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Origin of the hematopoietic system in the human embryo

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Abstract

The continuous generation of blood cells throughout life relies on the existence of hematopoietic stem cells (HSC) generated during embryogenesis. Given the importance of HSC transplantation in cell-based therapeutic approaches, considerable efforts have been made towards understanding the developmental origins of embryonic HSC. Adult-type HSC are first generated in the aorta-gonad-mesonephros (AGM) region between days 27 and 40 of human embryonic development, but an elusive blood forming potential is present earlier in the underlying splanchnopleura. It is relatively well accepted that the HSC emerge in the AGM through a hemogenic endothelium, but the direct precursor of this cell type remains to be clearly identified. This review is intended to summarize the recent advances made to understand the origins of hematopoietic stem cells in the early human embryo. In addition, we discuss in detail the discovery of the angiotensin-converting enzyme (ACE) as a novel marker of human HSC and of pre-hematopoietic precursors inside the embryo.

Introduction

Hematopoietic stem cells (HSC) are at the foundation of the adult hematopoietic system. These rare cells are characterized by the dual ability to self-renew, thereby maintaining their numbers, and to differentiate into all the lineages of the blood and immune system. HSC reside in the bone marrow (BM) in adult mammals, and produce immature progenitors that gradually and progressively, become restricted in lineage differentiation potential. These progenitors, through a series of proliferation and differentiation events, yield mature blood cells, including erythroid cells, megakaryocytes, myeloid cells, and lymphocytes. The tight regulation of HSC self-renewal and differentiation ensures the appropriate balance of blood cell production throughout life. The hematopoietic system is a prime example of successful applied regenerative medicine. Bone marrow transplantation, and more recently cord blood transplantation have become a routine treatment for blood disorders and malignant diseases.

However, the insufficient number of immune-compatible donors as well as transplant engraftment failure, graft-versus-host disease, and delayed reconstitution still remain a major constraint in clinical applications. The *ex-vivo* expansion of HSC represents one potential source of additional transplantable cells. However, even the most robust current protocols achieve only a modest expansion (or even maintenance) of long-term repopulating HSC. Recently, the possibility to activate primitive pluripotent genes within adult human cells that take them back in time to a pluripotent state (called induced pluripotent stem cells, iPS) provided a major breakthrough for the field of regenerative medicine. Thus, in parallel with the efforts to expand HSC, many studies have aimed to generate HSC from alternative sources. Although the generation of specific types of hematopoietic cells for specific applications is now becoming feasible, generating robust numbers of *bona fide* HSC that are capable of long-term self-renewal and with normal lineage potential so far remains impossible [1, 2]. 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.

In this review we present the latest advancements in the understanding of the early blood cell development in the human embryo. We focus on the still controversial cellular origin of

definitive HSC and point out the analysis of the expression of ACE, a novel marker of HSC, we have shown to identify pre-hematopoietic cells inside the early human embryo.

Establishment of the blood system during human embryonic ontogeny: an overview

Anatomic locations of blood emergence

The hematopoietic system is one of the first complex tissues to form in the incipient embryo, and in contrast to most other organs, different cell types emerge from some discrete anatomical niches that change temporally and spatially before eventual stabilization in the bone marrow and thymus. The transient generation of different embryonic cell types before the establishment of HSC was at the base of decades-long debates on how exactly this tissue is generated. It is now well accepted that in humans and other Mammals likewise, the ontogeny of hematopoietic system is characterized by two waves: *primitive hematopoiesis*, which generates transitory hematopoietic cell populations, and *permanent, definitive hematopoiesis*, which arises later in development from definitive multipotent hematopoietic stem cells.

Extraembryonic hematopoiesis: The yolk sac

The first hematopoietic emergence, a process named *primitive hematopoiesis*, starts during the third week of human development, before the establishment of the blood flow, in an extra-embryonic tissue - the yolk sac (YS) - which is composed of visceral endoderm and mesoderm [3]

In higher vertebrates, mesodermal cells of the YS form cell-aggregates, which are simultaneously at the origin of the vascular system and extra-embryonic hematopoiesis: the peripheral cells acquire endothelial markers and central cells disappear to create the lumen of the first blood vessels. Some mesodermal cells remain attached to the newly formed vascular endothelium, and constitute the blood islands at the origin of extra-embryonic hematopoiesis [4-6]. This overlapping ontogenic appearing of both hematopoietic and endothelial cells and the co-expression of several molecular makers suggested a common mesodermal precursor, *the hemangioblast* at the origin of both lineages (discussed later).

Primordial mesodermal aggregates appear in human YS at about 16-days of gestation [7] and blood island formation, observed from day 19 [3, 8], produces essentially short-lived primitive erythrocytes [9], as well as macrophages and primitive megakaryocytes [10]. Clonogenic pluripotent and committed progenitors are functionally identified in the human YS at 4-5-weeks of development [11-13]. However, all assays were carried out at stages when blood already circulates between the YS and embryo, and the presence in the YS of precursor cells of extrinsic origin could not be ruled out. Separation of the yolk sac and embryo at the 19-day stage - before the onset of the blood stream [14] - shows that the pre-circulation yolk sac is limited to the erythromyeloid lineage and lacks lympho-hematopoietic stem cell potential [15]. These findings, confirmed by similar results obtained in the mouse [16-18], suggest that adult mammalian hematopoiesis has an intraembryonic origin, in agreement with earlier observations made in avian embryos [19].

Intra-embryonic hematopoiesis: The AGM

A second wave of hematopoiesis, named *definitive hematopoiesis*, takes place inside the embryo proper and originates later during development in vertebrates.

The first evidence for the existence of an intrinsic blood cell activity inside the human embryo came from *in vitro* colony assay performed with hematopoietic CD34 expressing cells – known to identify human immature hematopoietic progenitors [20] - purified from either YS or intraembryonic tissues [13]. Whereas erythroid progenitors were equally distributed between the YS and the embryo, non-erythroid progenitors were predominantly found in the embryo after the liver was removed, indicating that intraembryonic hematopoietic activity was not conferred by the hepatic rudiment.

