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Embryonic development of the human hematopoietic system

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ABSTRACT Human hematopoiesis is initiated in the yolk sac during the third week of development. At the same time the capacity to produce blood cells also arises in the embryo, within the splanchnopleura, but this potential is not expressed before day 27, when clustered hematopoietic stem cells emerge from the ventral wall of the aorta and vitelline artery. Budding of hematopoietic cells from vessel walls reflects the re-differentiation of local endothelial cells, which are likely derived from angio-hematopoietic mesodermal ancestors emigrated from the splanchnopleura. Yolk sac-derived stem cells are limited to myelo-erythroid development, whereas those born in the embryo are, in addition, lymphopoietic and therefore represent the first multi-potent, adult-type blood progenitors that appear in human ontogeny, preceding shortly the onset of liver hematopoiesis. These results allowed the establishment of a novel hierarchy of blood-forming tissues in human development and induced an in depth reconsideration of the very origin of definitive human hematopoiesis. These results also fully corroborate the outcome of experiments performed in parallel in avian and mouse embryos and point to the conservation in all higher vertebrates of an ancestral route of blood cell production via embryonic vessel walls.

KEY WORDS: *human embryo, stem cell, hematopoiesis, lymphocyte, endothelium*

Introduction

In adult mammals, hematopoiesis normally occurs in the bone marrow, which supports simultaneously, within distinct cellular environments, the life-long maintenance of stem cells and the regulated production of end-stage lymphoid, myeloid and erythroid cells. Hematopoietic stem cells (HSC) encountered in the adult bone marrow arise by replication and amplification of a stock of HSC that emerged early in ontogenesis, when the bone marrow had not yet formed.

Indeed, a succession of organs sustains blood cell production during the embryogenesis of vertebrates. In vertebrates, the earliest hematopoietic activity is indicated by the appearance of blood islands in the mesoderm of the extraembryonic yolk sac. The yolk sac, which sustains principally primitive erythropoiesis, is later relayed by the liver and spleen, the role of which in blood cell production varies along phylogeny and eventually by the thymus and bone marrow, where post-natal definitive hematopoiesis becomes stabilized. Blood cell tracing along embryonic development was pioneered in parabiotic sex-mismatched chicken embryos (Moore and Owen, 1967) and was later much refined and exploited in the quail-chicken chimera system (reviewed in Le Douarin *et al.,* 1984). These experiments revealed that the development of intra-embryonic blood-forming tissues depends on the colonization of their rudiments by blood-borne hematopoietic progenitor cells. This observation first set the basis for the prevailing idea that the YS be the unique provider of HSC, since no intrinsic hematopoietic potential could be attributed to any other tissue inside the embryo (Moore and Owen, 1967). According to this theory, development of the blood system in vertebrates was long described as a *monophyletic* process with a unique organ of hematopoietic cell emergence – the yolk sac – colonizing other organs: first the liver, then the thymus, spleen and finally the bone marrow (Moore and Metcalf, 1970).

The scarcity of available human tissues at early embryonic stages as well as the very narrow range of assays in which human cells could be analyzed have hampered the study of the emerging human hematopoietic system, which for a long time only reposed on incidental morphologic observations (Rosenberg, 1969). This situation changed at the end of the 1980ies with the advent of a rapidly expanding array of markers for human hematopoietic cells, the development of long-term human HSC cultures and the successful engraftment of human hematopoietic cells in immune-

Abbreviations used in this paper: AGM, embryonic region comprising the aorta, genital ridges andmesonephros; HPP-CFC, high-proliferative potential colony forming cell; HSC, hematopoietic stem cell.

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deficient mice (reviewed in Peault *et al.,* 1992; Peault, 1995; Peault *et al.,* 1992; Vormoor *et al.,* 1995). Such technical improvements permitted to get novel insights into human developmental hematology, including the identification of human HSCs at pre- and post-natal stages (Baum *et al.,* 1992, Charbord *et al.,* 1996a, Kyoizumi *et al.,* 1993, Peault *et al.,* 1991, Uchida *et al.,* 1997). At the same period, access to earlier stages of human gestation turned easier because the anti-progestative compound RU486 became routinely used to electively terminate early pregnancies in France. Our laboratory at the Institut d'Embryologie then launched a program aimed at investigating incipient hematopoiesis in the first two months of human development.

