Ontogeny of the Hematopoietic System

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Abstract

Blood cells are constantly produced in the bone marrow (BM) of adult mammals. This constant turnover ultimately depends on a rare population of progenitors that displays self-renewal and multilineage differentiation potential, the hematopoietic stem cells (HSCs). It is generally accepted that HSCs are generated during embryonic development and sequentially colonize the fetal liver, the spleen, and finally the BM. Here we discuss the experimental evidence that argues for the extrinsic origin of HSCs and the potential locations where HSC generation might occur. The identification of the cellular components playing a role in the generation process, in these precise locations, will be important in understanding the molecular mechanisms involved in HSC production from undifferentiated mesoderm.

INTRODUCTION

Hematopoiesis: the process that gives rise to all mature blood cells through the production, by hematopoietic stem cells, of precursors that expand and differentiate

BM: bone marrow

HSCs: hematopoietic stem cells

LTR: long-term reconstitution

Niche: specialized environment required for a given cell type (e.g., HSC) to perform a function (e.g., self-renewal or differentiation)

Blood cells are produced throughout life. In mammals, hematopoiesis, defined by the expansion and differentiation of hematopoietic precursors, occurs in the bone marrow (BM) in the abluminal side of sinusoids that invade the bone cavity made by osteoclasts shortly after calcium deposits in the cartilaginous matrix.

It has been long known that BM cells can regenerate the blood compartment. Thus, BM transplantation was among the first cell replacement therapeutic approaches attempted.

Blood cells comprise multiple cell types and have accordingly variable life spans that go from a few days to several years. Hematopoietic cells undergo constant renewal and consequently have an active regenerative compartment. Only in the mid-twentieth century, however, did seminal work by Metcalf and Moore (1, 12) and by Till & McCullough (2) introduce the notion that multipotent progenitors can be found in adult BM and that these cells are responsible for the constant production of blood. Progress in the phenotypic and functional characterization of these precursors led to the isolation of hematopoietic stem cells (HSCs) and has made the hematopoietic system a paradigm in stem cell biology. Thus, HSCs have been characterized by two main properties, multipotency and self-renewal, at the single-cell level. Multipotency, as defined by the capacity of a single cell to give rise to a differentiated progeny comprising different cell types, was tested in vitro by clonal differentiation protocols. Self-renewal, the property that allows the maintenance of the HSC pool (probably through asymmetric cell division), is usually assessed by in vivo long-term reconstitution (LTR) experiments. This experimental approach requires irradiation of the recipient animals with the consequent perturbation of the natural environment. It measures not only self-renewal and multipotency, but also the homing capacity of the cell to find the appropriate niche in which it can exert a normal function. Self-renewal implies the maintenance of cell numbers in a given compartment: If one HSC is injected, one HSC should be recovered. However, expansion of the HSC pool is probably obtained under the irradiation ablative protocols conventionally used in LTR experiments.

In vitro assays have improved in efficiency and reliability in the past few years with the isolation of hematopoietic cytokines and supporting stromal cell lines, and multipotency is easily demonstrated. In contrast, self-renewal remained an elusive property, and the conditions that allow in vitro self-renewal of HSCs have been difficult to establish. Although it is generally accepted that the HSC pool is stable in numbers during life (implying a self-renewing capacity), few direct demonstrations of this property exist in the literature (3, 4). There is compelling evidence, however, that most HSCs in adult animal BM are in the G0 stage of the cell cycle (5). Thus, although they eventually will enter G1, most are resting, and the regeneration of the peripheral compartment is done by extensive proliferation of intermediate precursors. Therefore, self-renewing HSCs are difficult to detect.

Pioneering experiments in the late 1980s used retroviral integration as a marker of clonality and followed the progeny of single cells in vivo (3, 4). Sequential transplantation experiments demonstrated that single hematopoietic cells could generate all blood cell types in recipient mice for longer than six months, and on secondary engraftment the same viral integration was found in blood cells belonging to all lineages. These experiments showed for the first time the in vivo self-renewing nature of multipotent cells present in the adult BM. These experiments remain the most compelling direct demonstration of in vivo HSC renewal, with the possible caveat of clonal dominance conferred by particular retroviral integrations that might have favored the expansion of single infected cells in a nonphysiologic manner.

In the past few years, researchers have identified and characterized the regenerative compartment of other adult tissues. Cells within these compartments are generally designated as adult stem cells. Whereas all stem cells exhibit the properties of self-renewal and lifelong contribution (through maturation) to the differentiated cell pool, multipotency is a property only found in the stem cell pool of some tissues (e.g., hair follicle, intestinal epithelium, liver, central nervous system) and is probably absent in others (e.g., skeletal muscle).

Experimental evidence shows that there is a pool of tissue-specific, resident, self-renewing, and differentiating progenitors within many analyzed tissues. This finding suggests that adult stem cells are limited in their differentiation capacity to the tissue in which they reside and to which they are the restricted precursors. When isolated from their natural environment and placed in culture, stem cells eventually differentiate into mature cells belonging to the tissue of origin. The self-renewing capacity of stem cells ensures the integrity of the compartment throughout life. The additional contribution to a distinct adult tissue of other less differentiated progenitors (with broader potential) or of cells initially affiliated with other developmental programs is a possibility of limited physiological relevance and one that has been difficult to demonstrate formally (6, 7). It follows that the adult stem cell compartment of individual tissues is established possibly only once in the lifetime, in most cases during embryonic or perinatal life. As a consequence, investigators have concentrated on identifying the timing and anatomical location of stem cell generation to clarify the molecular mechanisms underlying their establishment.

HISTORICAL PERSPECTIVES

As mentioned above, identifying the embryonic site from which the adult pool of stem cells originates is crucial for understanding the underlying mechanisms responsible for the generation of adult stem cells. In the case of HSCs, the discussion was for a long time dominated by the direct observation of mammalian and avian embryos, in which an active erythropoietic activity is first detected in the yolk sac (YS). Within the mesodermal layer of the YS, homogeneous cellular aggregates appear in 7–7.5 days postcoitus (dpc) in the mouse embryo and rapidly form a confined structure surrounded by cells that evolve to resemble endothelial cells morphologically, while the inner part of the aggregate comprises erythrocytes. This cellular formation has been called a blood island (**Figure 1*a***). The erythrocytes in blood islands are large, nucleated cells that resemble erythrocyte precursors in the BM and erythrocytes in lower (primitive) vertebrate groups, such as birds, fish, and amphibians. They never reach the final enucleated stage in situ, and for this reason they have been called primitive. Primitive erythrocytes are the first detected hematopoietic cells of embryonic origin. It is logical to conclude that, because the first identifiable hematopoietic cells in embryogenesis are present in the YS, HSCs would originate in the YS and lead to hematopoiesis at this site.