The presence inside the intraembryonic compartment of progenitors presenting a HSC phenotype was corroborated by our group who identified a dense population of CD34⁺ blood cells in the aorta-gonad-mesonephros (AGM) region, in association with the ventral side of the aortic endothelium and vitelline artery [3, 21] This cell population displays a cell-surface and molecular phenotype typifying primitive hematopoietic progenitors (CD34⁺, CD45⁺, Ve-Cad⁺, CD43⁺, CD38⁻, negative for lineage markers) and also express proto-oncogenes and transcription factors (Gata-2⁺, Gata-3⁺, c-myb⁺, SCL/TAL1⁺, c-kit⁺, flk-1/KDR⁺) known to regulate the initial stages of blood cell development [3, 21-23].

The crucial role of the AGM in the establishment of definitive hematopoietic cells implies that this organ has the *autonomous* capacity to generate hematopoietic progenitors. The intrinsic origin of these blood progenitors was confirmed by the development of a three-dimensional culture that allowed the development of organs *in vitro*, in the absence of the rest of the embryo [24]. Investigations performed at earlier developmental stages, when blood does not yet circulate between the YS and the embryo, showed that an elusive blood forming potential is present earlier in the embryo, in the splanchnopleura (P-Sp) - the presumptive region of the aorta - confirming that blood cell progenitors are autonomously generated in the embryo [15]. Moreover, P-Sp-derived blood cell progenitors are multi-lineage whereas the YS only generates progenitor cells with limited, erythromyeloid potential [15]. Therefore, the first multipotent, myelolymphoid stem cells are generated in the splanchnopleura, within the embryo proper. These stem cells, or their undifferentiated progeny, are retrieved within trunk arteries during the fifth week of development, at the origin of definitive hematopoiesis [15]. Even though a recent study suggested the presence of B-lymphoid ability inside the YS in mice [25], it is generally accepted that in the human embryo, adult-type hematopoietic progenitors that also exhibit B- and T-lymphoid potentials are generated only in the AGM region [14]. Recent *in vivo* transplantation experiments have further corroborated these findings, confirming that the AGM region, and more specifically the dorsal aorta, is the first generator of highly potent HSC in the human [26]. These HSC provided long-term multilineage hematopoietic repopulation when transplanted into immunodeficient mice, with retransplantation ability, indicating their self-renewal capacity. Furthermore, it was shown that the rapid expansion of HSC from the early human embryo is related to their strong replication, which occurs every 3.5-4 weeks (while this takes place every 40 weeks on average in the human adult [27]). This high regenerative capacity of every HSC from the AGM, demonstrates that they are at the origin of the complex hematopoietic system found in the adult human.

Recent reports also describe the placenta as a site of early embryonic hematopoiesis outside the AGM [28-30]. These intriguing observations would further document the opportunistic, adaptable nature of incipient hematopoiesis, sustained in multiple, embryologically distinct cellular environments. However, the *de-novo* HSC generation in this organ has not been experimentally

proven, and the placenta still remains a transient hematopoietic organ with a unique function as a specialized “niche” for supporting development of exogenous HSC.

Spatial restriction of HSC emergence: the hematopoietic environment

During embryogenesis, the first hematopoietic cells appear as emergent clusters closely attached to the ventral endothelial floor of the dorsal aorta in the AGM region, a process well conserved in other vertebrate species [21, 31, 32]. In the human embryo, hematopoietic cell progenitors are first detected at 27 days of gestation as small groups of 2-3 cells, then rapidly proliferate to form clusters of many thousands of cells and extend to vitelline arteries [21]. The restricted appearance of these aggregates to the ventral aspect of these truncal arteries indicated the existence of a specific and localized microenvironment controlling hematopoietic cell initiation [21, 32-35]. These observations suggest that the ventral aortic mesenchyme provides the microenvironmental niche that regulates the development of definitive hematopoietic stem cells.

A number of circumstantial observations have been already made in this respect in human embryo such as the presence of an accumulation of smooth muscle actin-positive cells and the high expression of tenascin-C indicating the presence of local changes in the mesoderm underneath the clusters [3, 34]. In line with that, the implication of the ventral domain of the aorta to the hematopoietic emergence in the AGM is also suggested by the expression of a number of key transcription factors and molecules known to be involved in hematopoietic development during embryogenesis in many vertebrate species (reviewed in [36]).

One of them is BMP4, a member of the bone morphogenic protein family, known to play an important role in the development of the blood cell lineages. In the human AGM region, BMP-4 expression follows a gradient across the dorsoventral axis with a high polarization to the ventral wall of the dorsal aorta, more specifically in the mesenchyme underlying the intra-aortic clusters [37]. In agreement with these findings, functional studies in zebrafish and mouse embryo demonstrated that the ventral expression of BMP4 is crucial in the induction of hematopoiesis in the AGM region [38, 39]. Subsequently, a recent study in mouse embryo, has reported that downregulation of BMP4 is required for HSC generation and that the decrease of BMP4 activity is achieved by the overexpression of a number of BMP antagonists (Noggin, Chordin and Chordin-like2) as well as inhibitors (Smad6 and Smad7) in the ventral domain of the dorsal aorta (at E10.5-E11.5) [40]. These authors suggest that maturing HSC in intraaortic clusters are exposed to a low concentration of BMP4, which, by contrast, required for HSC development at earlier stages as previously described in zebrafish embryo [39].

In the mouse embryo, colony forming progenitors have also been detected in the aortic roof but only floor explants support the expansion of definitive HSC [41]. Accordingly, sub-dissections of the human aorta have further documented the presence of a strong dorsoventral polarization of hematopoietic activity in the human AGM, confirming that the ventral site contains significantly more hematopoietic cells than the dorsal side. Single cell suspensions prepared from the ventral domain of the aorta showed long-term, multilineage hematopoietic engraftment when injected into sublethally irradiated adult immunodeficient recipient mice. By contrast, no hematopoietic engraftment was detected from the dorsal side. Thereafter, these cells were identified as the first HSC by the expression of CD34, VE-Cadherin, CD45, c-kit, THY-1, Endoglin, Runx1, a low expression of CD38 and the absence of CD45RA expression [42].

