A view of human haematopoietic development from the Petri dish

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Abstract | Human pluripotent stem cells (hPSCs) provide an unparalleled opportunity to establish *in vitro* differentiation models that will transform our approach to the study of human development. In the case of the blood system, these models will enable investigation of the earliest stages of human embryonic haematopoiesis that was previously not possible. In addition, they will provide platforms for studying the origins of human blood cell diseases and for generating *de novo* haematopoietic stem and progenitor cell populations for cell-based regenerative therapies.

The differentiation of human pluripotent stem cells (hPSCs) towards a haematopoietic fate has been the subject of intense investigation in recent years, as the ability to generate human blood cell progenitors, in particular haematopoietic stem cells (HSCs), *in vitro* has broad applications in basic research and clinical medicine. Given that HSC transplantation and the transfusion of terminally differentiated blood cells are effective therapies for the treatment of haematological disorders[1], the generation of these cell types from hPSCs would overcome the current shortage of compatible donors and expand the patient groups that can be treated with cell-based strategies. Moreover, with our ability to generate patient-specific induced pluripotent stem cells (iPSCs), and to create specific mutations in hPSCs with CRISPR–Cas9 technology, it will be possible to model a wide range of blood diseases *in vitro* and to identify novel cellular targets for the development of new therapeutics[2]. Finally, recapitulating the early stages of human development *in vitro* will enable detailed investigation of the establishment of the human embryonic haematopoietic system.

The design of precisely staged protocols for the derivation of haematopoietic cells from hPSCs has been guided largely by our understanding of haematopoietic development in model organisms. This approach has been remarkably successful in generating progenitor cell populations that are difficult to isolate from early embryos. However, deriving HSCs — the ultimate goal of many researchers in the field — has been unsuccessful, probably owing to the complexity of the embryonic haematopoietic system and the lack of specific markers to distinguish the different stages of embryonic blood cell development. Generation of HSCs from hPSCs will ultimately require more refined approaches that accurately recapitulate, *in vitro*, the precise developmental stages of HSC specification in the early embryo. Insights from developmental biology will most certainly continue to guide us in these efforts.

In this Review, we first describe our current understanding of embryonic haematopoiesis and the structure of the embryonic haematopoietic system that serves as the roadmap for hPSC differentiation studies. We then review new insights into the emergence of the earliest embryonic haematopoietic populations and the relationship between the haematopoietic and vascular lineages obtained from studies with hPSC models. In the final section, we discuss HSC development from hPSCs and the challenges that lie ahead in achieving this goal.

Embryonic haematopoietic development

Our understanding of embryonic haematopoietic development has been shaped by decades of research using different model organisms, including the chick, frog, fish and mouse (reviewed in REFS 3,4). As the principles of blood cell development are remarkably conserved, these studies have led to a model of vertebrate embryonic haematopoiesis that comprises distinct developmental stages, which we refer to here as programmes. These programmes have different spatial and temporal patterns during development and different haematopoietic potential. The discovery, which was made more than 40 years ago, that haematopoiesis occurs at two independent sites[5] — the yolk sac and embryo proper — led to an early two-stage model of embryonic haematopoiesis that consisted of a primitive and a definitive programme. Although most observations over the next 3 decades fit into this model, studies during the past 10 years have revealed that the system is more complex and contains yolk sac-derived erythro-myeloid progenitors (EMPs) and lymphoid-primed multipotent progenitors (LMPPs) that cannot be easily assigned to either the definitive or the primitive programme. Given this, we propose that these progenitor populations are generated by independent programmes initiated within the yolk sac (FIG. 1).

Induced pluripotent stem cells
(iPSCs). Pluripotent stem cells generated from adult somatic cells through genetic reprogramming with transcription factors. Similar to human embryonic stem cells, iPSCs can be propagated indefinitely in culture while retaining the capacity to differentiate and give rise to virtually all cell types found in the human body. iPSCs should theoretically be an identical immunological match to the patient they are derived from.

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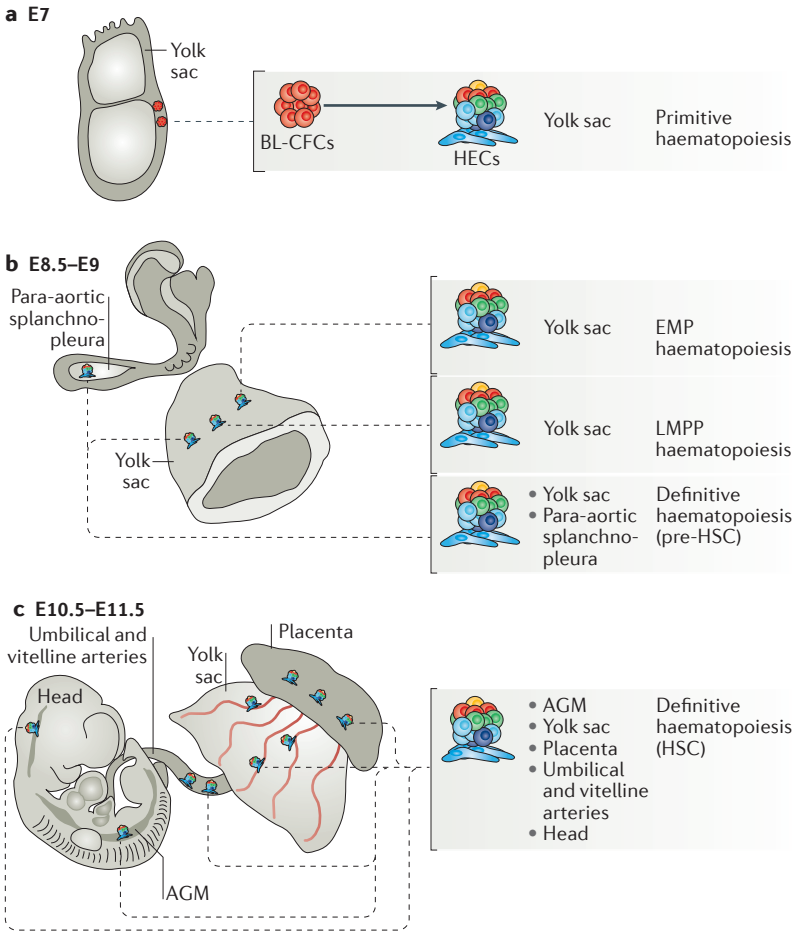

Figure 1 | **A model of mouse development depicting the origin and temporal development of the different embryonic haematopoietic programmes.** **a** | The primitive haematopoietic programme is the first to be initiated and is specified by embryonic day 7 (E7) in the presumptive blood island in the yolk sac region of the early embryo; the earliest detectable primitive haematopoietic progenitor is the haemangioblast, which is depicted as the blast colony-forming cells (BL-CFCs). As they differentiate, the BL-CFCs give rise to haemogenic endothelial cells (HECs). **b** | A schematic representation of E8.5–E9 embryos that indicates the emergence of the erythro-myeloid progenitor (EMP) and lymphoid-primed multipotent progenitor (LMPP) programmes in the yolk sac, and the definitive programme defined by the emergence of pre-haematopoietic stem cells (pre-HSCs) in both the yolk sac and intra-embryonic para-aortic splanchnopleura. **c** | These three programmes are specified from HECs between E10.5 and E11.5; HSCs that are able to engraft adult recipients are detected at multiple sites in the embryo, including the aorta–gonad–mesonephros (AGM), the umbilical and vitelline arteries, the placenta, the head and the yolk sac. Note that although HSCs are found at the indicated sites, it is still not clear whether they are all sites of *de novo* generation of HSCs.