The idea that HSCs originate in the YS from which (via the circulatory network that forms thereafter) they could reach the fetal liver (FL), the major embryonic hematopoietic organ, and then the thymus and BM prevailed in the 1970s. Several experimental lines of evidence reinforced this hypothesis. Embryonic cells of YS origin isolated shortly after blood island formation were injected in vivo into mouse embryos of related stages (8). Histological analysis, done after birth, indicated that a variable, albeit sizable fraction of cells of donor origin, belonging to the hematopoietic lineage, was present in the thymus and BM of the recipient mice. Auerbach's (9) group reinforced these results by showing T cell differentiation potential in YS cells after fetal thymus organ culture. Interestingly, the peak of hematopoietic progenitors with lymphoid potential occurred at 11 dpc, whereas the

YS: yolk sac
dpc: days postcoitus
FL: fetal liver

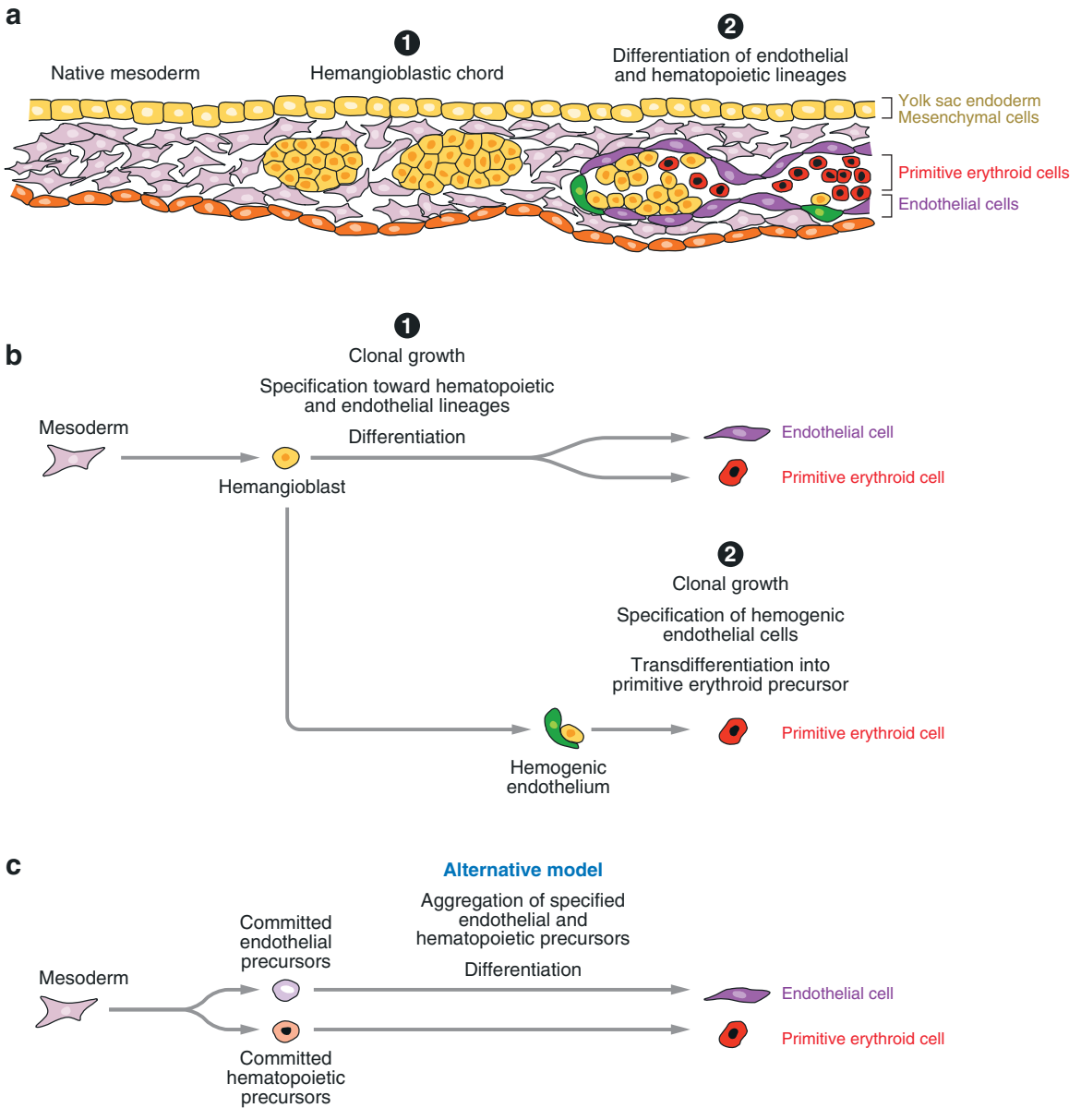

Figure 1

Mechanism of yolk sac (YS) blood island formation. (*a*) Schematic representation of the progressive evolution of blood island mesodermal cells to a functional vascular network and primitive erythroid cells. Two alternative mechanism of YS blood island formation are shown. (*b*) Endothelial and hematopoietic cells may be generated directly from progenitors restricted to both differentiation pathways, the hemangioblast, or via the intermediate production of hemogenic endothelial cells. (*c*) Alternatively, the specification into endothelial and hematopoietic cells might occur at earlier stages of differentiation (*c*).

intraembryonic compartment was consistently devoid of T cell differentiation potential.

At the time, these observations were taken as evidence that HSCs are present in the YS before or around the time circulation is established. However, hematopoietic cells enter circulation rapidly between the four- and five-somite stage (10, 11), from which they reach the hematopoietic organs. Therefore, isolating embryonic hematopoietic tissues after the five-somite stage does not allow the precise identification of the origin of HSCs.

Colony assays brought additional support to the theory of the YS origin of HSCs. Moore & Metcalf (12) developed the first in vitro assays designed to reveal multipotency by analysis of the progeny of single precursors. Limited cell types could be distinguished morphologically, but erythrocytes and polymorphonuclear cells could be discriminated from mononuclear elements. The authors used cytokines present in conditioned media to stimulate these cultures as the main mitotic agent responsible for colony formation in semisolid cultures. A careful time-course analysis of single hematopoietic progenitors and their progeny indeed showed that the first multilineage colonies were obtained from YS populations isolated shortly after the onset of blood island formation. These experiments showed that progenitors with multilineage differentiation potential are of YS origin and are the first to be generated.

In the late 1970s, Herzenberg's (13) laboratory used cells from different fractions of the embryo to reconstitute the B cell compartment of adult irradiated recipient mice. The results of this experiment were puzzling. They obtained B cells of donor origin, as distinguished by their immunoglobulin allotype, with YS derivatives with similar if not lower efficiency than that of the cells isolated from the intraembryonic compartment in a region confined to the subdiaphragma. This experiment, done with embryos after the establishment of circulation, raised the possibility that lymphocyte precursors could be present in the intraembryonic compartment before the colonization of the FL. This observation remained largely forgotten until experiments done in the avian model, using chicken/chicken or quail/chicken chimeras, showed that long-lasting contribution to the hematopoietic compartment was obtained from progenitors present in the body of the embryo rather than in the YS (14). The prevailing hypothesis in the late 1980s was thus based on evidence from experiments on mouse; it stated that HSCs first originated in the YS.

EXPERIMENTAL APPROACH OF DEVELOPMENTAL HEMATOPOIESIS: STRENGTHS AND LIMITATIONS OF ANIMAL MODELS

Recent progress in understanding the steps involved in HSC development relies on data gathered in different animal models. Here, we briefly describe the major features of each model and their respective contribution to the progress and breakthroughs in developmental hematopoiesis.

Amphibian and Avian Models

Amphibians and avians constitute the oldest vertebrate models in developmental biology, owing to the availability and large size of embryos. They are also amenable to the transplantation of embryonic territories, thus allowing developmental fate analyses. The construction of interspecific chimeras (first in the avian model, then in amphibians) allowed the definition of cell-fate analyses because the in vivo migration of small cell groups as well as their contribution to the developing tissues can be followed through donor-recipient markers (15).