As detailed elsewhere in this volume, the *Institut d'Embryologie du CNRS et du College de France* has made major contributions to developmental hematology and immunology. By creating multiple tissue combinations in quail/chicken chimaeras, N. Le Douarin and her team have deciphered the principle and chronology of HSC seeding to the embryonic thymus, bursa of Fabricius and bone marrow and ascertained the role of thymic epithelium in the induction of self tissue tolerance (reviewed in Le Douarin *et al.,* 1984). One of us participated in these studies by defining molecular markers for avian hematopoietic cells that were used to characterize the ontogeny of blood cell lineages in normal embryos and quail-chicken chimeras (Guillemot *et al.,* 1986, Peault, 1987, Peault *et al.,* 1983). On the other hand, the group led by Françoise Dieterlen in the same Institute has challenged the monophyletic theory of blood cell development by discovering that stem cells emerging inside the embryo proper and not in the yolk sac, are at the origin of definitive hematopoiesis in birds (Dieterlen-Lievre, 1975). Similar conclusions were reached by the same group regarding the origin of HSC in the mouse embryo (Cumano *et al.,* 1996). Both groups at the Institut d'Embryologie have also pioneered the study of vascular development in the avian embryo, making use of original markers of the hemangioblastic cell lineage (Pardanaud *et al.,* 1987, Peault, 1987).

We herein describe how we have, in this modern developmental hematology context, revisited the ontogeny of the human hematopoietic system. Our observations have pointed to the existence of a conserved scheme of hematopoietic stem cell emergence in vertebrate embryos (Tavian *et al.,* 1996). The intra-embryonic site of HSC generation we have identified represents also an original model whereby the induction and regulation of human hematopoiesis can be approached.

Extra-embryonic generation of hematopoietic cells

As is the case in other higher vertebrates, human hematopoiesis starts outside the embryo, in the yolk sac, then proceeds transiently in the liver before getting stabilized until adult life in the bone marrow. Only T cells are produced in the same tissue at embryonic, fetal and post-natal stages.

The yolk sac

In higher vertebrates, a network of mesoderm cell aggregates at the origin of both vascular and hematopoietic systems develops shortly after gastrulation in the extra-embryonic area. In these initially homogeneous solid clusters, peripheral cells acquire the morphology and markers of endothelial cells, while internal ones simultaneously disappear to open the first vessel lumens. Clumps of primitive mesodermal cells remaining adherent to the newly formed vascular endothelium – called *blood islands* – are at the origin of extra-embryonic hematopoiesis (Maximov, 1909, Sabin, 1920). Since both endothelial and hematopoietic cells develop from the same clusters of mesoderm, the existence of an ancestral precursor common to both lineages was postulated: the angioblast (Sabin, 1920), later renamed *hemangioblast* (Murray, 1932, Sabin, 1920). This term (that was initially coined to designate a group of blood- and vessel-forming mesoderm cells and not a single cell), fell into oblivion until the existence of such angio-hematopoietic stem cells was supported experimentally (Choi *et al.,* 1998, Eichmann *et al.,* 1997, Peault, 1987). The mesoderm of the human yolk sac has been shown to exhibit localized thickenings that probably represent the primordial blood islands, at about 16 days of development (Luckett, 1978). This is too early a stage to be accessed from elective pregnancy interruptions. Yet, occasional histological investigations have indicated that the human yolk sac produces mostly erythroid cells (Bloom and Bartelmez, 1940). The occasional presence of macrophages and primitive megakaryocytes has been also described (Fukuda, 1973). Although studies on hematopoietic cell emergence at these early stages still remain scarce, our own observations from day 19 of development have suggested that the sequence described in animal embryos also applies to the human yolk sac (Cortes *et al.,* 1999, Tavian *et al.,* 1999). Mesoderm-derived clusters of primitive hematopoietic cells - or *blood islands* - develop in close association with the endothelium of emerging yolk sac blood vessels. The co-expression of the CD34 surface molecule by hematopoietic cells within solid blood islands and by adjacent developing endothelial cells is consistent with the existence of a common hemangioblast precursor for blood and endothelial cell lineages (Tavian *et al.,* 1999). It remains to be determined, though, whether CD34 marks such a putative hemangioblast in the undifferentiated human yolk sac mesoderm prior to the onset of vasculogenesis. In this respect, CD34 expression in the early human yolk sac is reminiscent of that of MB1 (aka QH1) in the quail yolk sac. Similar to CD34 in man, the MB1 glycoprotein is expressed at the surface of quail endothelial and hematopoietic cells throughout embryonic and post-hatching life (Peault *et al.,* 1983). However, MB1 is first expressed in the yolk sac by emerging endothelial cells rather than by earlier, undifferentiated hemangioblastic mesoderm (Peault, 1987). This may suggest that yolk sac endothelial cells are the forerunners of extra-embryonic hematopoietic cells, as is the case for intra-embryonic hematopoiesis (see below). Earlier authors already hypothesized the existence of blood-forming endothelial cells in the avian yolk sac (Sabin, 1920).