One explanation for the polarization of HSC emergence inside the AGM has been offered by quail-chick grafting experiments showing that dorsal and ventral endothelial cells in the aorta have distinct origins in the somitic and splanchnopleural mesoderms, respectively [43]. Gradual replacement of the ventral hemogenic endothelium with non-hemogenic endothelial cells derived from the somites would eventually interrupt HSC production in the AGM [44].

The transcription factor Gata-3 is also highly upregulated in the AGM region, specifically localized in subendothelial cells underneath the lateral and ventral aspect of the dorsal aorta, at the time of intraaortic cluster emergence [45, 46]. Interestingly, HSC production and specification are impaired in absence of Gata-3 and that has been associated with the role of this transcription factor in the production of catecholamines, the mediators of the sympathetic nervous system [47]. These findings demonstrate that the sympathetic nervous system plays a critical role in the homeostasis of AGM hematopoiesis as was shown for the adult marrow [48, 49]. Gata-3 expression has been also described in the human AGM underneath the aortic clusters implying that the sympathetic nervous system could participate in human HSC development through a conserved mechanism [22].

A very recent study has documented an accumulation of primitive macrophages in the ventral domain of the AGM region of both human and zebrafish embryos [50]. Macrophages play a major role in the establishment of definitive hematopoiesis permitting the mobilization of HSC and the colonization of hematopoietic organs, via metalloproteases (Mmps) secretion and extracellular matrix remodeling. Beyond the mere confirmation that the mesoderm surrounding the aorta secretes molecules, and contains factors and cells involved in the hematopoietic emergence, these intriguing observations show that YS-derived blood cells affect the production of the subsequently emerging definitive blood cells, highlighting their possible collaborative role, not yet described in vertebrate blood system development.

Cellular origin of hematopoietic stem cells

The cellular and molecular processes implicated in the initial generation of hematopoietic stem cells in the vertebrates have been a matter of intense debate that remains, to date, not fully resolved (Figure 1). The close association between vascular endothelium and hematopoietic cells during ontogeny, both in the YS and AGM, set the base of a possible direct ontogenetic affiliation between these two cell lineages.

The hemangioblast

In the last century, anatomic studies suggested the physical association of blood forming cells with endothelial-lining in developing vascular structures across several species. In 1917, the pioneering studies of Sabin in the chick embryo observed blood cells that bud off from vascular endothelial cells into plasma circulating through the primitive vascular plexus of the developing embryo [51]. These mesodermal clusters that give rise to hematopoietic cells and endothelial cells were defined as ancestral precursor cells, common to both endothelial and hematopoietic lineages, initially called “*angioblasts*” [6] and later renamed “*hemangioblasts*” [52].

It is now commonly accepted that hematopoietic cell generation involves an intermediate and transient bipotent precursor cell giving rise to both endothelial cells and hematopoietic cells within YS blood islands. Recent supporting evidence for a lineage relationship includes the observation that the two cell types share a number of surface markers, such as Flk-1 - a receptor for

vascular endothelial growth factor (VEGF) [53], CD34 (mucosialin) [21, 35], VE-cadherin [54] and CD31 (PECAM) [21]. For instance, null mutations of the Flk-1 receptor result in the loss of both endothelial and blood cell lineages [55]. Runx1, a transcription factor critical to hematopoietic programs [56] is also expressed in endothelium, as is Gata-2 [57]. Although Runx1 and Gata-2 are both required for the generation of HSC, it has been recently shown that the developmental requirements for these transcription factors differ during HSC emergence and Gata-2 is further required for HSC survival [58, 59].

An *in vitro* model of murine YS hematopoiesis was first used to identify the hemangioblast as a blast colony forming cell (BL-CFC), derived from mouse embryonic stem (mES) cells, which generates colonies containing both endothelial cells and hematopoietic cells [60]. Equivalent cells have been identified *in vivo* within the primitive streak of gastrulating mouse embryos by co-expression of Brachyury and Flk-1 [61]. Hematopoietic differentiation has also been modeled from human embryonic stem cells (hESC) (reviewed in [62]). Zambidis et al. first developed a hESC model that recapitulates the human YS stages of hemato-endothelial development, following kinetics which predict the emergence of a hemangioblastic progenitor. Hematopoietic activity arises in human embryoid bodies (hEBs) from CD45-negative mesodermal hemato-endothelial precursors that give rise to hemato-endothelial colonies with the potential to generate primitive and definitive erythro-myelopoiesis [63]. These hemato-endothelial precursors injected into the yolk sac mesoderm of an avian embryo participate in the formation of vitelline vessels and hematopoiesis [64]. Recently, the same group have described a robust system in which human induced pluripotent stem cells have a hemato-endothelial capacity and can differentiate simultaneously into endothelial and hematopoietic lineages in a manner comparable to hESC [65].

The hemogenic endothelium

Despite the debate about the existence of the hemangioblast, it seems that this concept is only appropriate to the primitive hematopoiesis in the yolk sac. There is now strong evidence that the definitive hematopoiesis originates directly from a specialized vascular endothelium called “*hemogenic endothelium*” within the AGM. The first experimental indication for the existence of this transient endothelium came from chick embryos, in which endothelial cells were marked prior to the emergence of HSC [66]. As the marker was subsequently detected in hematopoietic cells, it was concluded that these were the progeny of the originally labelled endothelial cells. Similar results were obtained in mice embryos, using the same tracing technique [67]. In agreement, mouse embryo-derived VE-Cad⁺CD45⁻ endothelial cells can generate myeloid and lymphoid cells *in vitro* [68], and give rise to HSC that can colonize embryonic hematopoietic sites *in vivo* [69].

In the human embryo, CD34⁺CD45⁻ endothelial cells, sorted from AGM (between 27 and 40 days) and cultured in the presence of a stromal layer that support human multilineage hematopoiesis, differentiate into hematopoietic cells [70]. The frequency of hemogenic endothelial cells in the AGM was directly correlated with hematopoietic activity *in situ*, culminating at about 1/100 at day 27, when HSC emerge in the floor of the aorta. This study supports the concept of hemogenic endothelium in human intra-embryonic arteries at the origin of the embryonic hematopoietic system [70].

