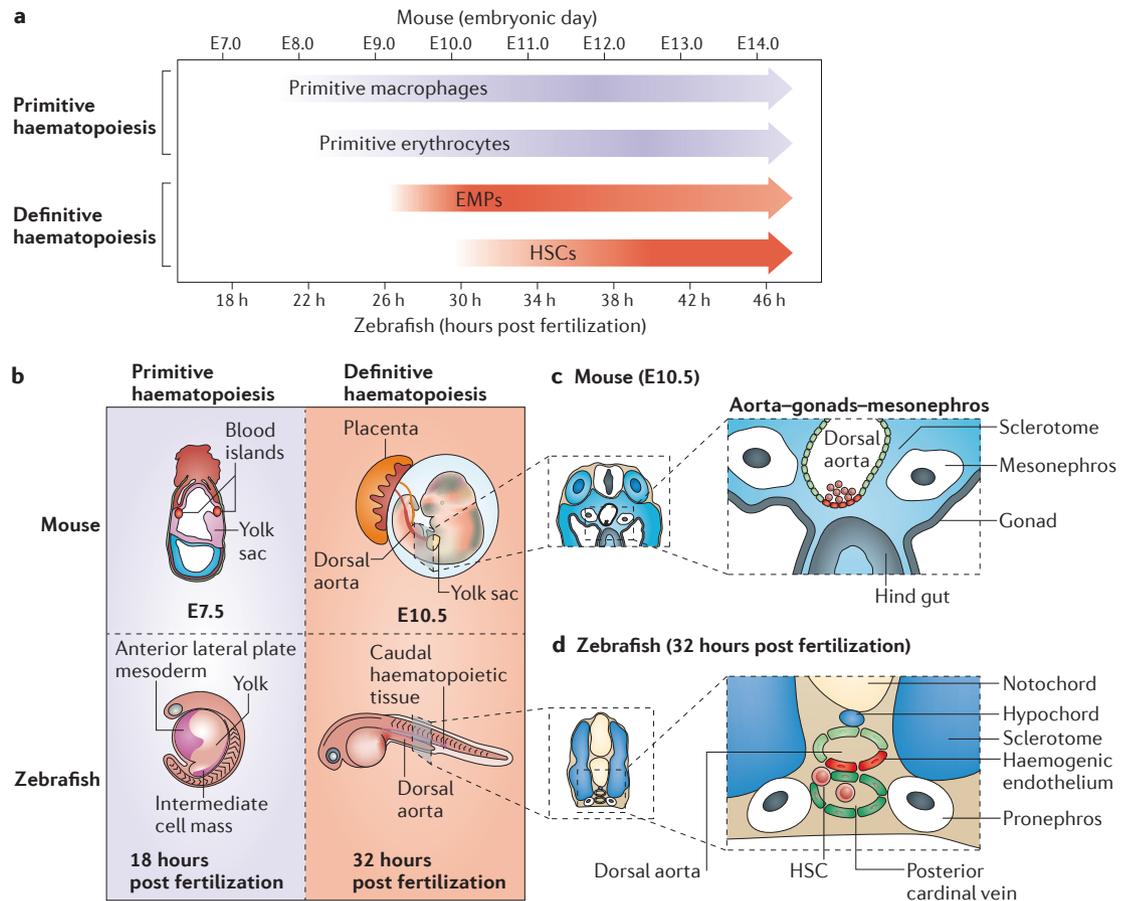


Figure 1 | Haematopoietic stem cells. **a** | Haematopoietic stem cell (HSC) specification is preceded by earlier waves of embryonic haematopoiesis. Primitive macrophages and erythrocytes arise first. A transient definitive erythromyeloid progenitor (EMP) population also precedes HSCs. **b** | Primitive blood in mouse derives from extra-embryonic tissue of the yolk sac in blood islands. In zebrafish, primitive red blood cells derive from mesoderm of the intermediate cell mass and primitive myeloid cells derive from anterior lateral plate mesoderm. EMPs are specified in the mouse yolk sac and zebrafish caudal haematopoietic tissue. **c,d** | HSCs (red) appear at embryonic day (E)10.5 in mouse and 32 hours post fertilization in zebrafish from haemogenic endothelium of the dorsal aorta, from where they bud into the arterial circulation (mouse; **c**) or the venous circulation (zebrafish; **d**).

Ventral blood islands

A ventroposterior region of the embryo in *Xenopus laevis* and other species that houses primitive haematopoiesis, in particular erythropoiesis.

Dorsal lateral plate mesoderm

(DLP mesoderm). Trunk mesoderm in *Xenopus laevis* that is lateral to the more axial notochord and somitic mesoderm. The DLP mesoderm contains tissue fated to form haemogenic endothelium (and thus haematopoietic stem cells) and is equivalent to zebrafish lateral plate mesoderm and mammalian splanchnic mesoderm.

Mid-neurula

In *Xenopus laevis*, a time in the middle of formation of the neural plate that occurs between the end of gastrulation (formation of the germ layers) and the initiation of somitogenesis.

Haemogenic endothelium

Endothelial cells found most notably in the ventral floor of the dorsal aorta that give rise to haematopoietic stem cells.

Yolk sac

An extra-embryonic tissue derived from the fertilized egg in mammals that ultimately surrounds the embryo and at early times functions as a signalling centre. The yolk sac is also the site of primitive and transient definitive haematopoiesis.

Dorsal aorta

The earliest trunk vessel and precursor to the adult descending aorta. Endothelium of the dorsal aorta forms from splanchnic mesoderm and contains the haemogenic endothelium that generates the first haematopoietic stem cells.

by the specification of HSCs that self-renew for the life of the individual and are capable of producing all adult lineages, including lymphocytes. That EMPs produce myeloid and erythroid lineages from a single progenitor and that murine EMPs produce erythrocytes expressing adult globins has caused some confusion, especially in *in vitro* assays seeking to generate HSCs, because it is impossible to distinguish EMPs and HSCs on the basis of assays with only an erythromyeloid readout. Furthermore, EMPs and HSCs cannot be distinguished by cell surface phenotype. These issues highlight the need to test haematopoietic precursors for their long-term reconstitution and lymphoid potential side-by-side with bone marrow HSCs when determining the success of *in vitro* HSC specification.

Anatomy of HSC specification

Across vertebrate phyla, HSCs arise from an endothelial precursor found specifically in arterial haemogenic endothelium. In mammals, there is evidence to support *de novo* HSC specification in the umbilical and vitelline arteries²⁰, placenta^{21,22} and, surprisingly, the head²³.

Some lineage tracing studies suggest that HSC specification also occurs in the yolk sac^{24,25}. However, the signalling events involved in HSC specification in the dorsal aorta are currently the best understood, and this site of emergence for HSCs is conserved in vertebrate models such as frog and fish, which has helped to illuminate the native processes controlling specification. It is likely that many (but probably not all^{26,27}) mammalian HSCs arise in the intraembryonic dorsal aorta^{20,28,29}. In chick, grafting experiments have shown that adult blood is entirely embryo derived³⁰, and in *X. laevis*, grafting experiments have established that adult blood comes from the tissue fated to form dorsal aorta¹⁰. Furthermore, in zebrafish^{31,32} and mouse explants³³ the emergence of HSCs from endothelium in the ventral floor of the embryonic dorsal aorta has been directly visualized, despite differences in the directionality of budding (into the venous circulation in zebrafish compared with into the arterial circulation in mouse). This Review therefore focuses on the conserved intraembryonic signalling environment that regulates the emergence of HSCs from the dorsal aorta.