The first functional study of human yolk sac hematopoiesis showed the presence of several generations of clonogenic progenitors at 4.5-weeks of development, including pluripotent (CFU GEMM), early (BFU E), or late (CFU E) erythroid and granulo-macrophage (CFU-GM) progenitors (Migliaccio *et al.,* 1986). The frequency of these progenitors drops dramatically thereafter, to disappear definitively after 6 weeks of gestation. These observations were confirmed by another group, which documented the presence of both erythroid and granulopoietic progenitors in the yolk sac as early as day 25 of development (Dommergues *et al.,* 1992, Huyhn *et al.,* 1995). However, these authors reported that the frequency of these precursors remains almost constant between days 35 and 50. Since all the assays were

carried out at stages when blood already circulates between the yolk sac and embryo, these discrepancies can reflect the presence in the yolk sac of precursor cells of extrinsic origin. In any case, these reports all agreed on the total disappearance of yolk sac hematopoiesis after the 60th day of development. Hence, the relative duration of yolk sac hematopoiesis during human gestation is much shorter than in birds and rodents.

Onset of blood circulation

The above-described observations have documented the hematopoietic *potential* of yolk sac-derived precursor cells in culture, which does not necessarily reflect the differentiation process that takes place during normal development. Blood cells produced in the yolk sac are predominantly nucleated erythrocytes, which synthesize embryonic hemoglobin (ζε). This first wave of erythrocyte production in the yolk sac is known as *primitive,* in contrast with the production of *definitive* erythrocytes, which takes place later in the liver. Primitive erythrocytes, already expressing the surface molecule glycophorin A, have also been detected in the cardiac cavity as early as the 3-somite stage (21 days) (Tavian *et al.,* 1999). Such observations indicate that vascular connections between the yolk sac and embryo are initiated at this stage, no blood cells being ever found inside the embryo proper at day 19 of development (MT, unpublished observations).

Colonization of embryonic hematopoietic tissues

The onset of circulation, which occurs in concomitance with the beginning of cardiac beating, allows yolk-sac derived blood cells to enter embryonic tissues. The first organ to be colonized is the liver, which remains the main blood-forming tissue in the embryo before the initiation of marrow hematopoiesis.

The liver

The liver develops from an endodermal diverticulum of the foregut, at the duodenal level, that migrates rostrally and penetrates the mesodermal *septum transversum*. These two tissues contribute hepatic parenchymal cords (hepatocytes) and vascular sinuses, respectively. In the human embryo the hepatic plate has been identified as an endodermal thickening at the rostral intestinal end, caudal to the heart at around 22 days of gestation (10-somite stage) (Severn, 1971). The assumption that the human embryonic liver rudiment is not able to produce blood cell progenitors, as previously shown in the mouse (Moore and Metcalf, 1970), but rather receives hematopoietic cells of yolk sac origin, which then proliferate and differentiate, was first suggested by Kelemen and coll. in 1979.

Yolk sac → liver transition

The transition from yolk sac to liver hematopoiesis was studied by analysis of the hemoglobin synthesis program and in vitro clonogenic assays. The embryonic → fetal globin switches (i.e. $\zeta \rightarrow \alpha$ and $\varepsilon \rightarrow \gamma$) occurring in the liver reflect the transition from *primitive* nucleated (*megaloblasts*) to *definitive* enucleated (*macrocytes*) erythrocytes (Peschle *et al.,* 1984). Primitive megaloblasts present in the early hepatic rudiment from week 4.5 to week 5 decrease rapidly in number, to be replaced simultaneously by definitive macrocytes. This phenomenon can imply a monoclonal model, where a single stem-cell pool would first give rise to primitive yolk sac erythropoiesis, then migrate to the liver to generate the definitive erythroblast lineage (Peschle *et al.,* 1985). Such a conclusion was also suggested by the analysis of in vitro colony-forming cells (CFCs) identified in yolk sac, liver and circulating blood cultures. In the fifth week, the yolk sac BFU-E pool undergoes a dramatic reduction, while these progenitors become rapidly detectable in the bloodstream and liver parenchyma (Migliaccio *et al.,* 1986). Therefore, stem cells and already committed progenitors would migrate from the YS to the liver at around 5 weeks of gestation. At the end of the first trimester and onwards, more primitive progenitors, CFU-GEMM and high-proliferative potential colony-forming cells (HPP-CFC) have also been detected in the liver (Hann *et al.,* 1983). In contrast, our recent work documents the appearance of hematopoietic cells inside the hepatic rudiment at earlier stages of development. At the 12-somite stage (23 days), we detected rare, scattered CD34-negative erythro-myeloid cells within developing hepatic sinusoids, suggesting that a first, previously unsuspected hepatic colonization occurs at this stage. The first CD34^{+} hematopoietic progenitors could be recognized in the liver anlage from day 30 only, the stage at which we propose that a second hepatic colonization takes place. The fact that this novel wave of hematopoietic cells entering the liver at three weeks of human development be composed solely of late-stage progenitors