In the avian model, such chimeras result from the combination of chick and quail tissues that can be discriminated from the morphology of nucleoli (condensed in quail and dispersed in chick) (16) and presently by using

species-specific antibodies (17). In some instances, investigators constructed chick-chick chimeras involving sex-mismatched (18) or allogeneic (19) embryos to analyze late stages of lymphoid development (including contribution to adulthood), as the initial synchrony of chick and quail embryonic development is eventually lost.

In the amphibian model, chimeras (first constructed in the frog *Rana*, then in the toad *Xenopus*) combine tissues from chromosomally distinct species (diploid versus triploid) (20, 21). Besides its usefulness in tissue-fate tracing, the amphibian model is the oldest model used to study the cascade of events leading to embryonic patterning (22), and as such it proves highly suitable for investigating mesoderm determination toward a hematopoietic fate (23), in particular in the animal cap assay in which a dissected part of the gastrula normally fated to produce ectodermal derivatives may be turned into blood-forming mesoderm on exposure to prospective inducing factors, such as BMP4 (see below).

Although antibodies against hematopoietic markers are available, ensuring the sorting and characterization of selected subsets, the limitations of these two models lie in the poorly developed in vitro culture systems—although clonogenic assays that allow erythromyeloid precursor characterization are available for avian cells (24)—and routine/up-to-date in vivo assays of LTR, as well as in the absence of tools to carry over genetic approaches (homologous recombination).

Zebrafish

Interest in the zebrafish as a hematopoietic development model lies first in the potential novel finding about the genetic control of developmental hematopoiesis, thanks to the large collection of chemically induced mutants generated by two laboratories (25, 26). The zebrafish constitutes a fast developing and promising model because LTR assays have been recently developed, as well as phenotypic FACS analyses (27, 28), which will complete the large panel of zebrafish assets, such as the availability of transgenic fish expressing an increasing number of fluorescent reporter proteins, the routine use of antisense technology, and lineage-tracing protocols. More importantly, the zebrafish provides the possibility, owing to the transparency of the embryo, of following in situ cell migration of selected groups or even single cells, without staining, at selected differentiation times and locations thanks to laser-driven fluorochromes uncaging. Nevertheless, studies of hematopoietic development in the zebrafish are complicated owing to the absence of hematopoiesis in the YS, with the first wave of hematopoietic cells appearing in a caudal site named an intercellular cell mass.

THE FIRST CHALLENGES TO THE CLASSICAL MODELS

Successive Hematopoietic Sites

Historically, investigators approached the understanding of hematopoietic development through observation of the sites at which differentiation occurs, an approach that proved somehow misleading as to the origin of HSCs because it was based on the assumption that the HSC generation site could be monitored by their differentiation. The main conclusion drawn from these analyses was that, in all species studied (**Table 1**), successive sites achieve the production of differentiated hematopoietic cells until the definitive (adult) hematopoietic organs are fully developed and ready to take over this function.

The first hematopoietic site, namely YS blood islands, is active from the earliest stage of organogenesis (soon after gastrulation). Before the development of the hematopoietic organs (the first being the thymus, then the spleen and, finally, the BM), hematopoiesis systematically occurs within an intermediate site, the FL in the mouse and human, as well as in the amphibian (23).

In the chick embryo, a function similar to that of the FL seems to be carried out by a

Table 1 Successive hematopoietic sites in vertebrate embryos: location and functions

	Initial generation site	Second generation site	Intermediate hematopoietic site	Definitive hematopoietic sites
Fish (zebrafish)	ICM: Generation + differentiation	AGM: Generation. No overt differentiation	Unknown	Thymus: T cell differentiation Kidney: HSC maintenance + differentiation?
Amphibian (*Xenopus*)	YS (VBI): Generation + differentiation	AGM (DLP): Generation. No overt differentiation	FL: HSC maintenance? HSC expansion? Differentiation	Thymus: T cell differentiation Spleen: differentiation Kidney: HSC maintenance + differentiation?
Avian (chick/quail)	YS: Generation + differentiation	AGM: Generation. No overt differentiation Allantois: Generation?	Para-aortic foci: HSC maintenance? HSC expansion? Differentiation	Thymus: T cell differentiation Bursa of Fabricius: B cell differentiation Spleen: differentiation BM: HSC maintenance + differentiation
Mouse	YS: Generation + differentiation	AGM: Generation. No differentiation. Expansion? Allantois/Placenta: Generation? Accumulation? Expansion?	FL: HSC maintenance. HSC expansion. Differentiation	Thymus: T cell differentiation Spleen: B cell differentiation BM: HSC maintenance + differentiation
Human	YS: Generation + differentiation	AGM: Generation. No overt differentiation. Expansion?	FL: HSC maintenance. HSC expansion. Differentiation	Thymus: T cell differentiation Spleen: B cell differentiation BM: HSC maintenance + differentiation

Abbreviations: AGM, aorta, gonads, and mesonephros; BM, bone marrow; DLP, dorsal lateral plate; FL, fetal liver; HSC, hematopoietic stem cell; ICM, intermediate cell mass; VBI, ventral blood island; YS, yolk sac.

structure called the para-aortic foci. In the fish embryo, no such intermediate hematopoietic site has been identified.

Extrinsic Origin of Hematopoietic Precursors in the Various Tissues Performing Hematopoietic Differentiation

The alternative hypotheses as to the origin of blood cell precursors in the successive organs sustaining hematopoietic cell production constituted a high controversy among embryologists for approximately one century, namely (*a*) each site independently produces its own precursors; (*b*) blood cell production in a given site depends on its colonization by precursors born elsewhere; and (*c*) hypothesis (*a*) or (*b*) applies, depending on the considered site. The first conclusive experimental data were obtained in the avian model. Using the chick-quail interspecific chimera setup,

researchers grafted the quail thymus to a chick embryo dorsal body wall and demonstrated that it was colonized by chick precursors, thus validating the extrinsic precursors hypothesis (29). The need for the developing hematopoietic organs to be colonized by extrinsic precursors via blood flow was further shown to be the general rule. Investigators demonstrated this using various experimental setups, such as an organ culture of embryonic anlagen [mouse thymus (30), FL (31), spleen (32), and BM (33)] and grafts of embryonic anlagen on the chick chorio-allantoic membrane [mouse thymus (30), chicken bone (34)]. Parabiosis experiments [chicken BM (35)] established that the colonization occurs via precursors migrating through the blood flow.

These observations raised a number of questions, some of which were recently revived: Where do the colonizing cells come from? Is there one or many sources of colonizing cells? How do the HSCs transfer from one site to another? Where do the stroma of all these organs come from? Is there one or various types of stromal precursors during ontogeny? When does it become competent in supporting hematopoietic differentiation and/or maintenance of HSCs?

The only tissue harboring differentiated hematopoietic cells that escape this extrinsic origin law is the YS, in which the progressive evolution of mesodermal cells toward differentiated hematopoietic cells in the blood islands occurs in situ. The first sign of mesodermal differentiation appears at 7.25 dpc in the mouse as homogeneous aggregates, called hemangioblastic chords, which soon evolve into erythrocyte-filled blood vessels as peripheral cells differentiate into endothelial and inner cells into erythroid cells (**Figure 1*a***). This sequence leading from early (undifferentiated) blood islands to the production of both endothelial and hematopoietic cells signals to an in situ production of hematopoietic cells in the YS. It also questions the lineage relationship between endothelial and hematopoietic cells, an issue we approach later.