Mouse ES (mES) cells provided further evidence for the clonal origin of hematopoietic cells from hemogenic endothelial cells *in vitro* [71, 72] and for the generation of HSC from hemogenic endothelium *in vivo* [69, 73].

A functioning hemogenic endothelium has been also recently visualized by live microscopy in zebrafish embryos [74-76]. In this study, by an *in vivo* imaging, Kissa and colleagues captured aortic endothelial cells that round up and delaminate from the aortic wall of the zebrafish embryo, undergoing what was defined as an endothelial-hematopoietic transition (EHT) [75]. A similar conclusion was also reached by another study from live imaging of cultured organotypic slices of the mouse AGM region [77].

Recent studies have functionally demonstrated that hemogenic endothelial cells are the precursors of hematopoietic cells derived from human pluripotent stem cells culture [78-82]. However, the molecular identity of hemogenic and non-hemogenic endothelial cells, and the mechanisms leading to hematopoietic commitment of hemato-endothelial cells remain unclear. Interesting observations in this context have been reported by investigating the role of Notch signaling, already known to be essential for definitive hematopoiesis in many vertebrate models [83-85]. Hemogenic endothelial cells have been characterized as a *Delta like ligand 4*-positive (a Notch ligand) cell population that undergoes a Notch-dependent endothelial-to-hematopoietic transition to generate the definitive hematopoietic progenitors [78]. Recently, Sox17 was shown to play a key role in priming hemogenic potential in endothelial cells in a model of human iPS. Depletion of Sox17 in these cells severely impaired their hemogenic activity [86]. Subsequently, analyses in human and murine hemogenic endothelium show that a defined Runx1/Sox17 ratio expression can predict the endothelial-hematopoietic transition stages [87].

This set of data marked a significant improvement in the functional and morphological, characterization of hemogenic endothelial cells as exclusive multilineage HSC precursors during definitive hematopoiesis. However, further investigations are needed to determine the mechanisms underlying hemogenic specification from non-blood forming endothelium and the molecular events that lead to the generation of HSC.

Mesodermal pre-hematopoietic precursors

Concepts of the hemangioblast and hemogenic endothelium at the origin of HSC have both received experimental confirmations. However, a recent report has reconciled these two pathways suggesting that they are not necessarily mutually exclusive but, rather represent sequential steps of the same developmental pathway (Figure 1). Based on a model of mES differentiation, this study shows that the hemangioblast generates the hemogenic endothelium which then produces hematopoietic cells [72]. In agreement with these observations, there is also evidence for a mesodermal origin of HSC *in vivo*. Indeed, it has been shown that patches of cells located in the subaortic ventral mesodermal of the mouse embryo (sub-aortic patches, SAPs), are involved in HSC generation from non-endothelial precursors [45] and have repopulation activity in immunodeficient recipients [46]. Furthermore, Runx1, a transcription factor required for the emergence of HSC, is expressed in the aortic endothelium as well as in ventral sub-aortic mesodermal cells [56].

Interestingly, HSC activity was also detected in sorted mesodermal cells of Runx1^{+/-} mouse AGM, suggesting that Runx1 haploinsufficiency may reveal a pre-endothelial origin of HSC [88].

Subsequently, lineage tracing endothelium, via an inducible VE-cadherin Cre line, confirmed that the

endothelium lining the ventral wall of the aorta was capable of HSC emergence in the mouse AGM and revealed that this hemogenic endothelium was derived from a transient early mesodermal cell population [69].

This concept of a mesodermal pre-hematopoietic precursor has also been demonstrated in human embryo. First, *in situ* hybridization results suggested that Flk-1⁺CD34⁻ cells migrate from the splanchnopleura into the sub-aortic mesoderm during the fourth week of development [8]. According to this model, HSC would be generated within SAPs of the human embryo, migrate toward the aortic floor and reach the blood flow to colonize the fetal liver. This transition across the ventral endothelium of the aorta could justify that the presence of the hemogenic endothelium has been confirmed only at certain stages - between 28 and 40 days - of human development - when HSC are present in the AGM [70]. On this line, subsequent analyses from our group indicate that at earlier stages, the hematopoietic activity in the splanchnopleura region is restricted to CD34⁻ non-endothelial cells [14]. These observations further suggested that the ventral wall of the dorsal aorta is colonized by mesodermal precursors, a hypothesis validated by the discovery that angiotensin-converting enzyme (ACE/CD143) is a marker of these pre-hematopoietic cells [14].

Angiotensin-Converting Enzyme

The expression of ACE/CD143 on blood progenitor cells was initially reported using the monoclonal antibody BB9 by the group of Paul Simmons [89]. BB9 was described to react to adult human bone marrow stromal cells, and with a subpopulation of CD34⁺ cells having phenotypic characteristics of immature progenitors. Subsequent transplantations into NOD/SCID mice demonstrated that human umbilical cord blood-derived CD34⁺ACE⁺ cells, but not CD34⁺ cells lacking the expression of ACE, sustained multilineage hematopoietic cell engraftment, a capacity defining *bona fide* human HSC [90].

ACE, a marker of HSC in all developing blood-forming tissues

In the attempt to track the earliest forerunners of intraembryonic blood forming activity, we have recently examined ACE expression in earlier human embryos. We reported that during ontogenesis, ACE is expressed in all blood forming tissues in the human embryo, namely the yolk sac, the para- splanchnopleura, the aorta, the fetal liver and the bone marrow (Figure 2) [14, 90]. In all sites, ACE⁺ cells are always associated with the developing endothelium, suggesting again direct ontogenic affiliation existing between these two cell lineages.