Aorta–gonads–mesonephros

The embryonic region in the embryonic day 9.5–11.0 mouse containing the primitive dorsal aorta and bounded by the mesonephros and gonads, where haematopoietic stem cells are specified.

Lateral

Anatomical adjective defining the direction away from the midline.

Anamniotes

An informal grouping of vertebrates (including frogs and fish) that develop external to the mother in eggs without an amnion, in contrast to birds and mammals, which have amnions.

Somites

Primitive mesodermal tissue found lateral to the notochord and axial to the lateral plate or splanchnic mesoderm. This tissue is segmented and contains precursors for adult skeleton, skeletal muscle, vascular smooth muscle and dermis.

Epiblast

In mouse and chick, the embryonic portion of the embryo before formation of the primary germ layers (ectoderm, mesoderm and endoderm).

Primitive streak

The site in the epiblast of primary ingression of cells that give rise to mesoderm.

Dorsal–ventral

Anatomical adjectives defining the back and belly directions, respectively.

Anterior–posterior

Anatomical adjectives defining the head and tail directions, respectively.

Dorsal organizer

A group of cells acting as a signalling centre that patterns local tissue to a specific fate, in this case dorsal and anterior. Sometimes known as ‘Spemann’s organizer’.

Intermediate cell mass

A region in the axial trunk of the 23–28 hours post fertilization zebrafish embryo that is ventral to the notochord. Primitive haematopoiesis occurs here, as well as formation of the haemogenic endothelium.

Box 1 | Primitive haematopoiesis

Although haematopoietic stem cells (HSCs) sustain the long-term production of all mature blood lineages during adult life, the emergence of HSCs during development is preceded by earlier and distinct haematopoietic ‘waves’ with limited or no self-renewal capacity and restricted lineage potential. These waves have been loosely grouped into primitive and definitive waves (FIG. 1a). Primitive haematopoiesis can be further divided into two waves that produce primitive erythrocytes or primitive myeloid cells (including macrophages, as well as possibly megakaryocytes¹⁶¹ and neutrophils^{38,39,162,163}). The primitive waves of haematopoiesis are distinct from definitive waves in at least four ways. First, they occur earlier than the definitive waves. Second, they occur in the absence of an identifiable self-renewing stem cell. Third, they do not yield the full distribution of adult mature blood cell lineages, notably not producing B and T cells. And fourth, primitive erythrocytes express a distinct set of globins from their adult counterparts. Primitive haematopoiesis occurs in the avian and mammalian yolk sac¹⁶³, and in the intermediate cell mass and on the anterior yolk ball of the zebrafish embryo³⁹.

Recent work in multiple species has conclusively demonstrated that HSC precursors transit through an endothelial stage. Conditional labelling in mouse shows that adult HSCs were previously positive for the endothelial marker vascular endothelial (VE)-cadherin (encoded by *Cdh5*)^{34,35}. Moreover, endothelial-specific deletion of the mouse runt-related transcription factor 1 (*Runx1*) gene (BOX 2), which is required for HSC specification, eliminates HSCs³⁵. The process of mouse and zebrafish endothelial cells becoming haematopoietic cells has been directly visualized in real time *in vitro*^{36,37} and *in vivo*^{31–33}. These results have established that HSCs derive from haemogenic endothelium of the dorsal aorta.

The dorsal aorta is part of the earliest trunk vasculature and in mouse lies in a region bounded by the primitive gonads and mesonephros, sometimes termed the aorta–gonads–mesonephros (FIG. 1b,c). The endothelium of the dorsal aorta is of mesodermal origin and derives from mesoderm termed ‘splanchnic’ in mammals or ‘lateral plate’ in anamniotes, which is initially found lateral to the somitic mesoderm. Endothelial precursors migrate axially below the developing somites either as two distinct developing endothelial tubes that merge to a single dorsal aorta (as in mouse; FIG. 1c) or as two populations of mesenchymal cells that will form a primitive vascular cord that subsequently lumenizes (as in zebrafish; FIG. 1d). Intraembryonically, only endothelial cells of the trunk dorsal aorta are competent to form HSCs, strongly suggesting that they are pre-patterned for cell-intrinsic competence to respond to later specification signals. Once HSCs have been specified from the haemogenic endothelium, they leave the dorsal aorta and move to transient proliferation tissues and finally to the adult haematopoietic organs: predominantly the bone marrow in mammals and the kidney in zebrafish^{7,38,39}.

Early embryonic patterning

Proper specification of the body axes and primary germ layers, as well as subsequent finer compartmentalization of the mesoderm into tissues and organs, are prerequisites for haematopoietic development. In many cases, *in vitro* attempts to make HSCs have been structured to duplicate early embryogenesis underlying formation of the posterior trunk mesoderm that houses haemogenic endothelium^{3,4}, so it is important to understand the signalling pathways underlying these events. Conceptually, at least five steps must occur endogenously to eventually produce HSCs: specification of mesoderm, ventralization and posteriorization of mesoderm, lateralization of mesoderm, production of trunk arterial endothelium, and specification of HSCs from haemogenic endothelium. The complex processes that generate the primary body architecture have been extensively reviewed elsewhere^{40–43} and only key events related to the generation of haematopoietic mesoderm are emphasized here.

Early development of lateral plate mesoderm. Mesoderm in mouse arises when cells of the epiblast ingress through the primitive streak (FIG. 2a), a process that requires Nodal⁴⁴, bone morphogenetic protein (BMP)^{45–47}, fibroblast growth factor (FGF)^{48,49} and WNT⁵⁰ signalling (BOX 2). Similarly, zebrafish that lack all Nodal signalling have a nearly complete failure of mesoderm specification^{51,52}. *In vitro*, the combined actions of WNT and Nodal signalling can produce mesodermal Brachyury-expressing primitive streak-like cells from mouse embryonic stem cells (ESCs) in embryoid bodies⁵³.

Mesoderm and other germ layer specification is integrated with dorsal–ventral and anterior–posterior patterning. Blood specification relies on this patterning as the dorsal aorta, and hence HSCs, develop from ventro-posterior lateral plate mesoderm. In anamniotes, specification of the earliest dorsoventral axis involves the Wnt pathway-dependent specification of a dorsal organizer and ventral Bmp signalling, which set up self-reinforcing genetic programmes that loosely partition the embryo^{42,43}. Across species, in subsequent anterior–posterior patterning, WNT signalling is actively suppressed in the anterior region by secreted antagonists, whereas WNT ligands and BMPs cooperate to specify ventroposterior mesoderm^{40–43} (FIG. 2b). FGF signalling is also required for specification of the posterior mesoderm^{48,49,54–56}. At this point, ventroposterior mesoderm contains the precursors for a fairly broad but finite number of tissues, including notochord, somite, pronephros, vasculature and blood, so further regulatory interactions must pattern these individual tissue fates.