Primitive haematopoiesis. Primitive haematopoiesis is specified and begins within the presumptive blood islands in the yolk sac of the mouse embryo by embryonic day 7 (E7), and in the human embryo during the first 3 weeks of gestation[6,7]. The primitive programme is transient and highly restricted, as it generates cells of only the erythroid, macrophage and megakaryocytic lineages[7]. Primitive erythroblasts can be distinguished from their definitive counterparts by their large size, by the fact that they retain their nuclei upon entry into the circulation and by their globin expression patterns (reviewed in REF. 8) (BOX 1). Macrophages and megakaryocytes generated during this stage of haematopoiesis also differ from those that are formed during the definitive programme. Primitive macrophages mature rapidly without progressing through a monocyte stage of development[9,10], and primitive megakaryocytes have reduced ploidy and produce fewer platelets than their fetal and adult counterparts[11,12].

Definitive haematopoiesis. Definitive haematopoiesis is initiated following the onset of primitive haematopoiesis at different sites within the developing vasculature of the embryo; the best-studied site is the dorsal aorta within the aorta–gonad–mesonephros (AGM) region of the embryo proper[13]. The defining feature of the definitive programme is its potential to generate HSCs. HSCs that can engraft adult animals are first detected in the AGM region of mouse embryos at E10.5 and in the same region of human embryos at day 32 of gestation[13–16]. HSCs are also found in the yolk sac, placenta and head of the mouse embryo at this stage[15,17–19]. These newly formed HSCs are identified by the co-expression of haematopoietic and vascular markers, including vascular endothelial cadherin (VE-cadherin; also known as CD144), CD45, CD93, KIT, stem cell antigen 1 (SCA1; also known as LY6A) and CD31 in mice[14,20,21] and CD34, VE-cadherin, CD117, CD90, CD45 and CD105 in humans[22].

Although HSCs that can engraft adult mice are first detected in the AGM, cells with the capacity to engraft neonatal recipients are present as early as E9 in both the yolk sac and the para-aortic splanchnopleura (pre-AGM) region of the embryo proper[23,24]. Despite the inability of these early-stage HSCs to directly engraft adult recipients, their progeny can acquire this capacity *in vivo*, as the marrow from the primary recipients is able to engraft secondary adult recipients[24]. These findings suggest that E9 cells are immature HSCs that require a 'fetal' environment for engraftment and maturation. Recent studies have shown that it is possible to recapitulate aspects of this fetal environment *in vitro*, as HSCs capable of engrafting adults were generated from E9.5–E10 AGM VE-cadherin$^+$ progenitors following co-culture with either OP9 stromal cells or endothelial cells[25–28]. Given their potential to generate HSCs, these VE-cadherin$^+$ cells were referred to as 'pre-HSCs'. This early AGM population has been further divided into three distinct categories of HSC precursors known as pro-HSCs, type I pre-HSCs and type II pre-HSCs that represent different stages of maturation[25,26]. Although this *in vitro* culture system promoted the maturation of AGM progenitors, it failed to support HSC development from yolk sac progenitors, indicating that it does not completely recapitulate the environment of the neonate. Understanding the developmental steps underlying the progression of pro-HSCs to pre-HSCs, and ultimately to mature HSCs, has important implications for the generation of hPSC-derived HSCs, as appropriate neonate transplantation assays and co-culture systems will need to be used to identify comparable progenitors in the human system.

Box 1 | **Globin expression during erythroid development**

The examination of the cells circulating in the bloodstream of developing mammalian embryos performed at the beginning of the twentieth century revealed the presence of two different populations of erythroid cells[130]. The earliest population is indicated as primitive and consists of larger nucleated cells. This erythroid programme is followed by the emergence of definitive erythroid cells, which are smaller and enucleated.

When assayed in methylcellulose cultures, both mouse and human primitive erythroid progenitors generate relatively small colonies of uniform size and morphology, suggesting that the lineage is specified as a cohort of cells with similar proliferative potential (reviewed in REF. 8). These progenitors are referred to as primitive erythroid colony-forming cells (EryP-CFCs) and emerge in a synchronous wave for approximately 36 hours, between embryonic day 7 (E7) and E8.5 in the mouse embryo[7]. This window sets the duration of primitive erythropoiesis and highlights the transient nature of this stage of embryonic haematopoiesis.

This pattern is distinct from the definitive erythroid lineage that contains progenitors representing different stages of development, including immature burst-forming unit erythroid (BFU-E) cells that give rise to large colonies that contain multiple clusters of erythroblasts and more advanced colony-forming unit erythroid (CFU-E) cells that generate medium to small, compact colonies consisting of a single cluster of cells (reviewed in REF. 8).

The pattern of expression of the haemoglobin genes of the β-globin locus is a peculiar characteristic of erythroid cells at different stages of development. These differences are very often used to define the haematopoietic programmes from which these cells originated. Mouse primitive erythroblasts express the embryonic εy- and βH1-globin genes, whereas the human primitive erythroid cells express predominantly the ε-globin genes of the β-globin cluster[8]. Definitive erythropoiesis in the mouse is characterized by the upregulation of β-globin and extinction of εy- and βH1-globin expression[131]. In the human, upregulation of γ-globin expression combined with the loss of ε-globin expression marks the emergence of the definitive erythroid lineage[132]. Human definitive erythropoiesis undergoes a further change with the switch from γ- to β-globin expression after birth[132].

EMP haematopoiesis. In addition to the primitive lineages and pre-HSCs, the yolk sac contains a population of erythroid, megakaryocyte and mast cell progenitors that is specified after the initiation of the primitive programme, but before the establishment of a fully functional circulatory system[7,20,29–31]. This cohort of yolk sac-derived progenitors, referred to as EMPs, was originally thought to represent the onset of definitive haematopoiesis in the yolk sac[7,31,32]. However, findings from recent studies have challenged this interpretation and suggest that EMPs are part of an erythro-myeloid-restricted haematopoietic programme that is distinct from primitive and definitive haematopoiesis. The strongest evidence in support of this latter hypothesis is the observation that mouse EMPs can be separated from the progenitors of the primitive and definitive programmes based on the expression of KIT, CD41 and CD16/32, and a lack of expression of SCA1 (REFS 33,34). When cultured *in vitro*, the KIT$^+$CD41$^+$CD16/32$^+$ progenitors gave rise to erythroid, megakaryocyte, macrophage, neutrophil, eosinophil and basophil progeny, demonstrating a greater degree of lineage diversity than that of the primitive programme. Lymphoid potential was not detected in this population. Transplantation experiments confirmed this restricted potential and showed that EMPs provide only limited, short-term erythroid, myeloid and platelet engraftment, in contrast to long-term multilineage engraftment observed following transplantation of HSCs. In addition to differences in surface markers, EMP and definitive haematopoiesis can be distinguished based on globin expression patterns in the respective erythroid populations. EMP-derived erythroid populations that are differentiated *in vitro* express low levels of embryonic βH1 globin together with adult β1 globin, a pattern distinct from that of the definitive lineage[32]. Interestingly, these EMP-derived erythroblasts were found to downregulate embryonic globin expression following transplantation *in vivo* and, in doing so, acquire a phenotype similar to that of the definitive lineage. Whether they can exchange oxygen with adult tissues as efficiently as definitive erythrocytes remains to be determined.