Indeed, at 4-5 weeks of development ACE identifies into the human AGM the CD34⁺CD45⁺ hematopoietic progenitor cell clusters emerging in the ventral side of the dorsal aorta, as well as the surrounding endothelial cells [14, 90]. Along with this line, analyses carried out throughout the developing embryo showed that ACE⁺ cells were detected in both the liver and BM at stages of hematopoiesis incipience. The results of long-term cultures and transplantations into NOD/SCID mice demonstrated that hepatic and medullary CD34⁺ cell progenitors expressing ACE, are endowed with long-term culture-initiating cell potential and sustain multilineage hematopoietic cell engraftment [14].

ACE, a marker of pre-hematopoietic precursors

Yet analysis conducted at earlier stages of human development have shown that ACE expression characterizes a subset of mesodermal CD34⁻CD45⁻ cells scattered in the hemogenic portion of the human P-Sp [14]. Indeed, CD34⁻CD45⁻ACE⁺ cells sorted from the P-Sp between 24 and 26 days of gestation, when HSC clusters are not yet developed within the vascular aortic wall, generate *in vitro* colonies of CD45⁺ hematopoietic cells at a frequency more than 40 times higher than that observed when plating ACE⁻ cells [14]. All these data strongly support previous observations [69] indicating that definitive hematopoiesis inside the embryo arises from transient mesodermal cell population and identify ACE as the first marker of pre-hematopoietic mesodermal cells inside the human embryo [14]. According with these observations, a study performed on hESC has shown that isolated ACE⁺ cells were highly enriched in BL-CFCs, known to represent bipotential hemato-endothelial progenitors (hemangioblasts) [91]. hESC-derived ACE⁺CD45⁻CD34^{+/-} cells are common yolk sac like progenitors not only for endothelium, but also for primitive and definitive human hematopoietic progenitors, suggesting ACE as a marker for the hemangioblast [64, 91].

The expression profile of ACE-positive cells inside the human AGM is depicted in Figure 3. Indeed, some of ACE⁺ cells co-express the transcription factor Runx1 (M.T., personal observation), which marks long-term repopulating hematopoietic stem cells in the midgestation mouse embryo, as well as mesodermal cells in Runx1^{+/-} mouse AGM endowed with HSC activity, suggesting a pre-endothelial origin of HSC [88]. Moreover, ACE⁺ mesodermal cells also express Oct-4A, a marker of stem cell pluripotency, and the surface antigen SSEA-1 indicating the immaturity of these cells [14]. These ACE⁺Oct-4⁺SSEA-1⁺ cells found within the human P-Sp present a phenotype compatible with that of migrating primordial germ cells. And some of them also express Flk-1, already identified as a marker of hemangioblast-like cells in the mouse model [60]. In correlation with the hematopoietic activity, the CXCR4 protein is present on the surface of ACE⁺ cells, and its chemokine SDF-1/CXCL12 is also detected in the ventral aspect of the AGM region, indicating the migratory capacity of ACE expressing cells into the human embryo. This pattern of expression supports the hypothesis of a hemangioblastic phenotype for ACE-positive cells and provides the first evidence for the presence of hematopoiesis committed pre-HSC inside the subaortic mesodermal layers, before the endothelium acquires hemoogenic capacity.

To further characterize pre-hematopoietic ACE-positive cells, we have recently analyzed the expression of the integrin alpha 6 (CD49f), also known as a HSC marker [92]. The group of K. Moore has recently reported that CD49f is early expressed during the hematopoietic reprogramming of murine embryonic fibroblasts (MEFs) [93]. This study shows that *in vitro* reprogramming of fibroblast cells proceeds through an endothelial-like intermediate. During this process, CD49f is expressed early in precursor cells and its expression is maintained in the emergent hematopoietic and endothelial-like cells. Interestingly, into the CD49f⁺ cell population, the blood-forming potential was entirely confined within the subset of cells which express ACE, further supporting the concept that ACE marks an intermediate state of hematopoietic specification before the appearing of the hemogenic endothelium.

In accord with this observation, our study on human embryo has shown that ACE expressing cells scattered in the mesoderm, ventrally to the dorsal aorta between 28 to 34-days of gestation, co-express CD49f (Figure 4). CD49f is not expressed on the HSC clusters, suggesting that this integrin could be a marker of hematopoietic specification inside the human embryo.

This pattern supports the hypothesis of a ACE⁺CD34⁺CD45⁺CD49f⁺Flk1⁺ mesodermal precursor cell that migrates from the P-Sp and give rise to hematopoietic stem cells emerging in the ventral aspect of the dorsal aorta through an endothelial cell intermediate (Figure 1).

ACE, a component of the Renin Angiotensin System

This enzyme is a key component of Renin-Angiotensin System (RAS) as it catalyzes the second step of the production of angiotensin II (Ang II) from angiotensinogen, well known for its effect in the control of blood pressure, cardiovascular regulation and fluid homeostasis. In addition to the systemic effect of the RAS, evidence for an autocrine or paracrine role has been well documented. The angiotensin peptides may act locally as growth factors or inhibitors and exert effects in cardiac, vascular, renal and other tissues, in a way similar to cytokines (reviewed in [94]). Newly reported data suggest that a local RAS also exists inside the bone marrow, where it exerts a role in the physiological regulation of hematopoiesis, either by a direct effect on hematopoietic stem cells or indirectly by stimulating the release of growth factors and cytokines from stromal cells (reviewed in [95]). Indeed, angiotensin II promotes the proliferation in the culture of bone marrow and cord blood-derived HSC and this proliferative effect is completely abolished by a specific angiotensin–receptor 1 (AT1) antagonist [96]. Inside the bone marrow RAS affects many cellular compartments involved in the physiological or pathological hematopoietic output, including the hematopoietic niches [94], the myelopoiesis [97] and erythropoiesis [98]. During ontogenesis, the RAS system is present in many organs in the human embryo [99], and has been shown to be implicated during primitive erythropoiesis in the avian embryo [100].

In a recent study, we show the presence of the key components of this system in the AGM - the same region of the embryo which expresses ACE - meaning that a local RAS exists in this region and highlighting its potential role in the establishment of definitive hematopoiesis (Julien et al. submitted).