Identification of the Compartments Involved in Hematopoietic Stem Cell Generation

Because the YS is the first embryonic site to harbor hematopoietic cells and because these cells are observed to be born in situ, researchers considered this site for years to be the source of HSCs that colonize further hematopoietic sites. Indeed, a few experiments supported this conclusion (see above).

Dieterlen's group (14) first tested the possible existence of an alternate HSC source in the avian model. They constructed chimeras combining a quail intraembryonic compartment to a chick YS (extraembryonic compartment). Early embryos, in which vascular connections between the two compartments are not yet established (hence forbidding a possible cross-contamination by circulating precursors), were used to allow for a clear-cut conclusion as to the origin of hematopoietic cells (14). The respective contribution of the two compartments to hematopoietic cells of the resulting chimeras was followed up to hatching. YS-derived cells made the major contribution to hematopoietic cells during the initial phase of embryonic development, but this contribution is transient because only cells derived from the quail intraembryonic compartment are present at development stages close to hatching. When chick-chick (sex-mismatched or allogeneic) chimeras were similarly built to investigate YS and intraembryonic contribution in adult chimeras, researchers found B and T lymphoid cells to be exclusively of intraembryonic origin (18, 19). These experiments thus uncovered a new source of hematopoietic cells that may be qualified as HSCs because they contribute to all lineages of the adult. They also established that YS-derived cells lack the capacity either to sustain the production of hematopoietic cells or to give rise to lymphoid cells, and hence they differ from bona fide HSCs.

Amphibian ploidy-distinct chimeras similarly built up led to mostly overlapping results (reviewed in 36). In particular, cells that

colonize the FL and produce an erythromyeloid as well as B lymphoid progeny derive from the dorsal lateral plate (DLP) (amphibian homologous to the intraembryonic hemogenic site) (37). However, in this group, the ventral blood islands (YS homologous) also produce T lymphocytes of the larva and adult (21), in addition to DLP-derived thymocytes. This discrepancy points to other standing questions in the field: Are mesodermal-derived hematopoietic precursors from the extra- and intraembryonic compartment initially identical? Is there a YS precursor limitation regarding the differentiation and self-renewal potential imposed by the environment? Alternatively, is the determination/generation process radically different for precursors of extra- and intraembryonic origin? The ability of amphibian YS precursors to give rise to a lymphoid progeny would favor the first hypothesis. More recently (38), fate-map tracing experiments showed that *Xenopus* ventral blood islands and DLP derive from distinct blastomeres of the 32-cell embryo, thus establishing the independent generation of precursors in both sites.

In mammals, the first evidence of an intraembryonic pool of hematopoietic precursors appeared in the early 1990s. Nishikawa's group (39) and Cumano et al. (40) showed that precursors endowed with a B lymphoid potential could be recovered first from the intraembryonic compartment and later in the YS. Godin et al. (41) established that the aortic region of 9-dpc embryos was capable of giving rise to B cells on transfer to irradiated mice, whereas the YS precursors could not. Medvinsky et al. (42) pointed to the same region—which encompasses the aorta, gonads, and mesonephros (AGM) at a latter stage (10.5 dpc)—as containing CFU-S before this precursor type can be recovered from the YS.

Investigators further characterized intraembryonic hematopoietic precursors as multipotent (32, 43) and capable of performing long-term multilineage hematopoietic reconstitution (LTR activity) of adult irradiated mice, when taken after 10.5 dpc (44). At earlier developmental stages (9 dpc), this activity could only be evidenced on the direct injection of intraembryonic precursors into the liver of myelo-ablated newborn recipients. Yoder et al. (45) concomitantly detected this activity (in larger amounts) in the corresponding YS that was similarly injected.

Studies combining phenotypic and cytological analyses looked into the possible occurrence of an intraembryonic hemogenic site during human ontogeny, which led to the conclusion that intraembryonic precursors are also present in the aorta region of human embryos (46, 47). However, a clear-cut homology to the two-generation events as found in lower vertebrate chimeras could not be drawn from these experiments: Intraembryonic hematopoietic precursors first appear at 8.5 dpc in the mouse (43), i.e., after the connection of YS and intraembryonic blood vessels, an event that occurs at 8 dpc (10, 48). Consequently, the recovery of intraembryonic precursors from the aorta region could result either from an in situ generation or from a selective aggregation of precursors born elsewhere in a suitable intraembryonic niche.

Hemogenic: property of a site that generates hematopoietic precursors by opposition to the hematopoietic site where maintenance and/or differentiation occurs

AGM: aorta, gonads, and mesonephros

Independent Generation of Hematopoietic Cells

Researchers undertook different approaches to answer the question of the independent generation of hematopoietic cells. An indirect approach involved organ culture of the intraembryonic hemogenic site explanted at 10 dpc (i.e., at stages when LTR activity on transfer to irradiated adult recipients is not detectable) (49). After organ culture, the investigators recovered a greater number of HSCs displaying LTR activity in adults (49), indicating either an increased de novo HSC generation or, alternatively, an amplification/maturation of the pool of precursors initially present in the explants (as the intraembryonic hemogenic site already contains multipotent precursors at the stage of AGM isolation) (43).

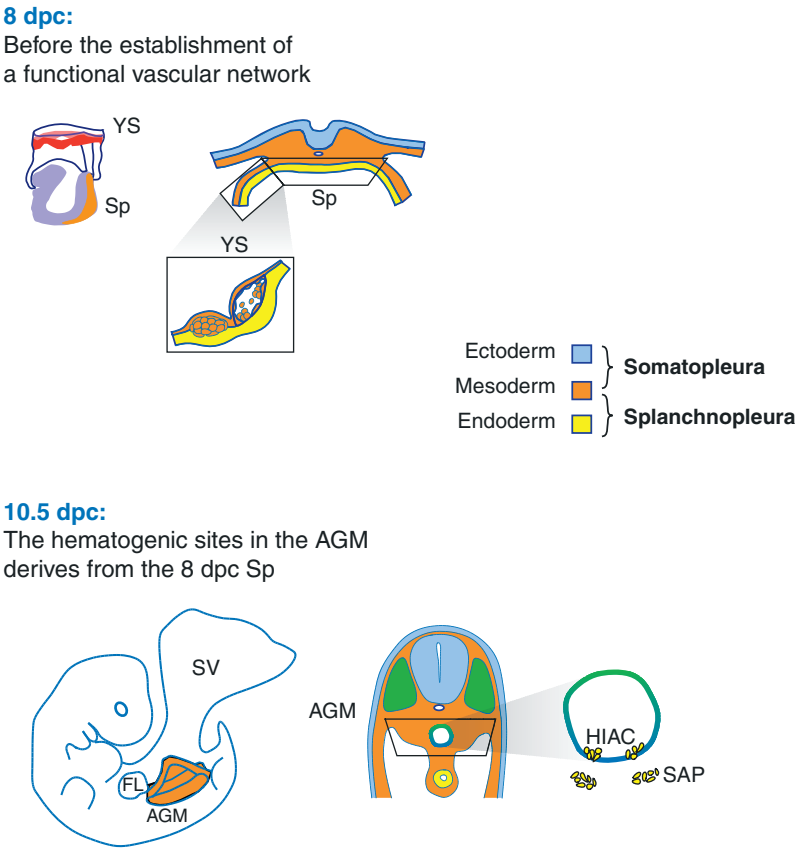

Figure 2

Hemogenic sites in the developing mouse embryo. (*a*) The respective localization and layer makeup of the extraembryonic yolk sac (YS) and presumptive intraembryonic splanchnopleura (Sp) are shown at the stage preceding the establishment of vacular connections between the two compartments. Both sites associate endoderm and mesoderm germ layers. (*b*) The Sp gives rise eventually to the para-aortic splanchnopleura/aorta, gonads, and mesonephros (P-Sp/AGM), whose localization within the embryo is shown, as well as the tissue structure in the schematic cross-section of a 10.5-dpc embryo. The respective localization of the hematopoietic intra-aortic clusters (HIACs) and subaortic patches (SAPs) is also shown in the enlarged aorta drawing.