HSCs develop from the dorsal aorta of the trunk mesoderm, and multiple lines of evidence indicate that trunk mesoderm is distinct from both head and tail mesoderm. Although all trunk mesodermal tissues are absent in zebrafish embryos lacking Nodal signalling, a few somites in the posterior do develop, which suggests that there are distinct mechanisms specifying the most posterior mesoderm^{51,52,57,58}. Similarly, the zebrafish *cloche* mutant, which lacks trunk endothelium and HSCs⁵⁹, nevertheless expresses multiple vascular markers in the ventral tail and later recovers limited blood marker

Box 2 | Key haematopoietic factors and pathways

RUNX1. The runt-domain transcription factor RUNX1 (also known as AML1 and CBF α) is a pivotal haematopoietic stem cell (HSC) marker. *RUNX1* was originally identified as a gene that is frequently rearranged in myeloid leukaemias¹⁶⁴. During development, it is expressed in the haematopoietic clusters in the dorsal aorta¹⁶⁵, marks long-term HSCs¹⁶⁶ and is required for definitive, but not primitive, haematopoiesis in mouse and zebrafish^{83,167,168}. RUNX1 is required for the transition from endothelial cells to HSCs but is not required later for HSC maintenance³⁵. This requirement apparently involves maintaining cell viability after emergence from the haemogenic endothelium, as live imaging in zebrafish shows that *runx1*-knockdown cells escape the endothelium, but quickly die³²; this phenotype is consistent with the absence of RUNX1⁺ cells in the fetal liver of *Runx1*-knockout mice¹⁶⁵. Thus *Runx1* expression in the dorsal aorta during HSC specification is one of the earliest markers of prospective HSCs.

Nodal and BMP signalling. Nodals and bone morphogenetic proteins (BMPs) are members of the transforming growth factor- β (TGF β) family of signalling molecules. They function through activin-like receptor family receptors, which homo- and heterotetramerize, cross-phosphorylate and lead to phospho-activation of downstream SMAD effector proteins^{165,169,170}. Nodal signalling additionally requires a co-receptor of the epidermal growth factor cripto FRL cryptic (EGF-CFC) family of proteins, which includes mammalian Cripto and Criptic and zebrafish One-eyed pinhead receptors. TGF β signals are transduced to the nucleus by the actions of SMAD proteins. Specific SMADs seem to be characteristically used by Nodal signalling (SMAD2 and SMAD3), whereas others seem to be used by BMPs (SMAD1, SMAD5 and SMAD8). SMAD4, the common SMAD, is shared by both sets. In mouse, there is a single *Nodal* gene, whereas in zebrafish there are two, *nodal-related 1* (*squint*) and *nodal-related 2* (*cyclops*).

FGF signalling. In human, mouse, and fish there are more than 20 fibroblast growth factor (FGF) ligands and five to eight FGF receptor (FGFR) or receptor-like genes. Dimeric FGFRs bind to ligand and activate multiple intracellular pathways, notably including the phosphoinositide 3-kinase (PI3K)-AKT and mitogen-activated protein kinase pathways¹⁷¹.

Notch signalling. The Notch pathway transmits signals between neighbouring cells and is heavily involved in embryonic patterning^{172–174}. In mammals there are three Delta family ligands (DLL1, DLL3 and DLL4) and two Jagged ligands (Jagged 1 and Jagged 2). In zebrafish there are five Delta family ligands (Dla, Dlb, Dlc, Dld and Dll4) and three Jagged ligands (Jagged 1a, Jagged 1b and Jagged 2). During normal Notch signalling, a transmembrane ligand on a presenting cell interacts with one of the Notch receptors, Notch 1 to Notch 4 in mammals or Notch 1a, Notch 1b, Notch 2 and Notch 3 in zebrafish. Functional signalling requires ubiquitin ligases of the Mind bomb family (MIB1 in mammals; Mib and Mib2 in zebrafish). Two sequential protein cleavage events mature the receptor and release an intracellular receptor fragment, the Notch intracellular domain (NICD), which binds DNA-binding CBF1/suppressor of hairless/Lag-2 (CSL; also known as Rbpjk), and mediates transcriptional activation in coordination with the Mastermind (Mam) co-activator. In the absence of NICD, CSL silences gene expression through the activity of co-repressors.

Canonical WNT signalling. The WNT signalling pathway regulates many aspects of development and disease¹⁷⁵. WNT signalling has been broadly separated into two arms: the β -catenin-dependent (canonical) and the β -catenin-independent (non-canonical) pathways. The canonical pathway has been characterized in far greater detail and involves regulation of the stability of a signalling pool of the multifunctional protein, β -catenin. In the absence of WNT signalling, β -catenin is phosphorylated by a 'destruction complex', targeting it for degradation by the proteasome. WNT ligation of its Frizzled and LDL-receptor-related protein (LRP)-family co-receptors leads to inhibition of the destruction complex and translocation of stabilized β -catenin to the nucleus, where it can activate transcription of WNT target genes in cooperation with DNA-binding factors of the lymphocyte enhancer factor/T cell factor (LEF/TCF) family.

expression specifically in this region^{60–62}. This differential patterning indicates that trunk mesoderm might carry forward distinct cell-intrinsic determinants of competence to respond to haematopoietic signalling.

The dorsal aorta and subsequently HSCs derive from lateral plate (splanchnic) mesoderm that is lateral to the axial, paraxial and somitic mesoderm (FIG. 2c). Thus, ventroposterior mesoderm must be 'lateralized' by the combined actions of WNT and BMP signalling. Ectopic expression of Wnt ligands, Nodal and Bmps in zebrafish can induce the formation of ectopic trunk and tail structures that contain lateral mesoderm, but not axial mesoderm or neural ectoderm, which indicates that these factors have a role in the induction of lateral plate mesoderm⁶³. In mouse as well, BMP signalling is required for distinguishing non-axial mesoderm, and embryos with mosaic deletion of BMP receptor 1A (*Bmpr1a*) form ectopic somitic tissue at the expense of lateral plate mesoderm⁶⁴.

But what are the WNT- and BMP-inducible factors that might mediate the haematopoietic competence of the lateral plate mesoderm? Studies in both mouse and zebrafish show that the cooperative actions of WNT3A, WNT8, BMP2B and BMP4 induce the expression of caudal-type homeobox (CDX) genes, including *Cdx1*, *Cdx2* and *Cdx4* (REFS 46,65–68), which regulate homeobox (HOX) gene expression (FIG. 2c). Key downstream HOX genes probably include *Hoxa9a* and *Hoxb6b*, which have been shown to be required for haematopoietic programming *in vivo* and *in vitro*^{69–73}. Although overexpression and *in vitro* data support a cell-autonomous role for these genes in regulating haematopoietic competence, their apparent lack of expression in haematopoietic precursors *in vivo*^{69,70} makes it important to consider that they might be required non-cell-autonomously (for example, by regulation of a relay signal).

Yolk ball

An extra-embryonic region of the zebrafish embryo that contains nutrients that sustain early development before feeding.