Conclusions and Perspectives

The production of HSC during development is a key step in making a healthy organism, and it involves multiple regulatory interactions during embryogenesis. It is now relatively well accepted that HSC emerge inside the embryo through hemogenic endothelial cells, however the direct origin of these cells still remains a matter of debate. Indeed, accumulating evidence suggests an alternative model to the hemogenic endothelium, a concept implying an endothelial-like intermediate [69, 72]. In accord with such an interpretation, our own findings put forth the concept that ACE likely marks an intermediate state of hematopoietic specification before the appearing of the hemogenic endothelium. This pattern supports the hypothesis of the existence of a ACE⁺CD34⁺CD45⁺ hemangioblastic precursor cell in the subaortic mesoderm, that migrates from the P-Sp toward the ventral aorta, and gives rise, through an intermediate hemogenic endothelial stage, to ACE⁺CD34⁺CD45⁺ hematopoietic progenitors and underlying ACE⁺CD34⁺CD45⁺ endothelial cells.

The expression of ACE has also been recently described in murine fibroblasts transfected with hemogenic inducing transcription factors [93]. ACE expression precedes the appearance of both hematopoietic (CD45) and endothelial (CD34) markers, as we have previously described to occur *in vivo* [14].

Understanding how HSC specification occurs natively during embryonic development goes beyond the mere comprehension of the fundamental ontogeny of the human blood system. Indeed, a clearer understanding of the embryonic signaling and morphogenetic processes which govern HSC

generation in the embryo will also be essential for the production of true HSC *in vitro* from ES cells or induced pluripotent stem cells, and therefore has important translational relevance.

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Figure Legends

Figure 1. Possible Cellular Origins of intraaortic HSC

(A) A hemangioblast gives rise separately to HSC and endothelial cells. (B) A transient hemogenic endothelial cell gives rise to hematopoietic stem cells and endothelial cells. (C) A combination mechanism reconciles A and B, as steps of the same pathway. A hemangioblast (or a mesodermal pre-hematopoietic precursor) gives rise to an intermediate hemogenic endothelium, that gives rise to hematopoietic stem cells and endothelial cells. Alternatively, mesodermal pre-hematopoietic precursor integrates into the endothelial layer and matures into HSC.

Figure 2. Chronology of appearance of ACE-positive cells in the developing human embryo.

The chronology of appearance of ACE (CD143) expression follows the hematopoietic development, and characterizes HSC as well as pre-hematopoietic cells. (A) During the earliest stages of development (19 days) in the yolk sac, ACE marks the endoderm and endothelial cells bordering the newly formed blood vessels, and also identifies rare solid blood islands present in the mesoderm. (B) At the same stage, ACE marks mesodermal pre-hematopoietic cells inside the P-Sp. (C) Later in development, ACE identifies, in the AGM region of the 4 to 5-week human embryo, CD34⁺ hematopoietic progenitors emerging in the ventral side of the dorsal aorta, as well as the surrounding endothelial cells. (D) In the hepatic rudiment, ACE marks endothelial cells but also rare CD45⁺ hematopoietic progenitor cells. (E) At later stages of development, in the fetal bone marrow as well as in the cord blood, ACE identifies *bona fide* HSC. P-Sp, paraaortic splanchnopleura; Ao, aorta; V, vein.

Figure 3. Molecular signature of ACE-positive cells in the AGM compartments.

Expression phenotype of mesodermal, endothelial and intraaortic hematopoietic cells expressing ACE into the human AGM region. *, indicates that only some cells express the marker.

Figure 4. Expression of ACE and CD49f in the human embryo.

(A) A cross section through the AGM region in a 34-day human embryo stained with anti-CD49f (green) and anti-ACE (red) antibodies. (B) Panels on the bottom show a higher magnification of the boxed area in the panels on the top. ACE is expressed in the emerging HSC clusters, in the surrounding endothelial cells as well as in mesodermal cells. All mesodermal ACE-positive cells express the integrin CD49f (white arrowhead), which is also expressed by other ACE-negative cells dispersed in the ventral wall of the aorta (white arrow). Scale bare, 10µm in the top, 100 µm in the bottom. Ao, aorta; Mn, mesonephros; NT, neural tube.

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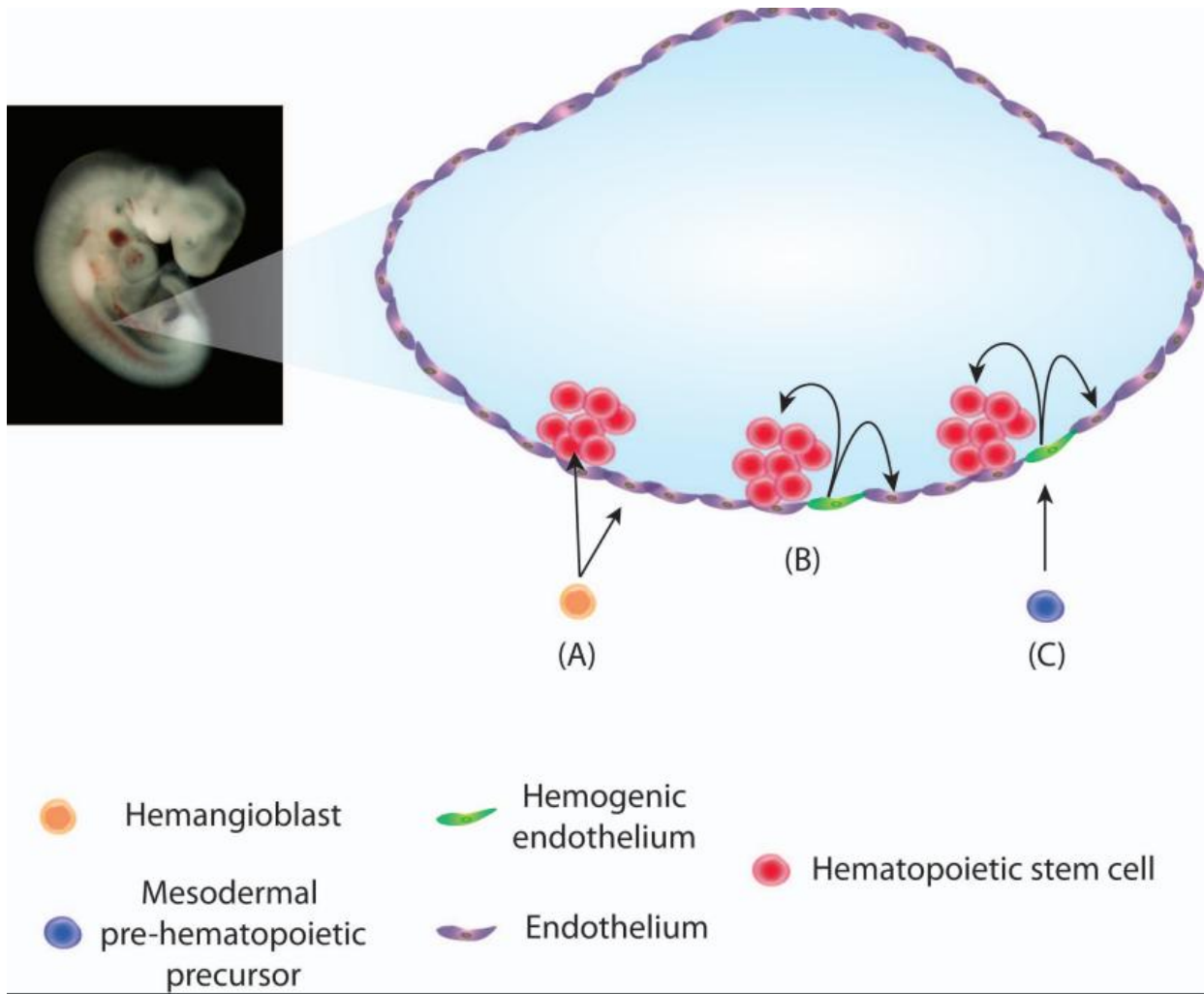