LMPP haematopoiesis. The observation that B cell- and T cell progenitors can be detected in the yolk sac as early as E8.25, before the establishment of circulation and the emergence of the pre-HSCs, suggested that this tissue also generates HSC-independent lymphoid lineage cells[35,36]. This concept was further supported by lineage-tracing studies and clonal analyses that identified a multipotent progenitor with B cell, T cell and myeloid potential in the yolk sac as early as E9.5, a stage that overlaps with EMP haematopoiesis[37]. Based on this restricted potential, these progenitors have become known as LMPPs.

The identification of yolk sac-derived LMPPs and EMPs indicates that the yolk sac has multilineage potential that derives from two independent progenitors. This division of potential is somewhat unexpected, as HSCs in the definitive programme give rise to the same variety of lineages. This raises the possibility that EMPs and LMPPs may not be distinct programmes, but rather represent independent lineages of a common yolk sac programme that derives from a multipotent progenitor. A better understanding of the ontogeny of EMPs and LMPPs is important for clarifying their relationship to HSCs and for identifying and characterizing comparable stages of haematopoietic development in hPSC differentiation cultures.

Taken together, these studies suggest that the yolk sac harbours considerable haematopoietic potential that is manifested as waves of haematopoiesis derived from distinct programmes (FIG. 1). Although the primary function of these different haematopoietic cells is to support embryonic development and growth, studies over the past 5 years have provided compelling evidence that the yolk sac haematopoietic contribution extends beyond fetal life. Lineage-tracing experiments have shown that the microglia of the brain are derived almost exclusively from primitive macrophages, whereas the macrophages found in many of the other tissues, including the liver and lungs, appear to be of EMP origin[38–40]. Along similar lines, evidence suggests that LMPPs could be a source of specific subsets of self-renewing lymphoid cells that persist in the adult, including B-1 cells in the peritoneum and γδ T cells that colonize different epithelial tissues[35,36,41,42]. Collectively, these observations force us to re-evaluate the model of adult haematopoiesis in which all cell lineages are maintained by HSCs, and to consider one in which the haematopoietic populations in the different adult tissues comprise both HSC- and long-lived embryo-derived cells. This distinction has important clinical implications, as macrophages play a pivotal

CRISPR–Cas9 technology
The enzyme Cas9 causes a DNA double-strand break at a site (or sites) within the genome that harbours complementarity to homologous short RNA sequences that can be easily synthesized and delivered to cells *in vitro*. This permits easy, cost-effective generation of in-frame deletions, or insertional mutagenesis, within human pluripotent stem cells.

Yolk sac
An extra-embryonic developmental tissue that harbours the first site of haematopoietic development.

Embryo proper
The anatomical structure that ultimately gives rise to the mature embryo and all tissues found in fetal and adult life. It does not include the extra-embryonic annexes.

part in tissue homeostasis (reviewed in REF. 43), and it is possible that the decline in organ function associated with ageing or degenerative diseases is due, in part, to a loss of or a change in function within this population. If true, transplantation strategies that replenish these embryo-derived lineages may prove to be effective therapeutic interventions. As these cell types are not derived from HSCs, the only current source for potential replacement therapy is hPSCs.

Progenitors of the different programmes

Along with developmental potential and site of origin, the different haematopoietic programmes are defined by the distinct progenitors they derive from. The identity of embryonic haematopoietic progenitors, in particular those of the primitive programme, has been of interest to developmental biologists for the better part of the past century[44–46]. This interest has led to concepts and hypotheses, some established many years ago, that continue to influence our thinking and experimental designs today.

Blast colony-forming cells: progenitors of the primitive programme. The best known and debated hypothesis is undoubtedly the one that proposed that the haematopoietic and vascular lineages develop from a common progenitor, the haemangioblast[45,46]. Progenitors with haemangioblast properties were first identified in haematopoietic populations generated from mouse embryonic stem (ES) cells through their ability to give rise to haematopoietic and vascular progeny, either in monolayers in liquid culture or in the form of blast cell colonies in methylcellulose cultures[47]. Given their colony-forming potential, these progenitors became known as blast colony-forming cells (BL-CFCs) and were further characterized as cells that express kinase insert domain receptor (KDR; also known as FLK1 and VEGFR2) and the primitive streak marker brachyury, but not the endothelial or haematopoietic markers KIT, CD31, VE-cadherin, CD45 or CD34. BL-CFCs with similar characteristics were identified in the posterior primitive streak region of E7 embryos, demonstrating that these progenitors were not an artefact of the *in vitro* differentiation model[48]. The fact that BL-CFCs display primitive haematopoietic potential and are specified before the primitive programme is initiated strongly suggests that they are the progenitors of primitive haematopoiesis. This interpretation is supported by lineage-tracing studies that showed that the primitive haematopoietic programme in embryos derives from KDR$^+$ progenitors[49].

Haemogenic endothelial cells. As with primitive haematopoiesis, the origin of the definitive haematopoietic programme has been the subject of extensive investigation over the past two decades. Through the use of viral marking, lineage-tracing and whole-mount live-imaging strategies, these studies have provided compelling evidence that the definitive haematopoietic programme and HSCs derive from embryonic progenitors that display endothelial characteristics; these progenitors are known as haemogenic endothelial cells (HECs)[49–56].

HECs express the endothelial surface markers VE-cadherin[21,57], CD31 (REF. 21) and KIT[58] and the transcription factors *Runx1* (REF. 57) and *Gata2* (REFS 59,60), and they are found at specific sites within the developing arterial vasculature of early embryos, the best characterized being the ventral wall of the dorsal aorta in the AGM. Given their close association with the arterial vasculature, it is generally assumed — but has never been proved — that they derive from the arterial vascular lineage. As they differentiate, HECs undergo an endothelial-to-haematopoietic transition (EHT) to give rise to non-adherent CD45$^+$ haematopoietic cells in culture or to haematopoietic clusters within the lumen of the aorta *in vivo*[55,61]. These intra-aortic clusters have been observed in different species, including mice, chicks, frogs and humans, suggesting that EHT is a common feature of vertebrate haematopoiesis[52,62–67].