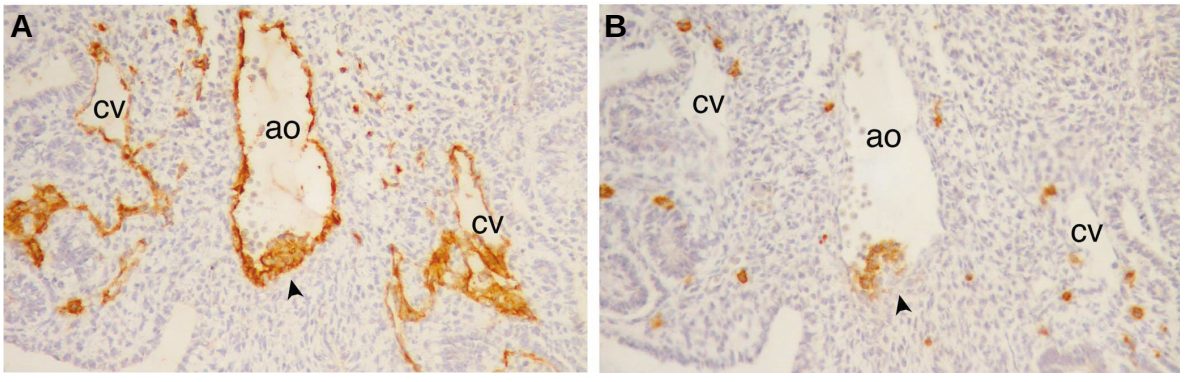

Fig. 1. *Hematopoietic stem cell clusters inside human embryonic arteries. Transverse sections of a 32-day human embryo stained with the anti-CD34* **(A)** *and anti CD45* **(B)** *antibodies. Arrows indicate the clusters of hematopoietic stem cells adhering to the ventral aspect of the aorta (a). Cardinal vein (cv).*

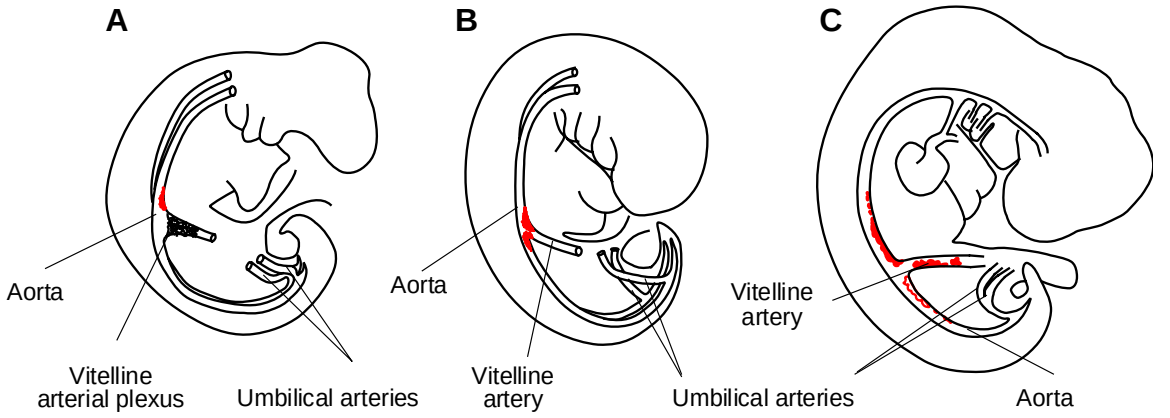

Fig. 2. Sequence of hematopoietic stem cell cluster emergence within the human embryo. (A) *Starting from 27 days of development, scattered groups of a few hematopoietic stem cells appear, adhering to the aortic endothelium in the pre-umbilical region. Groups of 2-3 cells are also often detected in a more rostral region, where the aorta is still bifurcated.* **(B)** *From day 30 of development the hematopoietic cell clusters increase in size and groups of cells are also encountered at the bifurcation of the vitelline artery, always associated with the ventral aspect of the vascular endothelium. The size of hematopoietic progenitor clusters attains several hundreds of cells at 36 days of development* **(C)**. *At subsequent stages stem cell clusters undergo gradual decrease till day 40, the latest at which they could be detected.*

was confirmed by cell culture experiments. Indeed, it is only after day 32 of development that the liver contains primitive precursors able to establish long-term hematopoiesis in vitro (Tavian *et al.,* 1999).

Bone marrow

The bone marrow, the main blood-forming tissue in the adult mammal, is also the last one that develops in ontogenesis, when hematopoiesis is already extinct in the yolk sac, transiently proceeding in the liver and actively developing in the thymus. Studies on the early ontogenesis of the mammalian bone marrow remain scarce. By virtue of an immuno-histochemical approach, we have previously explored the establishment of hematopoiesis within human medullary cavities (Charbord *et al.,* 1996b). Marrow hematopoiesis starts during the 11th week of human development in specialized mesodermal structures – or *primary logettes* - constituted by a loose network of mesenchymal cells supported by dense fibrillar material and surrounding a central artery. The earliest blood cells that differentiate within the bone marrow are CD15^{+} myeloid cells, closely followed by glycophorin A^{+} erythrocytes. Surprisingly, this process is not preceded by an influx of CD34^{+} hematopoietic precursor cells (Charbord *et al.,* 1996b). Of additional note, the primary logettes, where incipient marrow hematopoiesis protrudes inside medullary sinuses, hence are located at the farthest possible distance from ossifying cartilage trabeculae, at the surface of which lie the bone marrow osteoblasts. This is puzzling inasmuch as osteoblasts have recently been shown to represent a key component of the hematopoiesis-supporting bone marrow stroma and, notably, to participate in the *niche* that nurses hematopoietic stem cells (Zhang *et al.,* 2003). We can, however, possibly reconcile these diverging observations in light of a parallel study we have performed on fetal mouse bone marrow (Blazsek *et al.,* 2000). Although hematopoietic cells colonize the mouse marrow rudiment from about day 15 of development, these cells appear to be only late-stage committed progenitors. Conversely, it is only at 4-5 days after birth that genuine HSCs, capable of seeding long-term hematopoietic cultures, emerge within the mouse bone marrow.