Splanchnopleura (Sp): subdivision of the lateral plate mesoderm by the coelomic cavity that associates mesoderm and endoderm

Another strategy involved organ culture of the prospective territory that gives rise to the aortic region, the splanchnopleura (Sp) (see **Figure 2*a***), explanted at stages preceding both the establishment of blood vessel connections between the YS and the embryo proper, and the appearance of intraembryonic hematopoietic precursors (50, 51). In these experiments, the organ culture aimed to preserve the environment in which mesodermal cells may commit to a hematopoietic fate, such as a proper relationship with the endoderm, that is required to ensure the generation of hematopoietic cells from the nearby mesoderm, as discussed below. After the organ-culture step, hematopoietic cells could be recovered from the YS and from the intraembryonic hemogenic prospective territory, thus proving the existence of two independent waves of hematopoietic cell generation in the early embryo. Moreover, extra- and intraembryonic precursors differ strikingly in their

differentiation potential because, contrary to precursors derived from the intraembryonic compartment, YS-derived precursors proved unable to generate a lymphoid progeny. Before the organ-culture step, the intraembryonic, but not the extraembryonic, cells were devoid of hematopoietic potential in vitro, an observation that may account for the failure of Moore & Metcalf (12) to detect the intraembryonic precursors in their multilineage colony assays. Similar conclusions applied to human ontogeny because the early YS lacks both B lymphoid (on in vitro culture) and T lymphoid [on fetal-thymus organ culture in NOD-SCID (nonobese diabetic–severe combined immunodeficient)] potentials, whereas precursors derived from the intraembryonic hemogenic site display both lymphoid potentials (52).

To assay the in vivo engraftment capacity, Cumano et al. (51) transplanted early extra- and intraembryonic hematopoietic precursors, isolated before circulation, into the *Rag-2*$^{-/-}$ x $\gamma c^{-/-}$ mouse strain (53), which is alymphoid and also lacks natural killer cell activity. The authors chose these recipients because MHC class I molecules are hardly expressed in embryonic cells before 10.5 dpc (54), and hematopoietic precursors could therefore be eliminated by radiation-resistant host natural killer cells when transferred into conventional recipients. YS precursors were capable of engrafting these mice and transiently gave rise to a myeloid progeny. However, no lymphoid progeny was ever produced, and myeloid cell production was only transient because no contribution was ever detected three months after engraftment. In contrast, precursors derived from the organ-cultured intraembryonic hemogenic site generated myeloid and lymphoid cells that could be detected in the periphery and in the BM up to eight months after transplantation.

Because precursors displaying the multilineage and LTR activity that characterize HSCs could be derived from the prospective intraembryonic hemogenic site explanted at precirculatory stages and not from the corresponding YS, intraembryonic precursors may be responsible for the seeding of definitive hematopoietic organs. These conclusions point to an absence of lymphoid potential in the precirculatory YS (55, 56).

THE INTRAEMBRYONIC HEMOGENIC SITE

General Location: P-Sp/AGM

Both in vivo and in vitro functional approaches and analyses performed in situ point to the aorta and underlying mesenchyme as the source of intraembryonic HSC.

Experimental analyses. In the avian model, cytological (57) (see below) and in vitro (24) analyses found that intraembryonic HSCs are located in the region neighboring the dorsal aorta. Furthermore, on grafting a quail aorta into the dorsal mesentery of a chick embryo (58), investigators found that quail aorta–derived HSCs contributed to para-aortic foci (57), an intermediate differentiation site that may correspond to the FL of other vertebrate species as it harbors cells from all hematopoietic lineages, as well as transplantable lymphoid cells (in cycloheximide-treated chicken) (59). Interestingly, when the quail aorta was cleared of contaminating mesenchymal cells, no hematopoietic contribution was detected, an observation that led the authors to conclude that hematopoietic precursors likely arise from tissue surrounding the dorsal aorta (58).

As stated above, intraembryonic precursors are found in mammals in an area that includes the AGM (**Figure 2*b***). To determine precisely the site of intraembryonic HSC production, researchers subdivided the mouse AGM into its various components and assayed it for precursor enrichment in vitro. The isolated aorta and surrounding tissue harbored the majority of multipotent precursors (32). A similar approach, undertaken by Dzierzak's (60) group in an in vivo assay, established that the aorta region, rather than the urogenital

compartment, displays the highest frequency of LTR cells.

HIACs: hematopoietic intra-aortic clusters

SAPs: subaortic patches

Cytological analyses. Cytological analyses uncovered two structures that appear linked to the process of intraembryonic HSC generation (**Figure 2*b***). The first comprised hematopoietic intra-aortic clusters (HIACs) inserted inside the ventral wall of the dorsal aorta, which have been observed in bird (57, 61, 62), amphibian (38), mouse (63, 64), and human embryos (46). In situ phenotype analyses, in some instances linked to in vitro and in vivo potential assessment, indicate that the clusters comprise immature hematopoietic cells, as well as more differentiated cells, such as macrophages. In the mouse embryo, intra-arterial clusters are also detected in the umbilical and omphalo-mesenteric (also called vitelline) arteries (60, 63, 65); however, their characterization for in vitro and in vivo potentials, as well as phenotype, is less advanced than that of the aortic clusters. The only available functional data indicate that these two arteries harbor LTR HSCs. However, contrary to the aorta in which the LTR-HSC number increases on organ culture (49, 60), organ culture of either umbilical or omphalo-mesenteric arteries does not lead to an increased recovery of LTR HSCs (60). The cells that belong to the HIACs are inserted between endothelial cells, resulting in a discontinuity in the endothelial layer, but also in the basement membrane, which is disrupted at the HIAC level (46, 62, 63). The morphology of both HIAC cells and the underlying vessel wall suggests a migratory phenotype for these cells but gives no indication on the direction of a possible migratory route. Interestingly, HIAC are not detected from the beginning of intraembryonic hematopoietic precursor generation [8.5–9 dpc in the mouse (43)], but only when the number of generated precursors reaches a maximal level [10.5–11 dpc (32)].