Although HECs were considered to be exclusive to definitive haematopoiesis, analyses of the early stages of EMP and LMPP lineage specification revealed that these programmes also derive from VE-cadherin$^+$KIT$^+$CD31$^+$CD45$^-$ endothelial-like progenitors[33,35]. The presence of HECs in the yolk sac has been recently confirmed[68]. The existence of EMP HECs and possibly even primitive HECs calls into question the relationship of these progenitors to haemangioblasts. Insights into this relationship were provided by a study that showed that as BL-CFCs differentiate they give rise to HECs, which in turn generate haematopoietic progeny[69]. These findings support a model in which BL-CFCs represent the progenitor of the HEC in the primitive and/or EMP programmes. Whether a similar precursor–progeny relationship exists in the yolk sac of the embryo remains to be determined.

The existence of different HEC populations highlights one of the challenges associated with modelling hPSC-derived haematopoietic programmes *in vitro*, as it is currently impossible to distinguish these populations on the basis of surface marker expression alone. Although developmental potential can be used to identify the different programmes, the overlap of lineages between them creates a major technical difficulty and can lead to misinterpretation of data. An alternative approach to distinguishing the stages of haematopoiesis, which can be exploited to guide *in vitro* hPSC differentiation towards specific lineages, is the identification of signalling pathways and transcription factors that specifically regulate the different programmes (BOX 2).

Modelling embryonic haematopoiesis

The study of different model organisms has provided a detailed understanding of embryonic haematopoiesis and important insights into the origins of the adult haematopoietic system. The current picture of human haematopoietic development is largely extrapolated from these models, as comparable studies on the human embryo are not possible. hPSCs offer an unprecedented opportunity to complement this knowledge, assuming that differentiation in the culture dish can recapitulate the dynamics and complexity of haematopoietic development *in vivo*. Studies with

Aorta–gonad–mesonephros (AGM). A region in the caudal portion of the embryo containing the developing aorta, genital ridges and mesonephros that gives rise to haematopoietic stem cells.

OP9 stromal cells
A stromal cell line derived from the *op*/*op* mouse that does not produce macrophage colony-stimulating factor (M-CSF). These cells are commonly used to support differentiation and expansion of mouse and human haematopoietic progenitor populations.

Ontogeny
The development of a specific cell type within an organism.

B-1 cells
A subclass of B lymphocytes that are not part of the adaptive immune system and lack the ability to form memory B cells.

γδ T cells
A subset of T cells that have a distinct T cell receptor composed of a γ- and δ-chain.

Primitive streak
A dorsally located structure that forms in the epiblast of the early vertebrate embryo. The primitive streak establishes bilateral symmetry and is commonly associated with the onset of gastrulation and the generation of the three germ layers. Undifferentiated epiblast cells adopt either a mesoderm or an endoderm fate as they move through the primitive streak.

Viral marking
The use of a virus that inserts randomly into the genome as a means of tracking the progression of a cell within a population of other cells.

mouse ES cells have provided reassuring evidence that it is possible to model haematopoietic development *in vitro*, as their differentiation recapitulates many aspects of embryonic haematopoiesis with remarkable accuracy[70]. Experiments with hPSCs similarly show that haematopoietic development *in vitro* follows the patterns predicted from *in vivo* studies, thereby laying the foundation for developing a model for human embryonic haematopoiesis.

Differentiation strategies. Two approaches have been used to promote haematopoietic differentiation of hPSCs. The first involves co-culturing hPSCs with stromal cells in serum-containing medium. Although straightforward, this method precludes the ability to accurately control differentiation stages because of the presence of poorly defined growth factors and pathway inhibitors derived from the serum or the stromal cells. In the second approach, differentiation occurs under defined conditions with specific signalling pathway agonists and antagonists, enabling recapitulation of key aspects of embryonic development in a reproducible manner. Both strategies have been used to generate a wide range of human haematopoietic cell types *in vitro*, including erythroblasts, macrophages, dendritic cells, natural killer cells, neutrophils, eosinophils, basophils, megakaryocytes, and T and B cells (reviewed in REF. 71). The generation of this diverse set of blood cell lineages strongly suggests that the different haematopoietic programmes identified in model organisms can be specified using hPSCs.

Characterization of hPSC differentiation. As primitive haematopoiesis is the first programme to be initiated, it was the easiest to characterize in culture, and was identified by the early specification of macrophage and primitive erythroid progenitors[72]. The onset of human primitive haematopoiesis was preceded by the development of a transient population of KDR^+ BL-CFCs with the capacity to generate both haematopoietic and vascular progeny, suggesting that, as for mice, the human programme derives from a progenitor with haemangioblast properties[72,73]. Analyses of the signalling pathways that controlled these early stages in the defined culture system revealed that human haematopoietic development, including the formation of the primitive streak and the induction and specification of mesoderm, was regulated by Nodal–activin, WNT–β-catenin, bone morphogenetic protein (BMP) and fibroblast growth factor (FGF) signalling, pathways that are known to regulate these stages in model organisms[72,74–76]. These findings indicate that

Box 2 | **Regulation of haematopoietic development**

The establishment of the haematopoietic system involves a progression through multiples developmental stages that are regulated by the coordinated interaction of different signalling pathways and transcriptional networks. Here, we only discuss a select number of pathways and transcription factors that differentially regulate specific stages of haematopoietic development (see the table).

Dependency on Notch is perhaps the best approach for distinguishing different haematopoietic programmes. Studies in mice, the mouse embryonic stem cell differentiation model and zebrafish have provided compelling evidence that Notch signalling is essential for the development of the definitive programme but dispensable for primitive and erythro-myeloid progenitor (EMP) haematopoiesis[92,93,133]. The Notch dependence of lymphoid-primed multipotent progenitors (LMPPs) has not yet been formally investigated. Although the precise stage (or stages) at which Notch functions within the definitive programme *in vivo* has not been fully established, recent studies suggest that it may be at the level of haemogenic endothelial cell (HEC) development and/or maturation within the aorta–gonad–mesenephros (AGM)[94,95].

The function and expression patterns of specific transcription factors, including RUNX1 and MYB, have also been used to distinguish different stages of embryonic haematopoiesis[39,56,134–136]. *Runx1* is of particular interest, as its pattern of expression differs depending on promoter usage. *Runx1b* expressed from the proximal promoter P2 is detected in both yolk sac and AGM HECs, whereas the message from the distal promoter P1, referred to as *Runx1c*, is present only in the definitive HECs[137]. Targeting studies have shown that RUNX1 is required for EMP and definitive haematopoiesis, but not for development of the primitive erythroid lineage[134]. The *Runx1c* isoform, by contrast, is not essential for any stage of haematopoietic development[138]. *Myb* is expressed in EMP[40] and definitive progenitors but not in any of the primitive lineages[7]. *Myb*-null mice undergo normal primitive haematopoiesis[135,139,140] and contain EMP-derived macrophages[39] and megakaryocytes[141], indicating that the generation of these cell types is MYB independent. By contrast, MYB is required for development of the EMP-derived erythroid lineage[141] and the definitive haematopoietic programme[135,140].