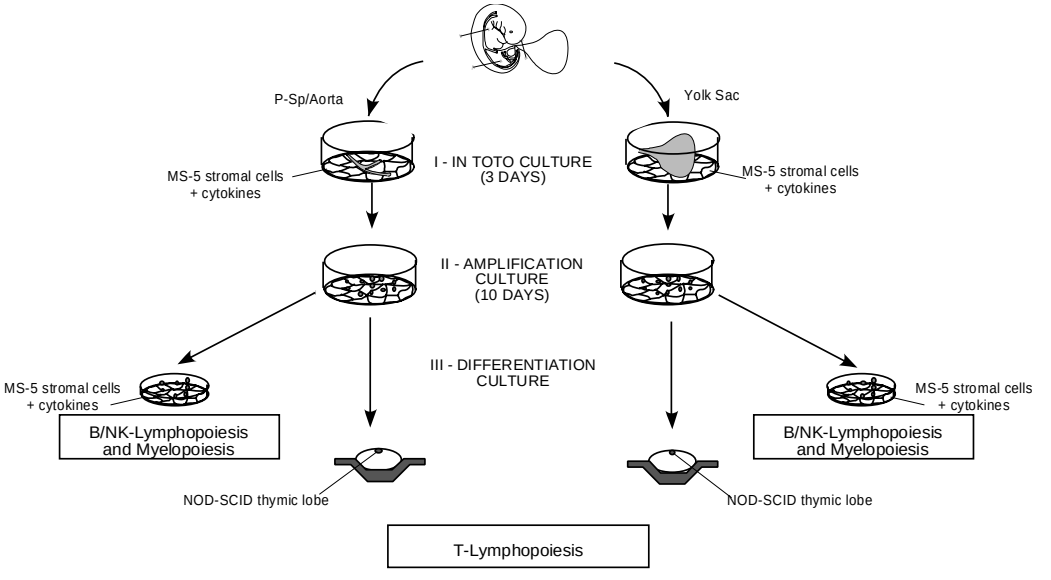

Fig. 3. Scheme of in vitro assays used *to detect myeloid-, NK-, B- and T-cell potentialities in the early human embryo.*

It therefore appears that in the fetal bone marrow (and possibly also, to some extent, in the

embryonic liver), a first line of rapidly developing hematopoiesis becomes established early in development by committed progenitor cells for accelerated procurement of vital myelo-erythroid blood cells. True HSC, as well as their supporting stromal environment, would settle only later and sustain hematopoiesis in the long run (Blazsek *et al.,* 2000).

Hematopoietic cells also emerge inside the human embryo

As previously introduced, all the observations described above may reflect a classic scheme for the development of the human hematopoietic system, along which all blood cells derive from precursors that were generated in the yolk sac but persist until adult life. However, this notion of a monophyletic development of the human blood system was challenged by our observation that an additional wave of HSC generation takes place within the human embryo between the differentiation of yolk sac blood islands and the colonization of the liver.

Embryonic arterial cell clusters

We initially observed the consistent presence of densely packed hematopoietic cells adhering firmly to the ventral endothelium of human embryonic arteries (Tavian *et al.,* 1996) (Fig. 1). These elements, detected from 27 days of development as small groups of 2-3 cells in the more rostral, duplicated section of the aorta, rapidly proliferate to constitute, at day 35, clusters of several thousands of cells that extend toward the umbilical region of the aorta and also in the vitelline artery, to disappear definitively after the 40th day of gestation (Tavian *et al.,* 1999) (Fig. 2).

Morphologic and phenotypic analyses

Immunohistochemical stainings and hybridizations on embryo sections revealed the primitive nature of these intra-embryonic hematopoietic cells. Indeed, these endothelium-adherent cells exhibit a surface phenotype, which typifies early blood progenitors, being $CD45^{+}$, $CD34^{++}$, $CD31^{+}$, $CD43^{+}$, $CD44^{+}$, $CD164^{+}$, but $CD38^{-}$ and negative for lineage-specific markers (Lin^{-}). They also express proto-oncogenes and transcription factors (GATA-2, GATA-3, c-myb, SCL/Tal, c-kit) that regulate the initial stages of blood cell development (Labastie *et al.,* 1998, Tavian *et al.,* 1996, Watt *et al.,* 2000).