The tissue below the aortic floor also appears potentially involved in the HSC generation process. It comprises the second structure discovered through cytological analysis, called subaortic patches (SAPs). SAPs were first identified through the expression of the GATA-3 transcription factor (66) and are present below the aorta for the whole duration of intraembryonic hematopoietic precursor generation, contrary to the HIACs, which are only detected at the peak of intraembryonic HSC production. SAPs, which are preferentially located below the HIACs at the time when these are present, from 10.5 to 11.5 dpc (63), disappear after 12.5 dpc, when intraembryonic precursors are no longer generated (32). Although a similar structure has also been identified in the human AGM (67, 68), SAPs are not characterized as such in other species, even if expression-pattern analyses report the expression in the area underlying the HIACs of a large array of genes and proteins involved in hematopoietic cell development (**Table 2**). Interestingly, these SAPs do not seem to occur below the umbilical or omphalo-mesenteric arteries (I. Godin, personal observation), so the above-mentioned difference between these two arteries and the aorta regarding in vitro HSC amplification on organ culture may be related to the different type of environment. The only direct evidence for the involvement of HIACs and the SAPs/subaortic area in the generation of intraembryonic HSCs comes from lineage-tracing experiments performed in *Xenopus* embryos, in which both intra-aortic clusters and the subaortic area are found to express hematopoietic markers, as well as the β-galactosidase reporter, on injection of the blastomeres that gives rise to the DLP (38).

Phenotypic Analysis and Precise Location of Hematopoietic Stem Cells

The observation that HSCs appear in a defined location around the dorsal aorta between 9–12 dpc led to an attempt to characterize the surface-marker expression pattern that characterizes them and to visualize emerging HSCs in the Sp/AGM. HSCs in the BM are

Table 2 In situ expression patterns in the various AGM compartment involved in HSC generation[a]

	Localization in the intraembryonic hemogenic site				
Marker	**CE**	**HIACs**	**HSCs in SAPs**	**SAPs**	**Reference(s)**
Adhesion molecules					
CD31	+	+	+	+	H: 46; M: 65, 84
CD34	+	+	+*	+*	H: 46, 95, 181; M: 64, 65; *I. Godin, unpublished
AA4.1	+	+	+	+	M: 66, 79, 84
Endomucin	+	+	?	–	M: 191
VE-cadherin	+	+	?	–	H: 67
V-CAM (CD106)	+	+	?	–	H: 192
CD41	–	+	+*	–*	A: 193; M: *84
CD43	–	+	?	–	H: 194
CD44	–	+	?	–	H: 194
CD164	–	+	–		H: 194
Tenascine C	– to +*	– to +*	+	+	H: 67; A: *195
ALCAM/CD166/BEN	–* to +	–	?	+*	H: *181; M: 196
H-CAM	–	+	?	–	H: 192
WASP	–	+	?	–	H: 192
Tyrosine kinases receptors					
c-Kit	+	+	?	?	H: 67; M: 98, 197
Flk-1/VEGF-R2/KDR	+	– to ±	?	?	H: 67, 95; M: 98; A: 62; Zf: 149, 198
Flt3/Flk2/STK-1	+	+	?	–	H: 67
Tie2/tek	+	+	?	?	M: 97
Growth factors/morphogens					
Flt3-ligand	+	+	?	–	H: 67
BMP-4	–	±	?	+	H: 105; M: A. Manaia & I. Godin, personal observation
VEGF	+	+	?	–	H: 67
TGFβ-1	±	+	?	–	H: 105
Transcription factors					
GATA-2	+	+	+*	+*	H: 67; M: *84; XL: 38; Zf: 149, 198
GATA-3	–	+	+*	+*	H: *199; XL: 38; Zf: 200; M: *66, 84
c-Myb	–	+	ND	–	H: 95; A: 201; Zf: 198
Lmo2	±	+	+	–	M: 66; Zf: 198
AML-1	±	+	?	–	M: 99; Zf: 38
Tal-1/SCL	+	+	+*	–*	H: 67, 95; M: 96 (*I. Godin, unpublished); XL: 38; Zf: 19, 149
Other					
vWf	+	–	ND	+	M: 65
CD45	–	*few cells to +	–	–	H: 46, 67; A: 12, 62; M: *66, 84
SMA	–	–	?	+	H: 47; M: 66

[a]Whenever contradictory expression patterns are found, * indicates the corresponding publication.

P-Sp: para-aortic splanchnopleura

P-Sp/AGM: intraembryonic hemogenic site from 8.5–11.5 dpc; the P-Sp 8.5–10 dpc evolves into the AGM at 10–11.5 dpc

characterized by the expression of c-kit and Sca-1 within the Lin^- fraction (75). Although it contains all HSCs, this fraction is far from homogeneous, and it was later fractionated with Flk2 and CD34 in $Flk2^-CD34^-$ long-term and $Flk2^+$ $CD34^+$ short-term reconstituting cells (76). HSCs can also be isolated by the expression of CD150 and the absence of CD48 (77). In the FL, HSCs express most of the above-mentioned markers, although they coexpress low levels of CD11b (78).

Surface-marker analysis. One of the first characterizations of hematopoietic cells in the para-aortic splanchnopleura (P-Sp; intraembryonic hemogenic site at 8.5–10 dpc) pointed to the expression of AA4.1 (43), later characterized by Lemischka et al. (79), as allowing a substantial enrichment of multipotent cells that differentiate in vitro. In the same study, multipotent cells were not enriched in the Sca-1^+ fraction. Subsequently, Sanchez et al. (80) showed that long-term reconstituting cells were all in the c-kit^+ fraction of the AGM and that they coexpress CD34 and low levels of Mac-1 (CD11b). The finding that some cells within this region also express CD31, VE-cadherin, and Tie-2 (expressed in endothelial cells) and that a fraction lack CD45 (a pan-hematopoietic marker) led some authors to propose that an endothelial compartment in this region contained HSCs (81–83, 170).

In an attempt to homogenize and extend the information on cell surface markers within the P-Sp/AGM region, a recent study (81) showed that 10.5-dpc multipotent cells, which also show LTR activity, were all indeed c-kit^+ but expressed low levels of CD45. They all also coexpress CD31, which was subsequently shown to be present in hematopoietic cells in the FL and BM and thus cannot be taken as a strict endothelial marker (82, 83). In this same region, Mac-1^+ cells are also present, and they correspond to monocyte/macrophage-committed precursors likely of YS origin (81). In situ labeling experiments have shown that CD41 is a marker of early hematopoietic cells but is absent in endothelial cells (81, 84–86). In adult BM, CD41 expression is restricted to megakaryocytes (87). The coexpression of CD41 (a strict hematopoietic marker) on all *c-kit*$^+$ cells also shows that no endothelial components are present in this fraction at this stage of development (71). *c-kit*$^+$ cells also express, as previously mentioned, a battery of endothelial markers (CD34, CD31, VE-cadherin, Tie-2), and some are even $Flk1^+$ (**Figure 3**). Whether this coincidence reflects a common precursor or a functional requirement remains to be determined. As development progresses, *c-kit*$^+$ cells express increasing levels of CD45, which labels most hematopoietic precursors in 11.5 dpc (88).

As circulation is established at the four-to-five-somite stage (8 dpc) (10, 48), no clear difference in surface phenotype was ever obtained between YS and AGM hematopoietic precursors. In YS, the first expressed markers are again *c-kit* and CD41 (85, 86, 89). By 10.5 dpc, the hematopoietic populations in both sites appear similar. However, a quantitative in vitro differentiation potential assay revealed that YS *c-kit*$^+$ cells exhibit a lower frequency of multipotent cells than AGM, while having a much higher frequency of myeloid progenitors (81). These myeloid progenitors originate in the YS, although some might also be of intraembryonic origin (90). A careful examination of the expression profiles shows that the frequency of AA.1, CD34, Tie-2 bright, and *c-kit*$^+$ cells is considerably higher in AGM than in YS hematopoietic cells (**Figure 3*c***). When isolated by flow cytometry, these brightly stained cells comprise multipotent cells at frequencies close to 1:1 (A. Cumano & I. Godin, unpublished observations). In conclusion, no particular phenotype distinguishes between the multipotent long-term reconstituting cells and YS-derived erythromyeloid precursors.