Gene	Primitive haematopoiesis	EMP haematopoiesis	LMPP haematopoiesis	Definitive haematopoiesis	Lethality	Refs
Runx1	Only primitive erythropoiesis observed	Absent	Unknown	Absent	E11.5	127, 129, 130
RBPJ (Notch signalling)	Unaffected	Reduced	Unknown	Absent	E10.5	126
Notch1	Unaffected	Reduced	Unknown	Absent	E11.5	92,134
Myb	Unaffected	Reduced erythropoiesis	Unknown	Absent	E16	39,128

E, embryonic day.

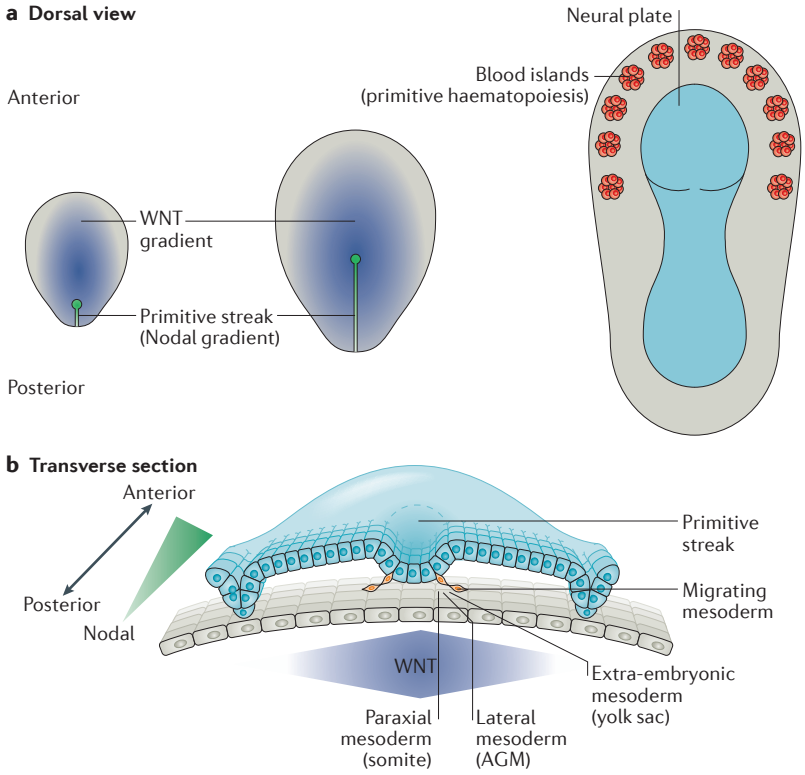

Figure 2 | **Schematic representation of the human embryo during early gastrulation. a** | Early human primitive streak has gradients of Nodal and WNT morphogens. The dorsal view of three different stages of embryonic development is shown. On the left is a day 15 human germ disc with a gradient of WNT ligands shown in blue. Nodal (green) is secreted by the primitive node, which migrates in a posterior-to-anterior direction. In the middle is a day 18 human germ disc, illustrating the anterior migration of the primitive node. On the right is a day 19 human embryo, with blood islands first appearing in the anterior yolk sac. **b** | Cartoon representing a transverse section of the human germ disc from days 15 to 20 of gestation. Migrating mesodermal cells pass through the primitive streak (centre) at various times throughout gestation. As cells pass through the streak, they are exposed to high (anterior) or low (posterior) concentrations of Nodal and to high or low concentrations of WNT ligands. Lateral plate mesoderm, which is exposed to low Nodal and high WNT signalling, will give rise to the definitive haematopoietic programme. AGM, aorta–gonad–mesonephros. Part **a** is adapted from Langman's Medical Embryology[87].

the regulatory pathways and patterns of lineage development that are associated with the onset of haematopoiesis in most vertebrate species are conserved in hPSC differentiation cultures.

Extended analyses of hPSC differentiation cultures documented the presence of CD45$^+$ erythroid and myeloid populations distinct from those of the primitive lineages, indicating that other programmes are induced in the cultures[77,78]. Although these progenitors were assigned to the definitive programme[79,80], it is possible that they represent EMP haematopoiesis, as the erythroid progenitors generated progeny that expressed ε-, γ- and β-globin[78,79]. The distinction between these different programmes in the human system is difficult, as human EMPs have not yet been characterized. Evidence for the existence of human EMPs *in vivo* is provided by the observation that the human fetal liver contains burst-forming unit erythroid (BFU-E) cells that generate erythroid cells that co-express ε- and γ-globin[81].

The most rigorous approach for distinguishing EMP from definitive haematopoiesis is the capacity to produce T cells[82–84]. Using this strategy, a hPSC-derived progenitor population that displays T cell, erythroid and myeloid potential was identified based on expression of the endothelial markers CD34, CD31 and VE-cadherin, and the lack of expression of the haematopoietic markers CD43 and CD45 (REFS 82,84,85). These progenitors developed in heterogeneous cultures that also contained a CD34$^+$CD43$^+$ population that had primitive erythroid and myeloid but no T cell potential, which is indicative of primitive and/or EMP haematopoiesis[82].

The overlapping development of different haematopoietic programmes in the same differentiated population highlights a major challenge in working with any PSC model, as primitive and EMP haematopoiesis emerge early and become predominant in culture. Analyses of the signalling pathways that control the earliest stages of haematopoietic development provided a solution to this problem by demonstrating that specification of the primitive and definitive programmes is differentially regulated by the Nodal–activin and WNT–β-catenin pathways[82,85]. These studies showed that human primitive haematopoiesis is temporally dependent on Nodal–activin signalling and inhibited by the WNT–β-catenin pathway. The definitive programme, by contrast, is specified in the absence of Nodal–activin signalling but is dependent on the WNT–β-catenin pathway. Interestingly, these opposing effects were only observed if the pathways were manipulated within 72 hours of the induction of differentiation, immediately after the formation of the primitive streak, suggesting that they function at the stage of mesoderm induction or patterning[82,85]. The observation that WNT–β-catenin signalling inhibits human primitive haematopoiesis[85] is at odds with findings in the mouse ES cell differentiation system that showed that this pathway is required for specification of the primitive programme from KDR$^+$ mesoderm[86].