Functional studies in vitro

The emergence of these cells within trunk arteries was also correlated with the appearance in that territory of functional hematopoietic progenitors. Indeed, preumbilical trunk tissues dissected from 5-week human embryos and cultured in the presence of MS-5 stromal cells established long-term cultures of hematopoietic cells and produced about 8 times more clonogenic progenitors than liver rudiments at the same stage of development (Tavian *et al.,* 1996).

These results confirmed the existence of a vigorous, precedently unidentified pool of hematopoietic stem cells associated with vascular endothelium in human embryonic truncal arteries. HSCs associated with vessel walls in the human embryo could be homologous to those present in and underneath the avian embryonic aorta (reviewed in Dieterlen-Lievre, 1994) and within the mouse embryonic aorta/gonads/mesonephros region (Garcia-Porrero *et al.,* 1995). However, the possible migration of mouse intra-embryonic HSCs from yolk sac blood islands, rather than their local origin within the embryo, has been a matter of debate and all of our initial investigations were performed at developmental stages when blood already circulates between the yolk sac and embryo (Tavian *et al.,* 1996). It was therefore essential to determine whether embryonic aortic tissues are truly endowed with blood-forming potential.

Hematopoietic potential is present in trunk mesoderm before the aorta develops

The demonstration of an independent generation of hematopoietic precursor cells inside the human embryo and the comparison of their potential with that of yolk sac-derived cells were performed by virtue of a new in vitro experimental assay (for details see Tavian and Peault, 2005) (Fig. 3). Three-dimensional organ culture of isolated embryonic explants, allowing organ development to proceed in the absence of the rest of the embryo, was carried out. Under these conditions, we have assayed blood-forming potential in the human embryo and yolk sac before and after the 21-day stage of human development, that marks the onset of blood circulation. Successively the splanchnopleura (Sp) - the presumptive region of the hematogenic aorta -, the para-aortic splanchnopleura (P-Sp) and the aorta itself were isolated and maintained in organ culture for 2-4 days, followed by in vitro analyses of hematopoietic potential. The corresponding yolk sac was analyzed under the same conditions in all experiments. From 27 to 40 days of gestation (i.e. stages when intravascular HSC clusters are present), the aorta was expectedly able to establish long-term hematopoietic cell cultures. However, the same potential was observed in the cultures containing the day-19 splanchnopleura, the earliest stage analyzed, at which the aorta has not yet developed. This indicates that 3 days before the onset of blood circulation and one week before the detection of

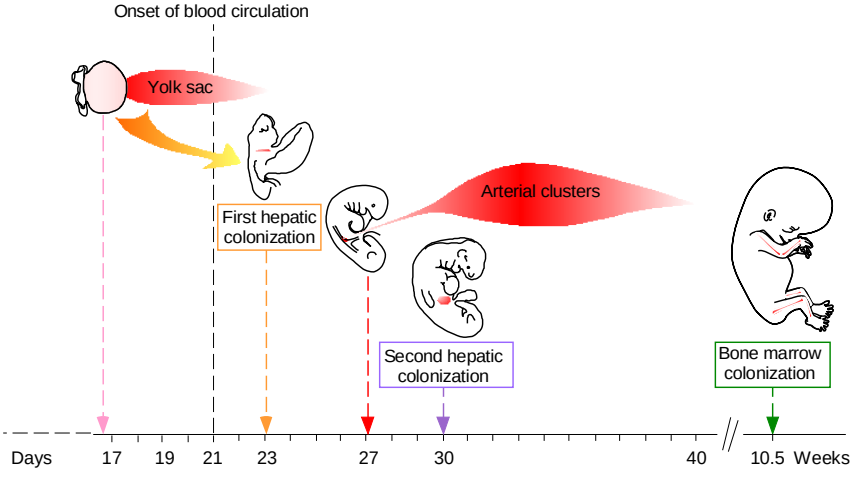

Fig. 4. Chronology of appearance of hematopoietic stem cells in the developing human embryo.

recognizable HSC clusters in the aorta, the splanchnopleura contains cells already instructed toward hematopoiesis. Therefore, during human development hematopoietic progenitors not only emerge in the yolk sac, but also intrinsically in the embryo proper, from the mesoderm of the splanchnopleura (Tavian *et al.,* 2001).