Figure 1

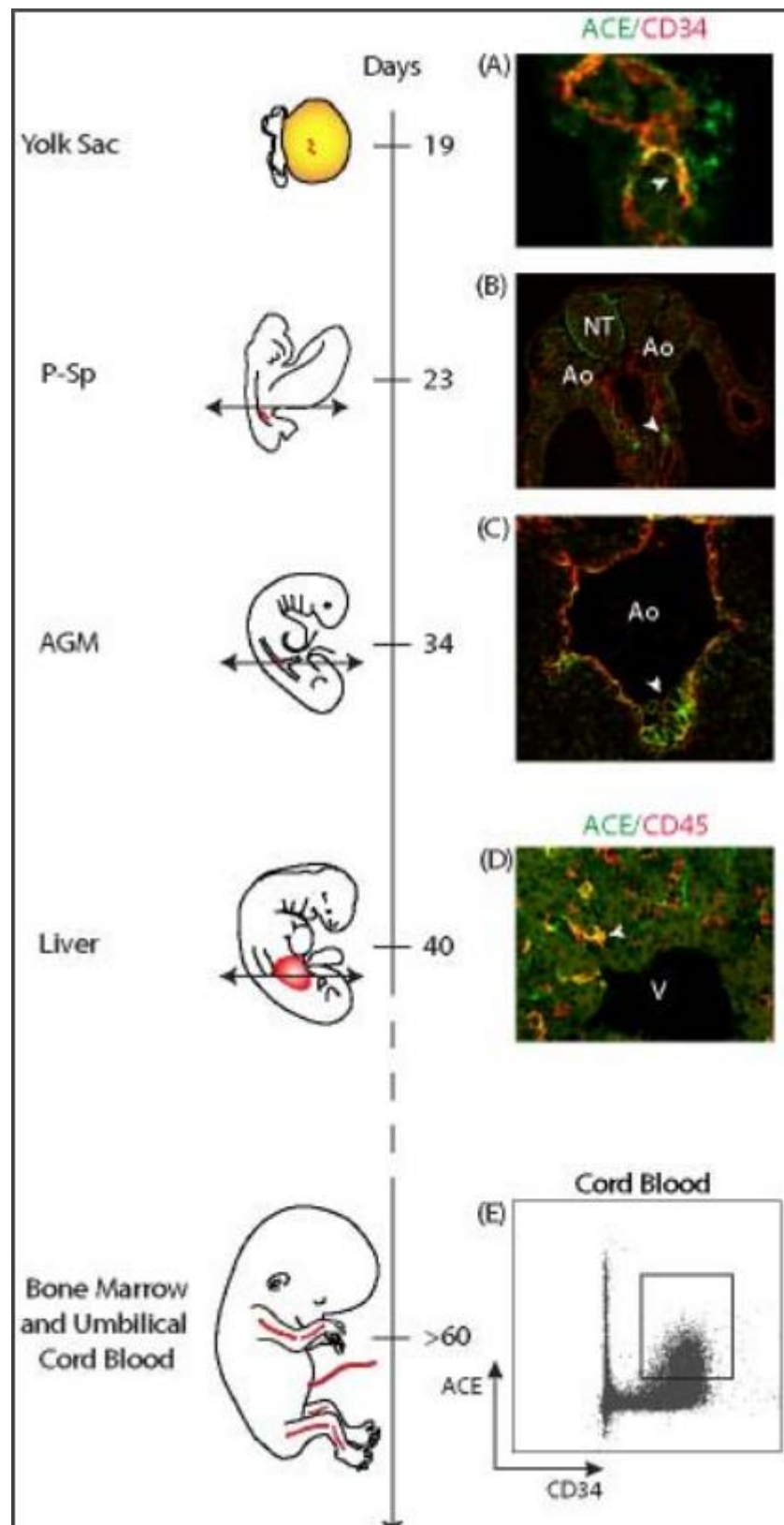


Figure 2

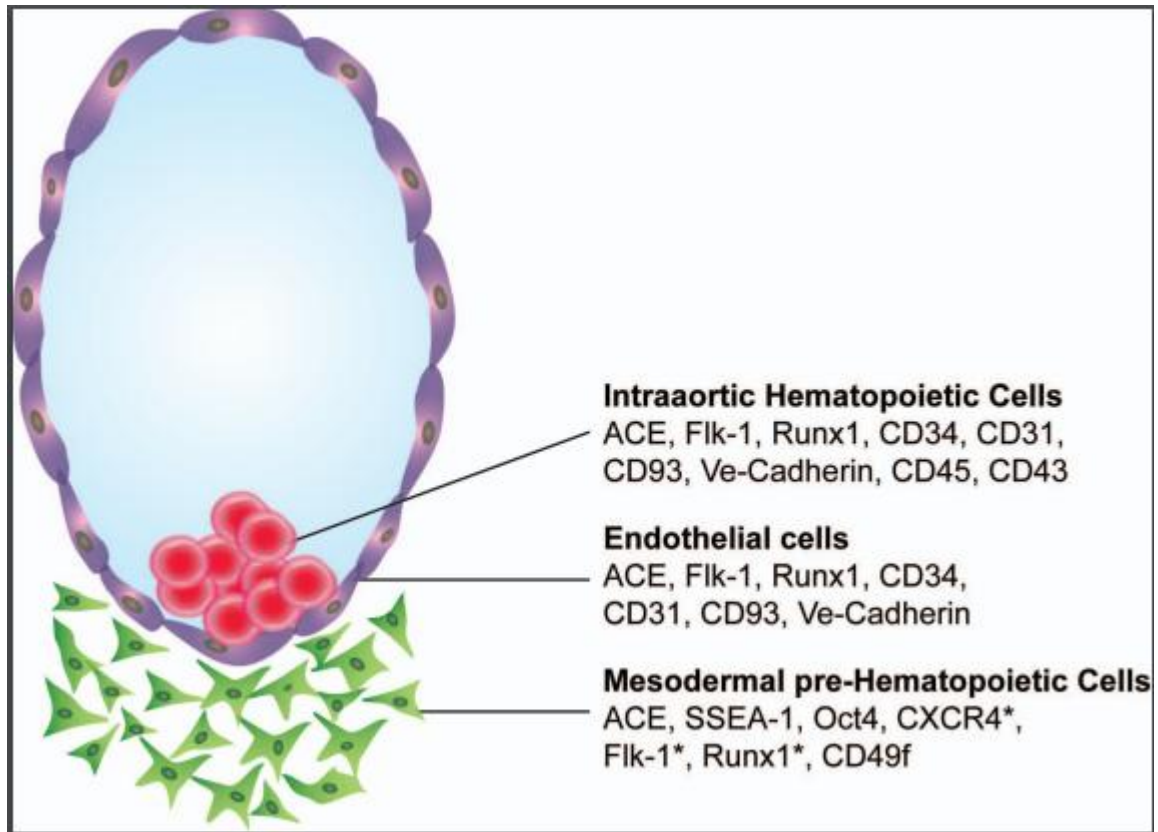