The regulation of primitive and definitive haematopoiesis observed in mouse and human PSC differentiation cultures is consistent with differences in patterns of mesoderm development and migration in the mouse and human embryo. The human primitive haematopoietic programme is thought to derive from mesoderm induced at the most posterior region of the primitive streak, where active Nodal signalling is initiated from the primitive node, which has not yet migrated anteriorly (FIG. 2). Once induced, these mesodermal cells migrate anteriorly to generate the blood islands that develop around the anterior region of the developing embryo[87]. As these cells migrate, they are exposed to WNT inhibitors secreted by the anterior (cranial) endoderm[88]. This pattern of cell movement is different from that observed in the avian and mouse embryos[89,90], in which the mesoderm that contributes to primitive haematopoiesis migrates posteriorly towards an environment of active WNT signalling. Mesoderm that will give rise to the human definitive programme migrates more posteriorly and ventrally, where the cells are exposed to WNT signalling and little, if any, Nodal signalling. These differences clearly show that not all aspects of developmental

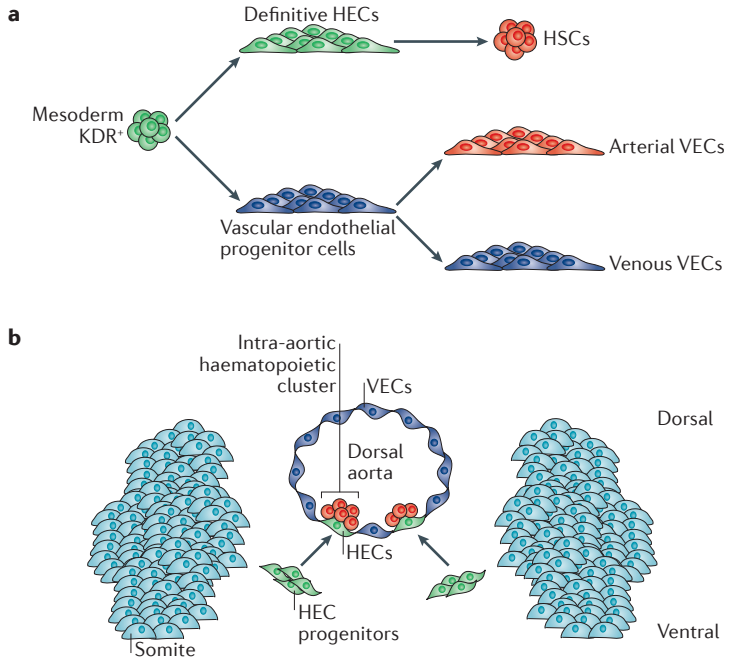

Figure 3 | **A model of human HEC development. a** | Haemogenic endothelial cells (HECs), which give rise to haematopoietic stem cells (HSCs), and vascular endothelial cells (VECs) are specified as distinct lineages from KDR$^+$ mesoderm. **b** | Following specification, HEC progenitors migrate in close contact with somitic cells to the aorta–gonad–mesonephros (AGM) region, where they integrate with the arterial vascular cells, and undergo endothelial-to-haematopoietic transition (EHT) to generate haematopoietic progeny that can be detected as distinct clusters in the lumen of the aorta.

biology can be translated from model organisms to humans and thus highlight the importance of developing an *in vitro* system that will enable us to model all aspects of human haematopoietic development.

When cultured under conditions that promote haematopoietic and vascular development, the CD34$^+$CD43$^-$ population derived from the mesoderm induced in the presence of either a Nodal–activin inhibitor or an agonist of the WNT–β-catenin pathway gave rise to cells with an endothelial-like morphology; these cells underwent EHT to generate a *RUNX1C*$^+$CD45$^+$ population that contained T cell, erythroid and myeloid progenitors[91]. Importantly, this transition was shown to be Notch dependent, a strong indication that the CD34$^+$CD43$^-$ population contains definitive HECs, as studies in the mouse and zebrafish have shown that establishment of the definitive programme, but not the primitive or EMP programmes, requires Notch signalling[92,93] (BOX 2). Despite the Notch dependency, the colonies generated from the erythroid progenitors in this population still expressed relatively high levels of ε-globin along with γ-globin, a pattern consistent with that of the EMP programme. These observations suggest that either a Notch-dependent human erythroid progenitor exists that is not representative of definitive haematopoiesis or, more intriguingly, that the human definitive erythroid lineage transitions through a stage during which the progenitors generate progeny that express this pattern of globin genes. Alternatively, it is possible that additional cell-non-autonomous signalling present in the *in vivo* environment is required to silence ε-globin expression. Distinguishing between these possibilities will require additional experimentation.

Defining lineage relationships. The identification of Nodal–activin and WNT–β-catenin as regulators of the human primitive and definitive haematopoietic fates underscores the potential of the hPSC system for investigating human embryonic haematopoiesis. Through our ability to specify either primitive or definitive haematopoiesis, it has been possible to study both programmes and, as discussed below, to identify progenitor populations and define lineage relationships that will provide the foundation for drawing a complete lineage map of the human embryonic haematopoietic system.

The T cell, erythroid and myeloid potential in the hPSC-derived CD34$^+$CD43$^-$ population described above is consistent with the presence of a multipotent definitive progenitor, a cell that could represent the human pre-HSC. Alternatively, this potential could indicate the emergence of separate EMP and LMPP programmes. To address this issue, the relationship of these lineages was investigated through clonal analyses, which were made possible by a cell-sorting strategy that identified a CD34$^+$CD73$^-$CD184$^-$DLL4$^-$ population that was highly enriched in HECs[79,91]. This strategy also identified CD34$^+$CD184$^+$ and CD34$^+$CD73$^+$ populations that gave rise to arterial or venous endothelium, respectively. Analyses of single CD34$^+$CD73$^-$CD184$^-$DLL4$^-$ cells revealed that a subset of these cells is multipotent and thus able to generate erythroid, myeloid and lymphoid progeny. The presence of multipotent progenitors demonstrates that the haematopoietic potential in this population is not simply reflective of a combination of EMP and LMPP haematopoiesis and further supports the notion that their emergence represents the onset of human definitive haematopoiesis.

In addition to identifying multipotent progenitors, studies using this enrichment strategy provided new insights into the origin and developmental potential of HECs. First, they showed that HECs and arterial vascular endothelial progenitors are found at day 8 in distinct hPSC-derived populations, providing the basis for the hypothesis that they represent separate lineages. This hypothesis is supported by findings in the mouse embryo showing that specification of AGM arterial vascular endothelial cells and HECs requires different levels of Notch signalling, an indication that they arise from different progenitors[94]. Based on these observations, we propose that HECs and vascular endothelial progenitors are specified as distinct lineages from KDR$^+$ mesoderm (FIG. 3a). Second, limiting dilution studies with the HEC-enriched population provided compelling evidence that hPSC-derived HECs are haematopoietic-restricted progenitors that are unable to generate endothelial progeny. The concept that human HECs are haematopoietic restricted is consistent with studies in the mouse embryo that showed that *Runx1*-EGFP$^+$VE-cadherin$^+$TER-119$^-$CD45$^-$CD41$^-$ progenitors prospectively isolated from the AGM region give rise to either haematopoietic or endothelial progeny, but never