Comparing the potential of intra-embryonic hematopoietic progenitors with that of yolk sac-derived cells

Having demonstrated that the intra-embryonic splanchnopleura, that includes the presumptive aorta, is a genuine hematopoietic tissue rudiment, we sought to compare, in terms of lineage potential, hematopoietic cells derived from this site with those originating in the yolk sac. To this end, cells derived from these two independent generation sites were cultured on the MS-5 stromal cell line. This line supports multi-lineage, myelo-lymphoid human hematopoiesis and maintains the T-cell potential that can be assessed by colonizing NOD-SCID mouse fetus thymus rudiments in vitro (Robin *et al.,* 1999a, Robin *et al.,* 1999b). In this setting, a radical difference in differentiation potential was observed between precursors derived from extra- and intra-embryonic compartments (Tavian *et al.,* 2001). Both yolk sac- and embryo-derived progenitors yielded myeloid and NK cells. In contrast, only HSCs of intra-embryonic origin also gave rise to T and B cells. These results led to the conclusion that two waves of hematopoietic cell emergence take place in early human ontogeny. The first multipotent, myelo-lymphoid stem cells are generated in the splanchnopleura, within the embryo proper, an observation we have recently confirmed in the model of mice expressing the GFP fluorescent protein under the control of the RAG-1 gene promoter (Yokota *et al.,* publication in preparation). We demonstrate that these HSCs encountered within trunk arteries do stem from the embryo and are not derived from the extra-embryonic yolk sac. These cells are responsible for the second hepatic colonization (Fig. 4) and, therefore, for the establishment of human definitive hematopoiesis (Tavian *et al.,* 2001).

Origin of intra-embryonic hematopoietic progenitors

The AGM is the region of the embryo that comprises the aorta, genital ridges and mesonephros. The aorta is derived from the splanchnopleura whereas the two latter rudiments originate in paraxial mesoderm, between somites and coelomic cavities. Since the mouse AGM is commonly referred to as the territory where intra-embryonic HSCs originate, we have subdivided the human AGM into its three main components, which have been separately assayed in culture. It was undoubtedly shown that, inside the human embryo, only the aorta is endowed with hematopoietic ability. The gonad and kidney rudiments were in all instances devoid of any detectable blood-forming potential (Tavian *et al.,* 2001). We next searched for the local origin, within the ventral wall of trunk arteries, of emerging hematopoietic stem cells.

Lineage relationship between hematopoietic and endothelial cells

The yolk sac mesoderm appears to differentiate simultaneously into vascular and hematopoietic structures. Yet the first recognizable blood cells – by either morphology or molecular markers – in the yolk sac are always organized as clusters (or *blood islands*) that adhere tightly to developing or newly-formed endothelial cells (Murray, 1932, Sabin, 1920). Even more obviously than in the yolk sac, intra-embryonic clusters of HSCs always emerge in close physical association with the differentiated ventral endothelium of trunk blood vessels. These observations set the basis for the hypothesis that endothelial cells themselves are at the origin of embryonic blood cells, which was already suggested a long time ago in the case of yolk sac hematopoiesis (Sabin, 1920). This would imply the re-differentiation of an already organized endothelial cell layer, an event that was indeed recently suggested to be at the origin of intra-embryonic hematopoiesis in mouse and bird embryos (Jaffredo *et al.,* 1998; Nishikawa *et al.,* 1998; de Bruijn *et al.,* 2002 and for review see Dzierzak, 2003).

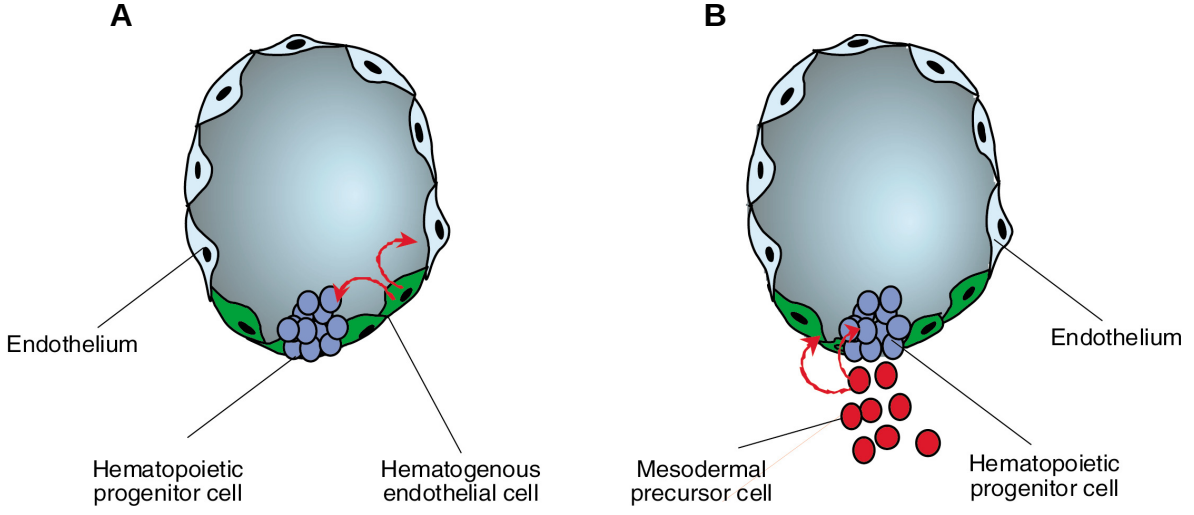

Fig. 5. Models of hematopoietic stem cell emergence within the human embryo. (A) *Re-differentiation model. Beginning at 27 days of development, pre-existing endothelial cells (green) in the ventral aspect of human intra-embryonic arteries differentiate locally into blood cell progenitors (blue).* **(B)** *Migration model. From 27 days of gestation, scattered mesodermal CD34-CD45- cell precursors (red) colonize the ventral vascular wall and give rise to (blood-forming?) endothelial cells (green) and hematopoietic cell clusters (blue).*