Direct visualization of hematopoietic stem cells in the aorta, gonads, and mesonephros. The direct visualization of the emerging HSCs in the AGM requires a

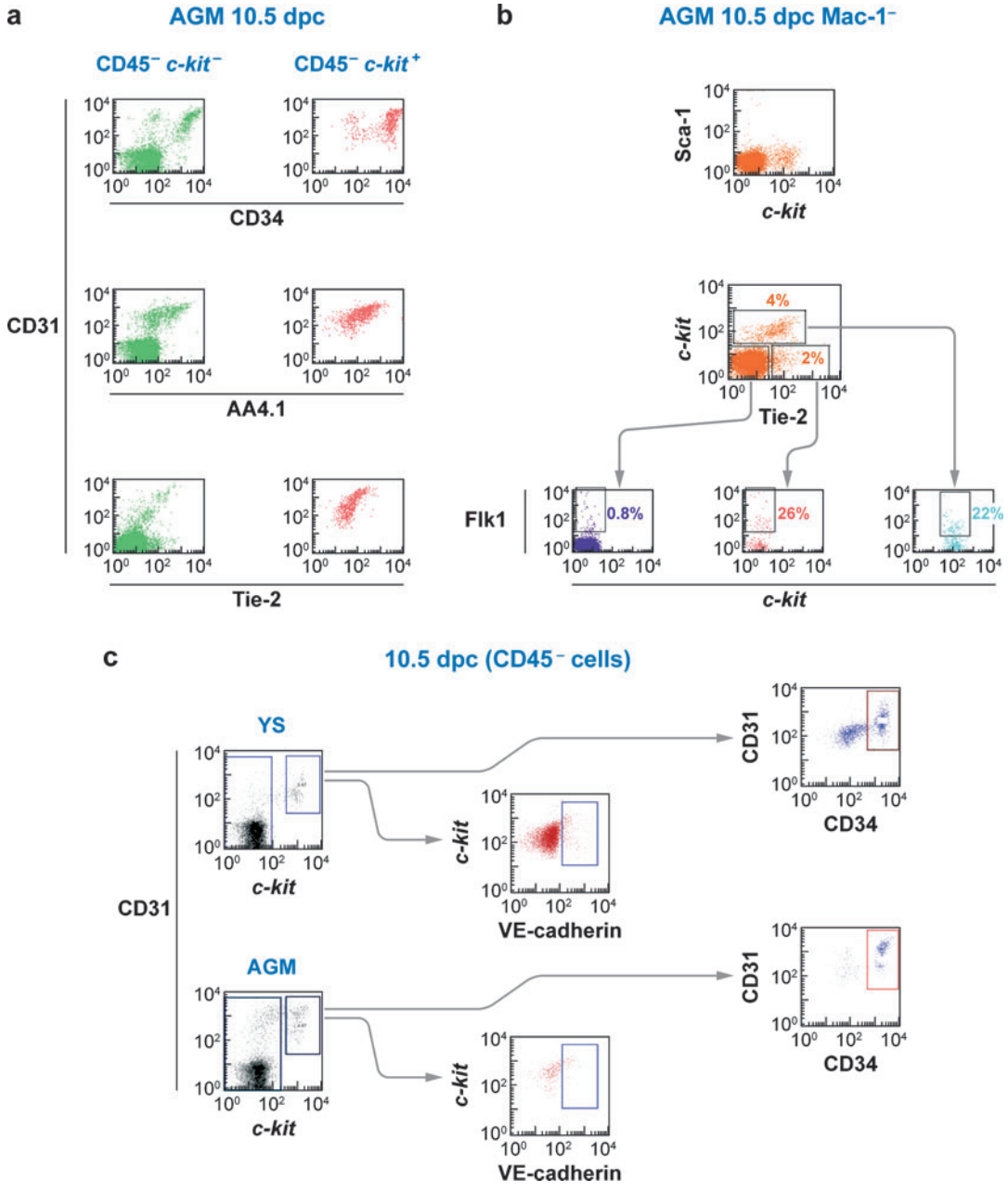

Figure 3

Flow-cytometric profiles of cell suspensions obtained from 10.5-dpc aorta, gonads, and mesonephros (AGM) and yolk sac (YS). (*a*) Profile of the staining of AGM cell suspensions with CD31, Tie-2, CD34, and AA4.1 gated on both *c-kit*$^+$ hematopoietic cells and *c-kit*$^-$ cells. Because most hematopoietic multipotent cells do not express CD45, these cells that comprise monocytes and macrophages at this stage were gated out. (*b*) Flow-cytometry profiles showing the representation of Sca-1, Tie-2, Flk1, and c-kit in AGM cell suspensions. In this experiment macrophages were gated out using the Mac-1 (CD11b) antibody. (*c*) Flow-cytometry profiles comparing AGM and YS cell suspensions for the expression of CD31, VE-cadherin, c-kit, and CD34.

precise phenotypic characterization that allows the discrimination of the different cell types present in situ. Classical histological analysis of sections of the dorsal aorta have shown that, as initially observed in the avian model, hematopoietic cell clusters appear in the ventral wall of the aorta in close association with the endothelium in both mouse and human embryos. Several laboratories have consistently observed the strict association of hematopoietic cell clusters with the ventral wall of the aorta, in a restricted region (**Table 2**). Cell-fate map experiments using avian chimera have shown that the ventral endothelium and the mesenchyme subjacent to the ventral aorta have a distinct embryonic origin from that of the dorsal aorta (91). This observation supports the notion that the ventral wall of the aorta, in contrast to the dorsal aspect, possesses a hemogenic property.

In the sequence of phenotypic characterization, in situ stainings aiming at detecting expression patterns associated with the emerging HSCs have shown the expression in this location of Lmo-2 (66), Tal-1 (67, 92, 93), Tie-2 (94), Flk1 (67, 92, 95), and Runx1 (96) (**Table 2**). All these cell surface markers and transcription factors expressed effectively participate in the formation of blood vessels, and their inactivation results in a drastic reduction of hematopoiesis (94, 97–101). Other proteins such as AA4.1, GATA-3, and BMP4 have also been detected (66, 102). Although AA4.1 inactivation has not been reported, BMP4 inactivation leads to embryonic lethality with reduced splanchnic mesoderm and abnormalities in the lateral plate formation (103). GATA-3 inactivation does not considerably affect hematopoiesis (104–106). Whether these proteins are expressed in the hematopoietic cells or in their environment remains uncertain (**Table 2**). It is striking, however, that the number of marked cells largely exceeds that of functional multipotent hematopoietic cells.