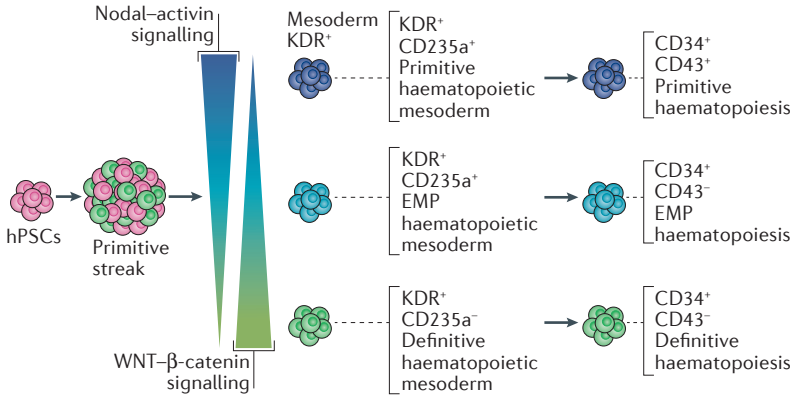

Figure 4 | **A model of human primitive, EMP and definitive haematopoietic mesoderm development.** We propose that the three mesoderm subtypes emerge as distinct populations as they are induced from the primitive streak. Primitive and erythro-myeloid progenitor (EMP) KDR$^+$ mesoderm is distinguished from definitive KDR$^+$ mesoderm by the expression of CD235a. Induction is regulated by the Nodal–activin and WNT–β-catenin signalling pathways.

to both[61]. These observations challenge the concept that HECs are of the endothelial cell lineage and rather support the interpretation that they are haematopoietic progenitors that express endothelial markers. With this interpretation, we propose that the HEC progenitors in the subaortic mesenchyme[20] migrate in close proximity to, and possibly in contact with, the somites, where they experience a first stimulus of Notch signalling[95–98], and then to the AGM region, where they integrate with the arterial endothelial cells in the ventral wall of the dorsal aorta and differentiate to generate the haematopoietic progeny detected as intra-aortic clusters (FIG. 3b).

In addition to providing new insights into the origin and potential of HECs, the hPSC model has been instrumental in mapping the emergence of different human haematopoietic programmes. The finding that both primitive and definitive haematopoiesis could be specified within 72 hours of differentiation through manipulation of the WNT–β-catenin and Nodal–activin pathways strongly suggests that these fates are established early. Evidence in support of this interpretation was provided by the finding that the mesodermal expression of CD235a identifies a subset of KDR$^+$ cells fated to the primitive lineage, distinguishing it from the CD235a$^-$ subpopulation that gives rise to the definitive lineage[85] (FIG. 4). The identification of KDR$^+$CD235a$^+$ primitive and KDR$^+$CD235a$^-$ definitive mesoderm populations suggests that these programmes are specified at the same time. These observations are not consistent with the classical temporal model of haematopoiesis in which primitive haematopoiesis initiates at least several days before the onset of definitive haematopoiesis. Rather, they are in line with lineage-tracing studies indicating that at least a subset of definitive haematopoietic progenitors in the mouse embryo derive from a *Runx1*$^+$ population specified in the extra-embryonic mesoderm by E7.5 (REFS 99,100). Subsequent studies showed that this early *Runx1*$^+$ population can be further subdivided by *Gata1* expression into a *Runx1*$^+$*Gata1*$^+$ progenitor population that displays primitive erythroid potential and a *Runx1*$^+$*Gata1*$^-$ progenitor population that migrates intra-embryonically and eventually gives rise to definitive haemogenic endothelium[101]. Although the interpretation of these studies remains controversial because haematopoietic development in *Runx1* heterozygous mice is accelerated[102], they provide the basis for a model in which the primitive and definitive programmes are specified as distinct mesodermal populations in the early embryo, probably at the stage of induction from the primitive streak (FIG. 4). The concept that human primitive and definitive haematopoiesis are specified early in development is in line with observations in *Xenopus laevis*, which suggested that these programmes develop from distinct blastomeres[62].

The separation of KDR$^+$ mesoderm on the basis of CD235a expression not only enables the distinction of primitive and definitive fates but might also provide an opportunity to identify and characterize the human EMP programme. In addition to CD34$^+$CD43$^+$ primitive progenitors, KDR$^+$CD235a$^+$ mesoderm generated CD34$^+$CD43$^-$ HECs that underwent EHT and gave rise to myeloid lineage cells and erythroid populations that expressed both ε-globin and γ-globin[96]. These HECs did not generate T cells, indicating a potential consistent with that of an EMP programme. Characterization of the Notch and MYB dependency (BOX 2) of these cells will determine whether they indeed represent human EMP haematopoiesis.

Generating HSCs from hPSCs. Although culture conditions have been established for the generation of most types of blood cells from hPSCs, it is not yet possible to derive HSCs. Several studies over the past 10 years have documented limited engraftment and a persistence of human haematopoietic cells in immunocompromised recipients following transplantation of hPSC-derived progenitors. However, in all cases, the levels of engraftment were exceptionally low and/or restricted to the myeloid lineages[103–106], indicating the absence of functional HSCs.

In an effort to overcome challenges associated with directed differentiation *in vitro*, two groups took advantage of the inherent capacity of hPSCs to produce differentiated progeny within teratomas formed *in vivo* as an approach to generate HSCs. They found that CD34$^+$CD45$^+$ teratoma-derived cells could provide multilineage engraftment for up to 12 weeks following transplantation into immunocompromised mice[107,108]. Limited myeloid-biased repopulation was detected in secondary recipients after 4 weeks[107]. Although the duration and levels of engraftment were less than typically used to assay cord-blood-derived HSCs, these findings nevertheless show that transplantable haematopoietic progenitors can be obtained from hPSCs. Whether they represent bona fide HSCs remains to be determined.

Although studies with mouse ES cells have been ongoing for much longer than those with human cells, they have not fared much better at generating HSCs. For many years, the only successful approach for reproducibly generating transplantable haematopoietic progenitors was through enforced expression of transcription factors, most notably homeobox B4 (HOXB4)[109,110]. A recent study, however, has provided

Somites
An embryonic tissue of paraxial mesoderm found in segments along the head-to-tail axis of the developing embryo. Somites ultimately give rise to the vertebrae, rib cage and occipital bone, as well as to cartilage, tendons and muscle.

Teratoma
A tumour that possesses tissue components derived from more than one germ layer.

the first compelling evidence that it is possible to generate engraftable cells from mouse ES cells without genetic manipulation[111]. A key aspect of this work was timing, as KIT$^+$ cells that showed engraftment potential were present in the cultures for only 24 hours. Although these findings are an important step forward and possibly provide a strategy for generating similar progenitors in hPSC differentiation cultures, it is not clear whether these cells are the equivalent of AGM HSCs, as haematopoietic engraftment waned over time and proof of engraftment in secondary recipients was not provided.