To test this possibility, we have first sorted by flow cytometry vascular endothelial cells from embryonic and fetal human blood-forming tissues. With the exception of CD45, most surface markers expressed on hematopoietic cells (CD31, CD34 and KDR/flk-1) are also present on endothelial cells at these early stages. A reliable marker combination to typify endothelial cells was recognized as CD34 or CD31 surface expression and absence of CD45, which indicates commitment towards hematopoiesis. $CD34^+CD45^-$ sorted cells were devoid of $CD45^+$ contaminating hematopoietic cells, as assessed by PCR analysis and did not give rise to blood cell colonies when seeded in methyl-cellulose assays. Yet, yolk sac- and AGM-derived endothelial cells (days 27-40) differentiated into hematopoietic cells when cultured in the presence of MS-5 stromal cells (Oberlin *et al.,* 2002). The frequency of hematogenous endothelial cells in these organs, at a given stage of ontogeny, was directly correlated with the hematopoietic activity of the tissue they were sorted from. In the yolk sac, where hematopoiesis begins around 16 days of development, the frequency of blood-forming endothelial cells was highest at day 19, the earliest stage assayed in this study, then decreased rapidly in parallel with the decline of vitelline hematopoiesis. In the whole embryo trunk, the frequency of hematopoiesis-endowed endothelial cells was about 1/100 at day 27, when HSCs emerge from the floor of the aorta. No hematopoietic activity was detected in cultures of endothelial cells sorted from the AGM region after day 40, when HSCs are no longer present in the lumen of the aorta. These experiments suggested that pre-existing endothelial cells in the human yolk sac and intra-embryonic arteries divide and differentiate locally into blood cell progenitors at the origin of primitive and definitive hematopoiesis (Oberlin *et al.,* 2002).

Conclusion: the ultimate human blood-forming cell

The main conclusions that can be drawn from the data digested in the present article are that multi-potent hematopoietic stem cells emerge within human embryonic arteries, autonomously from primary vitelline hematopoiesis, through a vascular endothelial cell intermediate. Hemogenicity appears to be acquired secondarily by aortic endothelial cells though. From 24 to 26 days of development, when the AGM is hemogenic in vitro but before the appearance of aortic HSC clusters, cell sorting and culture experiments have revealed that blood-forming potential in the AGM is confined to the CD34-negative, i.e. non-endothelial cell fraction (MT *et al.,* unpublished observations). This suggests either that ventral aortic endothelial cells switch suddenly to hematopoiesis at day 27, or that more primitive, CD34- angio-hematopoietic progenitors migrate through the peri-aortic mesenchyme and colonize the floor of the aorta at this stage. Several descriptive observations suggest that the latter hypothesis stands true. One is the expression of Flk-1, or VEGF-R2, in the early human embryo, which has revealed that a population of Flk-1+CD34- cells emigrates from the splanchnopleura into the sub-aortic mesoderm during the fourth week of development (Cortes *et al.,* 1999). More recently a surface protein named BB9, expressed in the adult marrow by HSCs and a stromal cell subset (Ramshaw *et al.,* 2001), was detected on a related population of aorta-colonizing cells in the 4-week human embryo. Strikingly, BB9 expression was then retrieved at the surface of both ventral aortic endothelial cells and associated HSCs (Jokubaitis *et al.,* submitted). Hence BB9 may represent the first marker of human angio-hematopoietic cells, or hemangioblasts.

We thus speculate that hemangioblasts, or even more primitive mesodermal stem cells migrating from the para-aortic splanchnopleura give rise in the floor of the embryonic aorta to blood-forming endothelial cells and, in turn, hematopoietic stem cells (Fig. 5B). Such differentiation cascades are assumed to be restricted to embryonic and early fetal stages of development. The fact that this may not be the case is implied by recent findings and opens interesting perspectives. Indeed, multipotent stem cells functioning far beyond hematopoiesis have been suggested to exist in adult tissues, among which the bone marrow. We have preliminary evidence that a small fraction of vascular endothelial cells sorted from adult bone marrow cavities can give rise to hematopoietic cells in culture (Souyri *et al.,* unpublished results) and hemangioblasts have been recently claimed to persist in the human adult (Pelosi *et al.,* 2002). It will be interesting to see whether an ancestral mechanism whereby hematopoietic cells are produced from the wall of embryonic blood vessels can be resumed in adult life in situations of emergency.

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