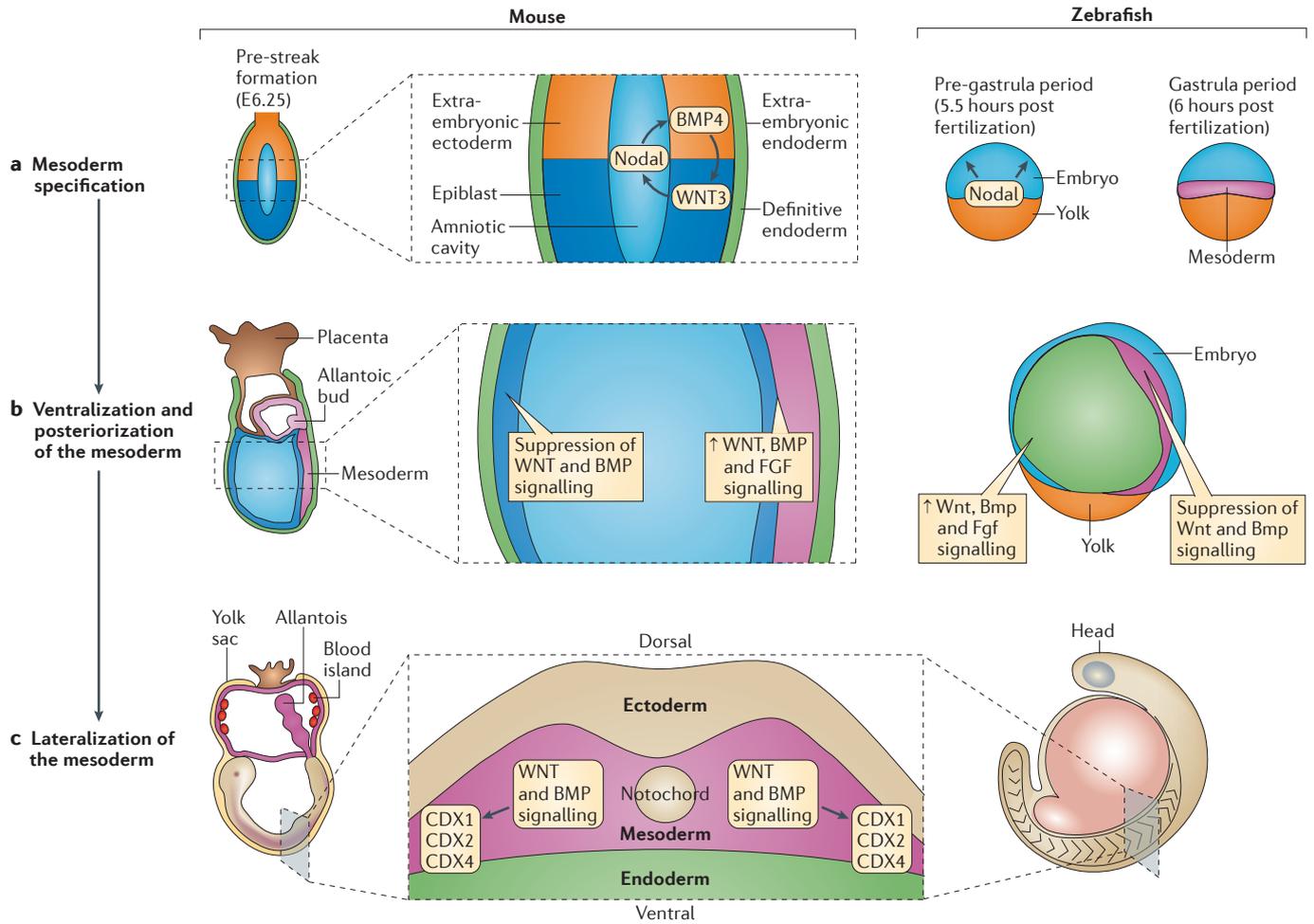


Figure 2 | Early signalling regulating HSC specification. Haematopoietic stem cells (HSCs) derive from haemogenic endothelium of the dorsal aorta, which is of mesodermal origin (pink). **a** | In mouse, Nodal, WNT3 and bone morphogenetic protein 4 (BMP4) are required for primitive streak formation (not shown) and mesoderm specification. In zebrafish, Nodal is required for mesoderm specification. **b** | HSCs derive specifically from ventroposterior mesoderm, specification of which requires WNT ligands, BMPs and fibroblast growth factors (FGFs). WNT and BMP signalling are actively suppressed in the anterior region by secreted antagonists, whereas WNT, BMP and FGF signalling cooperate to specify ventroposterior mesoderm. **c** | HSCs derive from lateral plate mesoderm. Mesoderm is lateralized by WNT ligands and BMPs, which regulate the expression of caudal-type homeobox 1 (*CDX1*), *CDX2* and *CDX4*.

Formation of the haemogenic endothelium

The step immediately preceding HSC emergence is the development of the haemogenic endothelium that lies in the ventral floor of the primitive dorsal aorta, which expresses the vascular endothelial growth factor (VEGF) receptor KDR (also known as FLK1 and VEGFR2; *Kdr* in zebrafish)^{31,32,74,75}. In mouse and chick, bilateral lumenized endothelial aortae converge to the midline and fuse before the emergence of HSCs⁷⁶. Before fusion, BMP antagonists, including chordin (CHRD) and noggin (NOG), prevent the aortae from migrating to the midline^{77,78}. Maintenance of the axial ‘avascular space’ is relieved by downregulation of these BMP antagonists, signalling the onset of fusion⁷⁹. By contrast, in amniotes such as frog and fish, unlumenized, mesenchymal angioblasts migrate to the midline, where they form a primitive vascular cord, which subsequently lumenizes to form the early dorsal

aorta⁸⁰. This migration happens despite axial expression of multiple Bmp inhibitors^{81,82}. In either case, HSCs do not appear until the formation of a single lumenized aorta at the midline, which indicates that trigger signals might be received at this point.

Work in zebrafish and frogs shows that Sonic hedgehog (Shh) and *Vegfa* are involved in regulating the medial convergence of haemangioblasts (which contain future endothelium and HSCs) in the lateral plate mesoderm (FIG. 3). In all vertebrates, *Shh* is expressed in specific axial tissues, namely in the floorplate of the neural tube and in the notochord. In mouse and chick, *Shh* is also expressed in the gut endoderm at the relevant times. Pre-haematopoietic *kdr*-expressing mesoderm exhibits altered midline convergence in zebrafish embryos with gain or loss of Shh signalling^{83–85}, and one of the *X. laevis Vegfa* isoforms is able to regulate angioblast migration⁸⁶. Thus, early on, Shh and *Vegfa* are

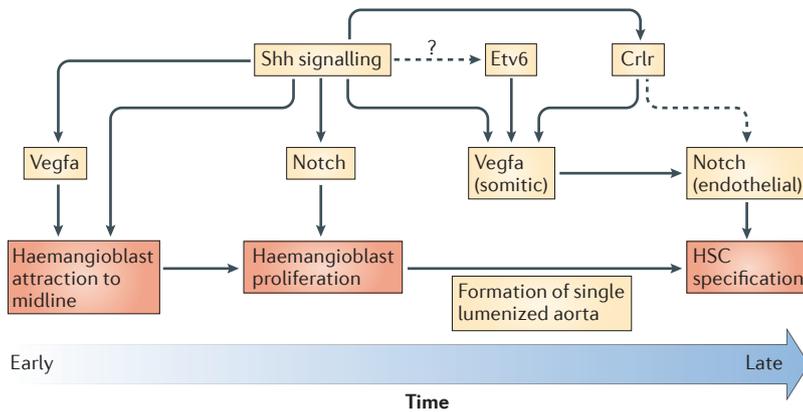


Figure 3 | Processes regulated by Sonic hedgehog. Sonic hedgehog (Shh) and vascular endothelial growth factor A (Vegfa) function as attractive cues to haemangioblasts converging to the midline in zebrafish. Shh induces Notch-dependent proliferation of haemangioblasts. Vegfa is required for the expression of Notch receptors in haemogenic endothelium. Shh regulates *vegfa* expression directly and through calcitonin receptor-like receptor (Crlr). The Ets transcription factor Etv6 (also known as Tel1) also regulates *vegfa* expression in *Xenopus laevis*. HSC, haematopoietic stem cell.

required for arteriovenous and HSC specification by regulating the convergence of precursors to the midline. In some cases of perturbation in the levels of Shh, although haemangioblast convergence is delayed, many cells do eventually reach the midline but fail to appropriately turn on haematopoietic markers^{83–85}, so conceivably the precursor population must arrive in time to experience an as yet unknown transient specification signal.