Induced HSCs as an alternative strategy

An alternative strategy to the derivation of HSCs from hPSCs is the reprogramming (also known as 'direct conversion' or 'transdifferentiation') of different cell types by enforced expression of transcription factors. This approach is dependent on the identification of appropriate combinations of transcription factors that will induce a switch in fate from the target cell to a HSC. If successful, reprogramming experiments will not only lead to the development of HSCs but could also identify key transcription factors that need to be induced during the directed differentiation of hPSCs. Proof of principle that this strategy can work has been provided by studies that demonstrate the conversion of human vascular endothelial cells to transplantable haematopoietic progenitors through the expression of *FOSB*, *GFI1*, *RUNX1* and *SPI1* (REF. 112) and by the finding that adult mouse pre- and pro-B cells and myeloid progenitors can be reprogrammed to HSCs by expression of *Run1t1*, *Hlf*, *Lmo2*, *Prdm5*, *Pbx1* and *Zfp37* (REF. 113). The reprogrammed cells in the latter study, known as induced HSCs (iHSCs), showed robust multilineage potential in both the primary and secondary recipients. By contrast, the reprogrammed human endothelial cells displayed a myeloid bias with little lymphoid potential, indicating that they differ from cord blood HSCs that show efficient B lymphoid engraftment in the NSG transplant model. The reprogramming strategy has been applied to hPSC-derived cells by expressing *ERG*, *HOXA9*, *MYB*, *RORA* and *SOX4* in primitive and/or EMP erythro-myeloid haematopoietic populations[114]. The resulting reprogrammed progenitors showed no long-term HSC potential but did display transient erythro-myeloid engraftment.

Collectively, these studies highlight both the strengths and the challenges of the reprogramming approach. Cells with engraftment potential can be generated, but it remains to be seen whether they have the same differentiation potential as those generated through normal developmental pathways. Many of the transcription factors used in these direct conversion studies are likely to play a part during HSC specification and development and therefore need to be activated during hPSC differentiation in order to generate HSCs. It is possible, however, that they will need to be expressed in specific target populations in a sequential manner that reflects normal developmental patterns in order to achieve this goal. Future experiments using strategies that enable stage-specific, controlled expression of transcription factors in appropriate hPSC-derived populations will be required to address these issues.

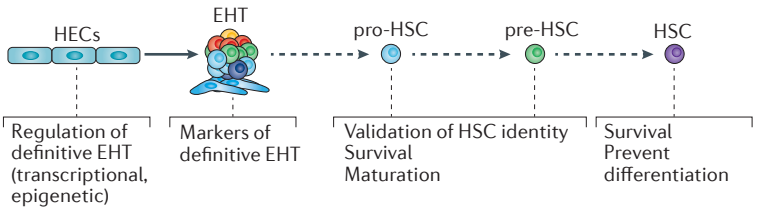

Figure 5 | **A model of human HSC specification from HECs.** The model highlights stages of differentiation from haemogenic endothelial cells (HECs) to haematopoietic stem cells (HSCs). Key challenges that need to be addressed for the successful generation of HSCs *in vitro* are indicated. Signalling pathways that regulate the development of HECs and their subsequent endothelial-to-haematopoietic transition (EHT) need to be identified. In addition to defining the key signalling pathways, understanding the epigenetic remodelling and transcriptional changes during this stage will provide new insights into the earliest molecular events that regulate HSC development. Given that HECs are associated with most stages of embryonic haematopoiesis, the identification of markers that specifically identify those stages of the definitive programme will be important. Through the EHT, HECs generate pro-HSCs and pre-HSCs as well as committed haematopoietic progenitors. Identification of markers that distinguish these haematopoietic populations will allow the development and maturation of the pro- and pre-HSCs to be monitored. As pro- and pre-HSCs differentiate, they will give rise to HSCs. Understanding the regulatory pathways that control this process will be essential to generate functional HSCs *in vitro*. Finally, it will be important to define conditions to promote survival of HSCs once they are specified from the pro- and pre-HSC progenitors.

Conclusions and future challenges

Despite years of effort, generating HSCs *de novo* remains one of the major challenges of modelling human haematopoietic development with hPSCs. Many of the failures to date have probably resulted from the use of primitive and/or EMP-derived populations for transplantation, as most were not generated with protocols that selectively promote definitive haematopoiesis. The ability to produce populations enriched in definitive HECs should overcome this problem and enable us to focus on the progenitors with the potential to give rise to HSCs.

Success in generating HSCs will depend on our ability to accurately model the steps that lead to their development from HECs (FIG. 5). Currently, little is known about the regulation of HEC development and the process of EHT that gives rise to the HSC in embryos. Expression analyses have provided insights into the regulatory networks that control the sequential stages of HSC maturation[115]; recent studies have shown dramatic changes in the transcriptional programme of HECs during EHT[116] and have provided evidence for epigenetic remodelling during this process[117]. However, it is not clear which events regulate HSC specification, as relatively few repopulating cells are detected among the haematopoietic progeny generated in the AGM[118]. The identification and validation of markers (such as G protein-coupled receptor 56 (GPR56)[119]) that distinguish the emerging HSCs from the other HEC-derived progenitors would provide a strategy for defining the pathways that regulate HSC development.

In addition to understanding the molecular events that guide HSC development, future studies will require precise stage-specific activation as well as inhibition of

combinations of signalling pathways to more accurately mimic the events *in vivo*. For example, recent studies in the mouse embryo have shown that sustained BMP signalling is detrimental for HSC specification in the dorsal aorta region[120]. This is an important observation, as BMP is used routinely in the hPSC differentiation cultures to induce mesoderm and, to date, little attention has been given to specific inhibition of the pathway at appropriate stages of haematopoietic development. It is well established that Notch signalling is essential for definitive haematopoietic development. However, in this case, the strength of the signal is crucial for specifying HECs[94]. Achieving appropriate levels of Notch signalling at the correct stage will be challenging, as regulating the activity of this pathway is difficult. Seemingly contradictory findings on the role of WNT signalling in HSC development suggest that tight temporal regulation of this pathway is required to specify this fate. Manipulation of the pathway in AGM explant cultures and the zebrafish embryo revealed that active signalling is required for HSC generation[121,122]. By contrast, *ex vivo* retinoic acid-mediated inhibition of WNT–β-catenin signalling in isolated E10.5 $CD93^{+}VE\text{-}cadherin^{+}$ HECs and pre-HSCs was shown to facilitate HSC development[123]. The differences in these outcomes can be explained by manipulation of the pathway at slightly different developmental stages.

Once specified, HSCs might differentiate immediately given the high doses of haematopoietic cytokines present in the cultures. To overcome this, optimization of conditions to promote HSC survival with appropriate factors and small molecules will be important[122,124–129]. If human haematopoietic development recapitulates that in the mouse, then pro- and pre-HSCs[25,26] will be the first definitive progenitors to emerge. As these progenitors will probably show poor or no potential to engraft adults, assays that can measure human pre-HSCs need to be developed. In addition, conditions that promote the sequential maturation of pro-HSCs to pre-HSCs and finally to HSCs *in vitro* need to be identified.

When these challenges are met, we will ultimately succeed in generating hPSC-derived HSCs, reaching an important milestone in human developmental haematopoiesis. This success will have a profound impact on the field, as it will provide an unlimited source of patient-specific HSCs for therapeutic applications, including transplantation for the treatment of various haematological disorders, the generation of blood cell products such as red blood cells and platelets for transfusion, and the production of tumour-specific T cells for immunotherapy. Finally, the ability to generate HSCs will provide a platform for modelling haematological diseases within the definitive haematopoietic lineages, which is the true origin for most of these diseases.

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Competing interests statement
The authors declare no competing interests.