Figure 3

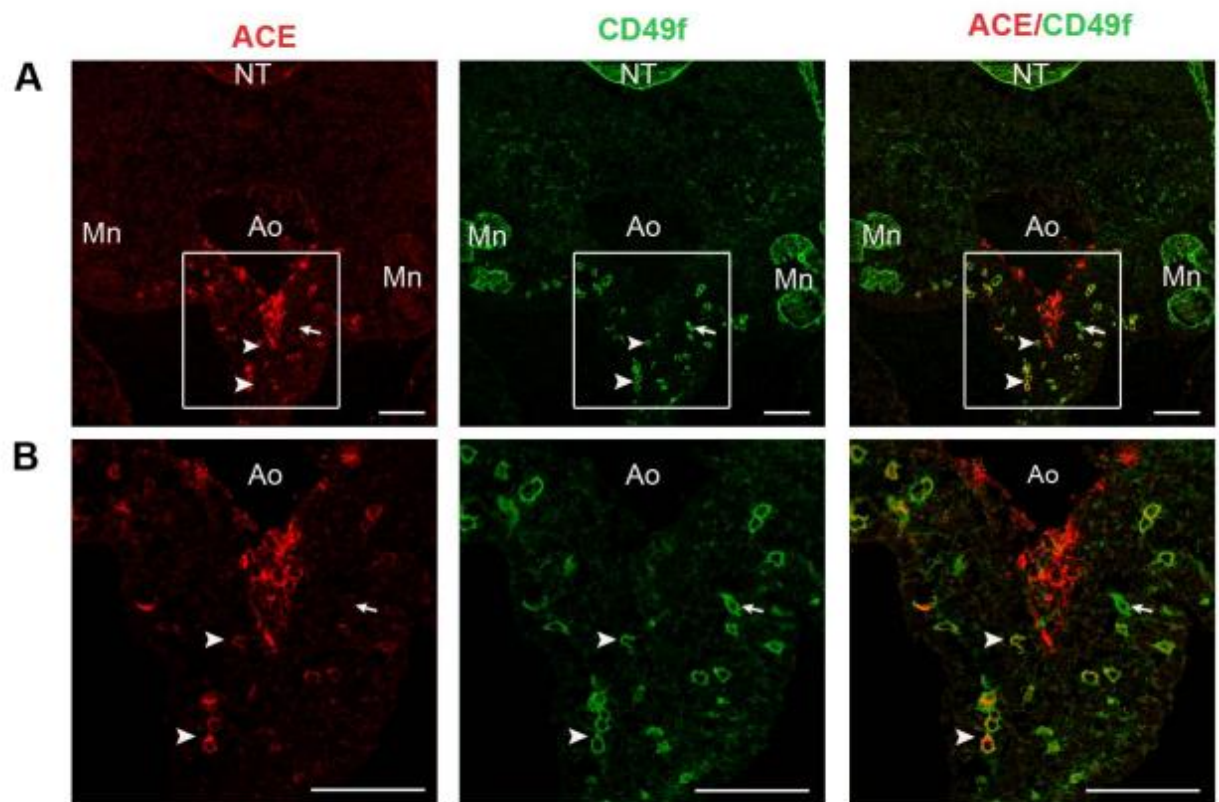


Figure 4