HSCs derive particularly from endothelium of the primitive dorsal aorta²⁰. This observation suggests the possibility that distinguishing artery from vein may be a prerequisite for initiation of the HSC specification programme. However, in at least one highly artificial situation, HSC markers are expressed by endothelium without arterial gene expression⁸⁷. Nevertheless, many of the pathways that are required for HSC specification, such as Shh, Vegfa and Notch signalling (BOX 2), are also required for the preceding arteriovenous specification (see below), raising the possibility that the requirement for these signals in HSC specification might, in part, reflect their requirement in arteriovenous specification.

Notch signalling is iteratively involved in patterning the early trunk vasculature of vertebrates, including the dorsal aorta that contains haemogenic endothelium. Before the formation of a primitive vascular cord in fish, Notch signalling downstream of Shh regulates the proliferation (FIG. 3) of lateral plate haemangioblasts positive for *friend leukaemia integration* (*fli*; also known as *fli1a*) and *ets1-related protein* (*etsrp*; also known as *etv2*)^{87–93,87}. Later, Notch signalling controls arteriovenous specification⁹⁴ (FIG. 4a,b). Loss of *Notch1* or of *Notch1* and *Notch4* (REF. 95), or of other Notch pathway components required for Notch activity (such as mind bomb (*Mib*)^{94,96,97} and CBF1/suppressor of hairless/Lag-2 (*Csl*; also known as *Rbpjk*)^{94,98}) leads to loss of arterial-specific marker expression in both mouse and zebrafish, as does

loss of specific downstream effectors of Notch signalling such as hairy/enhancer-of-split related with YRPW motif 2 (*Hey2*; also known as *Herp1*, *Herp2*, *Hesr2* and *Hrt2*)^{99–101}. Notch signalling leads to the expression of arterial-specific genes including ephrin B2 (*Efnb2*) (FIG. 4b), which has been identified as a direct target of Notch 1 in endothelial cell culture¹⁰². *Efnb2* is a ligand for Eph receptor b4 (*Ephb4*), which is expressed by venous cells, with reciprocal signalling partially mediating differential adhesion and arteriovenous segregation^{72,103,104}. After the initial patterning of the dorsal aorta and posterior cardinal vein, Notch signalling mediated by Delta-like 4 (*DLL4*) regulates angiogenic growth of the intersegmental vessels^{105,106}. Thus Notch signalling is a conserved integral part of maturation of the arterial endothelium from which HSCs derive.

The question of when the first cells of the dorsal aorta are specified relative to the posterior cardinal vein is crucial to understanding the signalling underlying specification of HSCs, because HSCs only derive from arterial endothelium. If the artery is specified earlier than at the midline, then HSC programming could occur when the vessels are en route to their axial destination. In mouse and chick, the endothelium of the early bilateral aortae derives from splanchnopleural mesoderm, whereas the bilateral veins come from somitic mesoderm. In chick, somite-derived endothelium later completely replaces the original aortic endothelium, but this second-wave endothelium is not haemogenic, and it is not clear whether this trait is conserved in other vertebrates. However, in both mouse and chick, arteriovenous segregation clearly occurs before aortic fusion at the midline, and HSC patterning signals might therefore be presented before fusion. Nevertheless, the timing of HSC appearance, after the formation of a single lumenized aorta at the midline, supports the likelihood of at least some signals for HSC specification being presented at the midline.

In fish, presumptive arterial and venous angioblasts are morphologically indistinguishable during their convergence. Experimental evidence as to when these cells are specified as artery or vein is mixed. Fate mapping⁹⁹ and live imaging⁸⁴ studies imply that individual lateral plate mesoderm cells 'know' their fate before arrival at the midline. By contrast, another study has shown that the first venous cells sprout from the primitive aorta, suggesting that they are mixed until then¹⁰³. One potential resolution of these data is that intermingled cells of the primitive vascular cord may already know their fate, and that specified venous cells sort (rather than divide) away from arterial cells in a ventral migration. It is also possible that different mechanisms operate in parallel: some mixed but pre-specified arteriovenous cells might first populate the primitive vascular cord before distinct lateral plate mesoderm cells migrate directly to the posterior cardinal vein region. Venous cells of the mixed cord might then sprout off and contribute to the existing posterior cardinal vein rudiment. In any case, it seems likely that arteriovenous specification is underway before mesoderm cells reach the midline in fish.

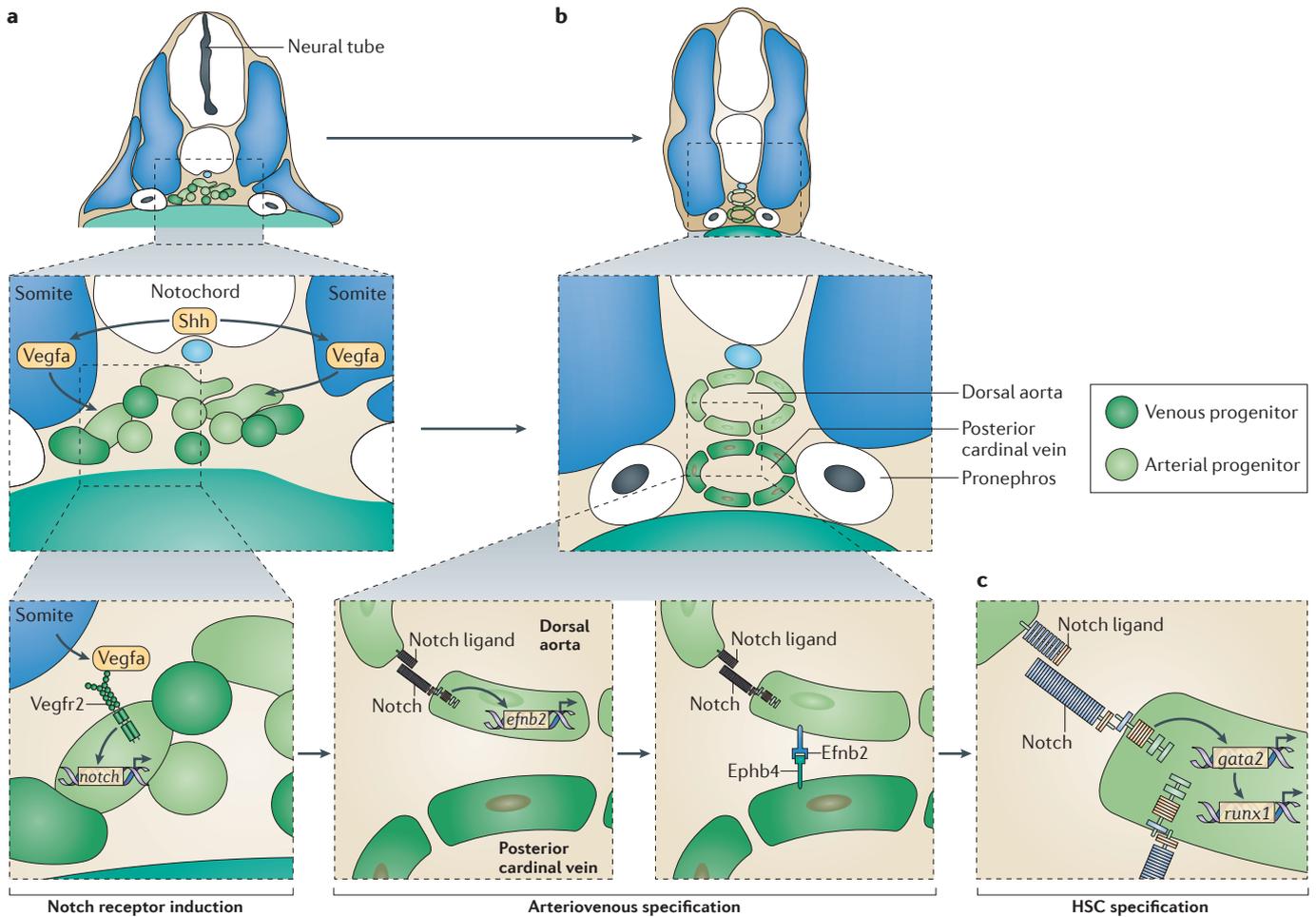


Figure 4 | **Processes regulated by Notch.** Arteriovenous specification in zebrafish. **a** | Sonic hedgehog (Shh) produced by the notochord induces somite expression of vascular endothelial growth factor A (Vegfa), which, in turn, through binding its receptor Vegfr2, induces expression of *notch* in haemogenic endothelial cells. **b** | Notch regulates arteriovenous specification by regulating ephrin B2 (*efnb2*) expression. Arterial cells (light green) express *Efnb2* and segregate from venous cells (dark green), which express the *Efnb2* receptor Eph receptor b4 (*Ephb4*). **c** | Notch ligands including *Jagged1* expressed by neighbouring cells activate Notch receptors to specify haematopoietic stem cells (HSCs) by activating *gata2* expression, which in turn activates *runx1* expression. The relevant *runx1* enhancer does not contain Notch-responsive binding elements, which indicates that Notch does not directly regulate *runx1* expression.

HSC specification from haemogenic endothelium
HSCs are ultimately specified from haemogenic endothelium in the ventral floor of the dorsal aorta at the midline. Several immediately proximal signalling events trigger this final specification step.

Notch signalling. As described above, Notch signalling is required for vascular patterning, particularly vascular morphogenesis and arteriovenous specification. Notch signalling is additionally required for HSC specification. Zebrafish and mouse embryos with a mutation or targeted deletion in *Mib*, which encodes a ubiquitin ligase required for Notch signalling, lack HSCs^{107,108}. Similarly, mice with a targeted deletion of the Notch pathway component *Csl* have a decreased number of HSCs¹⁰⁹. At least one Notch ligand, *Jagged1*, has been found to contribute to the HSC specification process, as *Jagged1*-knockout mice have decreased numbers of

Gata2- and *Runx1*-expressing HSCs in the dorsal aorta at embryonic day (E)10.5–E11 (REF. 110). Interestingly, these animals represent one of the few circumstances in which arteriovenous and HSC specification can be separated, as *Jagged1*-knockout animals retain expression of the arterial marker *EFNB2* (REF. 110). Of the Notch family receptors, Notch 1 is required for definitive haematopoietic development¹¹¹ upstream of *Runx1*, which when ectopically expressed can rescue haematopoietic gene expression in cultured para-aortic splanchnopleural cells deficient for *Notch1* (REF. 112). Notch 1-mediated activation of *Runx1* has been posited to work indirectly through GATA2, as the *Runx1* promoter does not contain Notch-responsive elements. Importantly, the requirement for Notch 1 is cell-autonomous; in other words, HSC precursors must experience a Notch 1-mediated signal at some point to become HSCs, as shown by the fact that chimeric mice

generated from wild-type and *Notch1*-deleted cells show no contribution of Notch 1-deficient cells to the adult haematopoietic system¹¹³. Together these findings have led to a model wherein Notch ligands, including Jagged 1, either in the endothelium of the dorsal aorta itself or in nearby cells, signal through Notch 1 for cellular commitment to an HSC fate (FIG. 4c). However, as noted above, Notch signalling is also required for arteriovenous specification, and it has not yet been established whether HSC specification through Notch signalling is a distinct process. Interestingly, reception of a Notch signal (or signals) is one of the few things that distinguishes HSCs from EMPs^{111,114}.

Shh–Vegfa signalling. From work in zebrafish, it is clear that Notch pathway-mediated activation of HSC specification (and arterial fate) is directly regulated by an Shh–Vegfa signalling axis (FIGS 3, 4a). Shh signalling regulates somitic expression of *vegfa*; Vegfa is a ligand for the *kdrl* gene product Vegfr2 (REF. 115). Vegfa in turn regulates endothelial cell expression in the presumptive arterial endothelium of arterial markers such as *efnb2a*, as well as expression of the Notch receptor *notch3* (previously known as *notch5*)^{71,115}. Shh, Vegfr2 and Notch signalling are all required for HSC specification⁸³. Taken together, these results suggest a model wherein Shh signalling from the floorplate, notochord and hypochord induces *vegfa* expression in the somites. Vegfa, in turn, activates expression of Notch receptor genes in the arterial endothelium, thereby potentiating the ability to receive the Notch signals that are required for HSC specification. There are still some pieces of information missing to fully validate this model. For example, the specific Notch receptor thought to receive the requisite HSC-specifying Notch signal is Notch 1 (REF. 113); however, although Shh regulates expression of arterial *notch1b*⁸⁴, no studies have shown that Vegfa is required for expression of either of the *notch1* paralogues in zebrafish.

Multiple additional factors also influence the Shh–Vegfa–Notch pathway (FIG. 3b). The Ets transcription factor *Etv6* (also known as *Tel1*) was recently shown to be required non-cell-autonomously for HSC specification by activating expression of *vegfa* in the somites of *X. laevis* embryos¹¹⁶. Knockdown of *etv6* results in a failure of arterial specification, including loss of *notch4* expression. Whether or not *etv6* is regulated by Shh remains to be determined. Another player in this pathway is the calcitonin receptor-like receptor (Crlr). In zebrafish, Shh positively regulates expression of *crlr*¹¹⁷, and Crlr is required for *vegfa* and arterial gene expression^{85,117}. Interestingly, under gain-of-function Shh signalling conditions, both Crlr and Vegfa seem to independently regulate arterial specification, as both must be knocked down to abrogate arterial gene expression, and Notch is required for the effects of both⁸⁵. As Crlr overexpression is unable to produce ectopic arterial gene expression on its own⁸⁵, these results suggest that Shh gain of function is able to upregulate as-yet-unknown Crlr cofactors that can cooperate to specify arterial gene expression independently of Vegfa.

BMP signalling. BMP signalling, as described earlier, is essential for patterning the ventroposterior mesoderm of the developing embryo in conjunction with WNT family members. Recently, *Bmp4* signalling has been shown to be directly required to trigger HSC specification from haemogenic endothelium of the dorsal aorta in zebrafish⁸². Secreted *Bmp* antagonists, including *Chd* and *Nog*, are expressed in the axial tissue surrounding the forming dorsal aorta until shortly before the appearance of HSC precursors marked by *runx1*. Immediately before the onset of *runx1* expression, expression of these *Bmp* antagonists is downregulated and the proteins are degraded by the pro-*Bmp* molecule *Tolloid*^{82,118}. Interestingly, although HSC specification fails in animals with conditional abrogation of *Bmp* signalling, arterial gene expression was left intact, demonstrating that arterial and HSC fate specification can be separated. A similar requirement for BMP signalling in mouse has been suggested by the identification of *BMP4* as a factor expressed by stromal cells of the aorta–gonads–mesonephros that augments HSC potential¹¹⁹.

Canonical WNT signalling. The effects of canonical WNT signalling on the homeostasis of the adult haematopoietic system have been highly controversial because of conflicting results based on gain of function^{120–125} and loss of function^{126–130} experiments involving central components of the pathway. Although increased canonical WNT signalling has significant effects on HSC self-renewal and lineage maturation, deletion of key WNT factors in mouse, such as β -catenin and γ -catenin, has in some cases produced no effect on HSC homeostasis^{126–128}. However, in these studies, residual WNT activity was observable, indicating that the deletions produced hypomorphic alleles or that alternative factors transduced WNT activity in the absence of β -catenin and γ -catenin. Using alternative approaches, abrogating canonical WNT signalling does produce HSC and lineage maturation effects^{124,130}. Recent studies have shown that the haematopoietic system is highly sensitive to the dosage of WNT signalling, for example by enhancing HSC self-renewal or skewing lineage distribution^{125,131,132}.

During development, WNT signalling is crucial for the initial specification and/or very early maintenance of HSCs in multiple ways. The requirement for WNT factors in patterning the ventroposterior mesoderm that gives rise to HSCs has been discussed above. In addition, it seems that WNT factors have a more direct role in signalling to early HSCs or HSC precursors. *Wnt3a*-knockout mice have decreased numbers of HSCs in the fetal liver and an impaired ability to support serial transplantation¹²⁹. Interestingly, the deleterious effects of a failure to experience early WNT3A signalling cannot be rescued by transplantation of those HSCs that do develop into wild-type hosts, which indicates that knockout cells might ‘remember’ their early environment, perhaps through epigenetic modifications^{125,129}. These results indicate a crucial requirement for WNT3A signalling in the early maintenance and proliferation of HSCs. Direct effects of WNT3A

Serial transplantation

A procedure to test the longevity and self-renewal potential of putative populations of haematopoietic stem cells by transplanting into primary, secondary, tertiary and higher-order recipients.

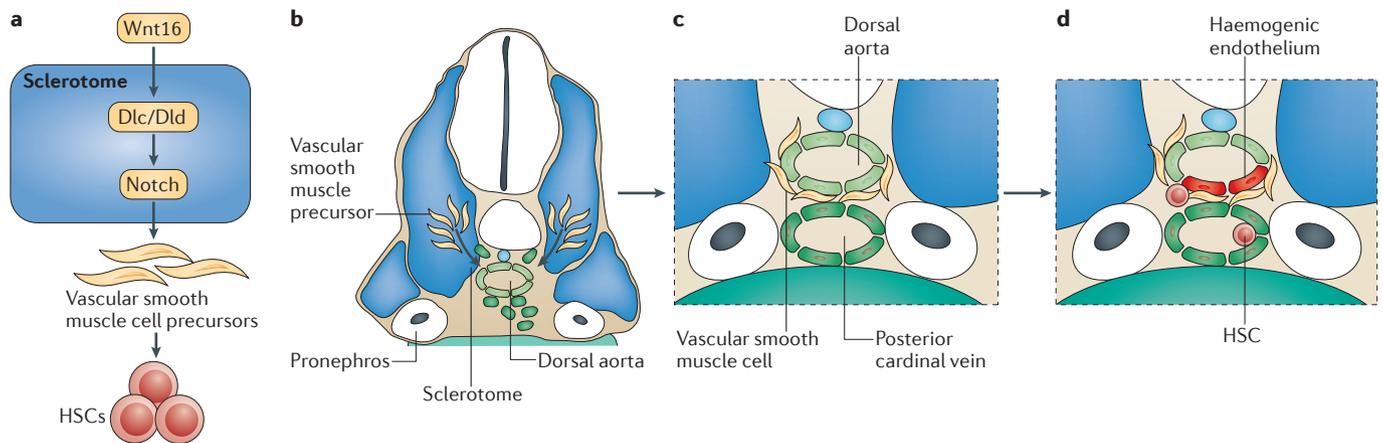


Figure 5 | Model for indirect Notch-mediated haematopoietic stem cell specification in zebrafish. **a** | Schematic of a model for Wnt16-mediated regulation of haematopoietic stem cells (HSCs) through Notch patterning of the ventromedial compartment of the somite, the sclerotome. Wnt16 regulates somitic expression of the Delta family ligand genes *dlc* and *dld*. Both Wnt16 and the combined actions of Dlc and Dld are required for the specification of sclerotome, which contains vascular smooth muscle cell precursors. These precursors emigrate from the somite (**b**), sheathe the dorsal aorta (**c**) and signal to haemogenic endothelium to trigger HSC specification (**d**).

on the original specification of HSCs in the haemogenic endothelium of the dorsal aorta have not been reported, but conditional deletion of the central effector of the canonical WNT pathway, β -catenin, in endothelial cells results in a strong loss of haematopoietic potential, but does not otherwise affect formation of endothelium in, for example, the liver¹³³. These results indicate that WNT- β -catenin signalling is required for haematopoietic commitment from endothelium.

Prostaglandins. An important role for prostaglandin E2 (PGE2) in HSC specification or early maintenance was uncovered in a chemical screen for molecules that alter the number of HSCs in zebrafish at 36 hours post fertilization¹³⁴, about 13 hours after HSC precursors first appear as a *runx1*-expressing group of cells in the ventral floor of the dorsal aorta. In these studies, PGE2 treatment increased HSC numbers, whereas an inhibitor of PGE2, indomethacin, led to loss of HSCs. In follow-up studies¹³⁵, this group showed that PGE2 mediates cAMP-directed stimulation of cAMP-dependent protein kinase (also known as PKA) activity, which augments Wnt-mediated inhibition of glycogen synthase kinase 3 (Gsk3). These conditions amplify β -catenin levels, resulting in greater canonical Wnt signal transduction, which in turn results in increased HSC numbers. Subsequent preclinical studies have verified the ability of PGE2 to augment the transplantation potential of human cord blood total and CD34⁺ cells in xenotransplants to mice, as well as of mobilized peripheral blood stem cells in autologous transplantation of non-human primates¹³⁶.

Non-canonical WNT signalling. Multiple β -catenin-independent (non-canonical) WNT signalling pathways have been described¹³⁷⁻¹³⁹. In some cases, these pathways make use of Frizzled receptors, whereas in others they seem to function through atypical receptors

such as members of the receptor tyrosine kinase-like kinase (RYK) and the receptor tyrosine kinase-like orphan receptor (ROR) families. These pathways activate diverse cellular responses and signal transduction pathways, from cytoskeletal changes to alterations in calcium signalling to antagonism of canonical WNT signalling.

We recently showed that non-canonical Wnt signalling by the conserved Wnt16 ligand is required for HSC specification in zebrafish¹⁴⁰ (FIG. 5). Wnt16 does not act directly on HSC precursors, but rather acts in the adjacent somites to activate expression of two Notch family ligand genes, *deltac* (*dlc*) and *deltad* (*dld*), which are required in combination for HSC specification (FIG. 5a). In the absence of either ligand, HSC numbers are decreased, and in the absence of both ligands, HSC specification is abrogated. As described above, Notch signalling is required cell-autonomously for HSC specification in mouse, but expression of Notch reporter transgenes and arteriovenous specification remain intact in *wnt16*-knockdown zebrafish, which indicates that HSC precursors in the dorsal aorta are still experiencing Notch signals. Moreover, rescue experiments in which Notch activation in HSC precursors was enforced in the absence of Wnt16 show that the Notch signalling event controlled by Wnt16-Dlc and Wnt16-Dld is earlier than and distinct from the cell-autonomous requirement for Notch signalling. Thus somitic Dlc- and Dld-mediated Notch signalling does not seem to act directly on HSC precursors. These results suggest that Wnt16-Dlc and Wnt16-Dld activate expression of a relay signal or are required for a morphogenetic event that potentiates HSC specification.

Animals with *wnt16* knockdown or *dlc* and *dld* knockdown also have defects in the ventromedial compartment of the somite, the sclerotome. Therefore, sclerotomal specification or morphogenesis could be required for HSC specification. The sclerotome has

Sclerotome

A ventromedial compartment of each somite containing a variety of tissue precursors, including vertebral column cells, breast bone cells and vascular smooth muscle cells.

been shown to give rise to multiple adult tissues, including the vascular smooth muscle cells (VSMCs) that sheath the dorsal aorta^{141–144}. In chick and mouse, VSMC precursors emigrate from the somite and surround the dorsal aorta to make mature vasculature (FIG. 5b,c). Notch signalling has been shown to control the emigration of a population of somitic cells that joins the dorsal aorta in chick, although it was not determined whether these cells are indeed future VSMCs¹⁴⁵. A single model that explains all these events is that Wnt16–Dlc and Wnt16–Dld signalling direct VSMC precursors to carry an HSC-specification relay signal to the dorsal aorta (FIG. 5d). Testing this model will require the generation of transgenic animals to determine the timing, origin and signalling capacity of VSMC precursors.

Blood flow, shear force and nitric oxide signalling. The effects of blood flow and pressure on the endothelium have an important role in both patterning the developing cardiovascular system and remodelling adult vasculature¹⁴⁶. Recent work indicates that blood flow is also required for the early maintenance of HSCs^{147–149}. A chemical screen for modifiers of HSC development in zebrafish showed that multiple compounds that regulate heartbeat and blood flow had effects on HSC numbers in developing zebrafish¹⁴⁸. Many of these effects can be explained by a lack of blood flow, as embryos mutant for the cardiac troponin T2 (*tnnt2*) gene, which lack a heartbeat, also had large decreases in the number of HSCs at 36 hours post fertilization. Similarly, less than a day after the initiation of blood flow, the E9.25 aorta–gonads–mesonephros region of mouse sodium/calcium exchanger 1 (*Ncx1*; also known as *Slc8a1*)^{-/-} embryos (which also have no heartbeat) had less-abundant expression of HSC markers and decreased haematopoietic potential *in vitro* compared with wild-type¹⁴⁷. Subjecting embryoid bodies to artificial shear stress *in vitro* augmented haematopoietic potential, even in *Ncx1*^{-/-} mouse-derived tissue¹⁴⁷. It is likely, however, that blood flow maintains the HSC programme rather than initiating it, because although the expression of HSC genes — for example, *Runx1* — was ultimately decreased in *Ncx1*^{-/-} mice¹⁴⁷, initiation of *runx1* and even *cmyb* expression occurs fairly normally in zebrafish with *tnnt2* knockdown¹⁴⁹.

Nitric oxide signalling has a key role in transmitting the effects of blood flow. Nitric oxide has well-established effects on the vascular system and endothelial cells¹⁵⁰, and loss of function of specific nitric oxide synthase genes in both mouse and zebrafish resulted in notable decreases in HSC numbers¹⁴⁸. Pharmacological manipulation of nitric oxide signalling also had strong effects on haematopoietic potential *in vitro*¹⁴⁷. Enforced nitric oxide signalling could rescue HSC specification in embryos with no heartbeat, and transplanted cells with knockdown of nitric oxide synthase genes could not contribute to HSCs¹⁴⁸. These results indicate that nitric oxide signalling cell-autonomously mediates the haemogenic effects of blood flow.

Catecholamines and GATA3. Signalling by the sympathetic nervous system to the dorsal aorta is also likely to regulate HSC specification. In a screen for genes that are expressed in the mouse aorta–gonads–mesonephros, *Gata3* was found to be upregulated¹⁵¹. *Gata3* is expressed by numerous tissues, including embryonic HSCs¹³, kidney¹⁵² and the sympathetic nervous system¹⁵³. By various criteria, fewer HSCs are specified during development in *Gata3*^{-/-} mice¹⁵⁴. Surprisingly, however, HSC precursors do not normally express *Gata3*, which indicates that the effects of GATA3 are non-cell-autonomous. Several tissues near HSCs in the dorsal aorta express *Gata3*, including the subaortic mesenchyme and neurons of the sympathetic nervous system derived from recently migrated neural crest. *Gata3* is required for generation of the sympathetic nervous system^{155–158}, which produces signalling molecules such as catecholamines. Exogenous catecholamines can rescue HSC defects when added to dissected aorta–gonads–mesonephros *ex vivo* explant cultures¹⁵⁴, suggesting that the requirement for GATA3 for HSC specification reflects a contribution by signalling from neurons of the developing sympathetic nervous system.

Conclusion

HSCs form the foundation of the adult haematopoietic system. The production of HSCs during development is a key step in making a healthy organism, and it involves multiple regulatory interactions during embryogenesis. The *in vitro* production of HSCs from iPS cells could provide a source of transplantable cells for regenerative medicine and the treatment of sickle cell anaemia, thalassaemia, leukaemia and other diseases with autologous, genetically corrected cells. *In vitro*-generated HSCs would also provide material for patient-specific disease studies. Although the generation of specific types of haematopoietic cells for specific applications is now becoming feasible, generating robust numbers of bona fide HSCs with normal lineage potential that are capable of generating erythrocytes expressing adult globins so far remains impossible^{5,6}. Understanding how HSC specification occurs natively during embryonic development and trying to carefully reproduce these events is one clear means of trying to develop improved techniques for directed differentiation protocols.

Current protocols for the haematopoietic differentiation of pluripotent cells involve treating cell populations with signalling factors *in vitro* for defined periods of time in order to induce a stepwise progression towards the desired fate outcome. These protocols necessarily involve selecting desirable cell fate intermediates from the total pool for further differentiation by treatment with additional factors over defined times, rather than the more difficult goal of directing a homogeneous cell fate. In some cases, cells have been modified to carry genes that augment their differentiation potential, but lasting genetic modification has unpredictable consequences and is therefore undesirable for protocols for therapeutic purposes. A clearer understanding of native embryonic signalling and morphogenetic processes

Sympathetic nervous system

A neural crest-derived component of the autonomic nervous system that regulates tissue responses including the 'fight-or-flight' response. The sympathetic nervous system develops in tandem with the adrenal glands and regulates secretion of cardiovascular system regulatory hormones including the catecholamines adrenaline and noradrenaline.

might enable the selection of a preferable intermediate or suggest additional molecular factors that could make the differentiation process more robust, efficient and realistic, and therefore come closer to generating large numbers of true HSCs. For example, the recent recognition that HSCs derive from arterial haemogenic endothelium suggests that one obvious step is to attempt to reproduce the formation of this tissue^{159,160}.

These advances highlight the fact that work in non-human vertebrates as diverse as mouse and zebrafish can productively inform strategies for human haematopoietic programming owing to the conservation of mechanisms between species. We hope that in the future, the complete set of native signals involved can be defined and incorporated into existing protocols to realize the ultimate goal of producing HSCs *in vitro*.

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Competing interests statement

The authors declare no competing financial interests.

FURTHER INFORMATION

Wilson K. Clements' homepage: <http://www.stjude.org/clements>
 David Traver's homepage: http://labs.biology.ucsd.edu/traver/Traver_Laboratory/Home.html

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