Ly6A/Sca-1 is a marker in HSCs present in the adult BM (75). A mouse carrying a transgene expressing GFP under the control of the Sca-1 promoter aimed at marking nascent HSCs. In this mouse, GFP can be found in some c-kit$^+$ 11-dpc AGM cells, although Sca-1 is expressed at low levels in 10.5–11-dpc HSCs in the embryo and is undetectable by conventional antibody staining. However, in addition to HSCs, other cell types express Sca-1, namely mesenchymal and endothelial cells (107). Nevertheless, cell-sorting experiments showed an enrichment for HSCs based on the expression of c-kit and GFP. The strict localization of GFP$^+$ cells in the endothelial layer of the aorta led these authors to speculate that HSCs indeed originated in the endothelial layer; this finding supports the hemogenic endothelium theory of HSC formation (107). Although compelling, these experiments present a number of intriguing observations. The identified HSCs that express CD31 and GFP are located in the ventral and the lateral wall of the aorta and appear randomly interspersed within the endothelial layer, inconsistent with the in situ localization of HIACs. No HIACs were observed in situ; thus, the identification of HSCs expressing GFP relies only on the cell-sorting experiments. That approximately half the cells that express GFP do not express c-kit suggests that, in this location, GFP expression is not restricted to HSCs, which could explain the unconventional localization of the putative HSCs.

More recent evidence on the precise location of HSCs in the P-Sp/AGM involved in situ multicolor confocal analysis. As previously reported, functional in vivo and in vitro experiments identified that HSCs express c-kit, AA4.1, CD31, and more importantly CD41 (81). CD41 is not expressed in endothelial cells and allows the unequivocal identification of hematopoietic cells. The in situ analysis revealed that hematopoietic multipotent cells were located in the intra-aortic clusters but also in the mesenchyme underlying the endothelium within clusters of CD31$^+$ CD41$^-$ cells, in the structures previously identified as SAPs. No double-stained CD31/CD41 cells were identified within the

endothelial layer of the aorta, as in the study previously described. We are therefore left with some contradictory results that nevertheless coincide to identify HSCs in close association with the ventral wall of the dorsal aorta both in intra-aortic clusters and some within a particular set of mesenchymal cells in the subjacent region (81). Still, all these experiments combined have not provided conclusive evidence for either the generation process or the exact location of the differentiating cells.

OTHER EXTRA-EMBRYONIC GENERATION SITES?

In the avian embryo, the allantois combines both mesoderm and endoderm and as such may potentially contribute to blood cells, as it harbors the same embryonic layer makeup as the YS and the early intraembryonic hemogenic site. Caprioli et al. (69) experimentally tested this possibility by grafting quail precirculatory allantoic buds into the coelom of chick recipients. The host embryo BM, analyzed at the onset of hematopoietic activity, harbored allantois-derived hematopoietic and endothelial cells, suggesting that both hematopoietic and endothelial precursors may emerge in situ in precirculatory allantoic buds and reach the BM via the blood flow.

Two recent publications (107a,b) indicate that such potential is present in the mouse allantois, although it is purely mesodermal: Previous results showed that no hematopoietic contribution could be obtained from precirculatory allantoic explants in vitro (70, 71). However, by bringing in an organ culture, both authors reveal an erythromyeloid potential in the allantois. Moreover, Zeigler et al. (107a) obtained a similar hematopoietic progeny from chorion maintained in organ culture.

At later stages, two groups (72, 73) reported that the mouse placenta might serve as a stem cell reservoir starting at 11 dpc (i.e., at the onset of FL colonization by AGM-derived HSCs) because cells recovered from this location display LTR activity when transferred into adult irradiated recipients. Placenta LTR HSCs are comprised in a population expressing c-Kit and CD34 (72, 73), as well as CD31, CD41, and CD45 (73), a phenotype typical of FL or circulating HSCs at the developmental time analyzed (12 dpc).

Three mutually nonexclusive phenomena may explain the formerly unnoticed presence of HSCs in the mid-gestation placenta. First, placental HSCs may simply reflect the presence of this cell type in systemic circulation, their recovery from this site being facilitated by the abundance of blood in this physiological sponge. Second, the placenta may provide an environment suitable for peripheral blood HSC accumulation/adhesion and expansion, the functional significance of which remains to be determined. Third, the placenta may perform de novo HSC generation, an issue difficult to assess owing to the presence of HSCs in the vascular network during the stages when these cells are retrieved from the placenta (11 to 15 dpc), from the AGM (up to 12.5 dpc), and later from the FL.

Two groups attempted a comparative quantification of LTR HSCs to approach these possibilities. The highest frequency of placenta HSCs occurs at 12.5 dpc (72), when the generation process in the AGM ceases (32), concomitantly with the beginning of FL activity in HSC expansion and differentiation. The low frequency of HSCs—1 per 49,000 cells; i.e., 12 HSCs per 12-dpc placenta (73)—argues in favor of a sequestration of circulating cells, as does the lack of HSC expansion on placenta organ culture, although this latter failure may also be attributed to inadequate culture conditions (73). This quantification strictly correlates with the number of multipotent hematopoietic precursors previously detected in the 12-dpc placenta in an in vitro analysis (74). In this later study, the number of multipotent precursors found in the peripheral blood and FL at this stage exceeded that of the placenta. In contrast, the comparison of LTR-HSC content in the AGM, placenta, peripheral blood, and FL (72) suggests that

the highest number is found in the placenta, leading the authors to conclude that this organ is a suitable niche for HSC accumulation and/or in situ generation.

MODELS OF HEMATOPOIETIC PRECURSOR GENERATION

Presently, only two sites of hematopoietic cell generation have been unambiguously identified, namely the YS–blood island mesoderm and the ventral aorta within the P-Sp/AGM. This identification was possible through experimental approaches carried out before the possible occurrence of cross-contamination through systemic blood flow.

Extra- and intraembryonic hemogenic sites share a set of common features—the germ layer components (both are Sp), the genes involved in HSC generation (*flk-1*, *Tal-1/SCL*, and *lmo2*), and the precursor phenotype—but they also display a number of differences (environment, gene expressed) that may be involved in the different potentials displayed by the generated precursors.

Does Mesoderm Commitment Toward a Hematopoietic Lineage Gives Rise to the Same Type of Hematopoietic Precursors in Both Extra- and Intraembryonic Compartments?

The striking opposing features of YS and intraembryonic hematopoietic precursors regarding the differentiation potential and maintenance capacity may reflect two alternative developmental pathways: (*a*) The path leading from extraembryonic mesoderm to YS-hematopoietic precursors would completely differ from that leading from intraembryonic mesoderm to HSCs; or (*b*) in both compartments, the sequence of events may be initially identical, the mesoderm giving rise to the same type of hematopoietic precursor. Further limitation in YS-precursor self-renewal and differentiation potential would thus result from constraints imposed on these YS-hematopoietic precursors by the environment.

A few experimental data suggest that extra- and intraembryonic native mesoderm have a similar competence to giving rise to HSCs. The strongest evidence again comes from experiments performed in the amphibian model. In the course of reciprocal transplantation experiments in which the ventral blood island (extraembryonic hemogenic site) was transferred to the DLP region (intraembryonic hemogenic site) and vice versa, the contribution of both precursor territories conformed to the site to which they were engrafted. This capacity is lost at the neural stage (108). These results, which imply that mesodermal precursors in both sites are equally competent, stress the differential ability of the environments in both hemogenic sites to modulate the potential of the hematopoietic precursors. The capacity of amphibian YS precursors to give rise to T cells also favors a similar process of hematopoietic precursor generation in the two compartments (21).

A common mechanism of mesoderm determination toward hematopoietic fate is suggested by the fact that the genes so far identified as absolutely required for hematopoietic cell generation (*Flk-1*, *Tal-1/Scl*, and *lmo2*; see below) similarly affect the extra- and intraembryonic compartments, whereas the genes only required for full HSC development (such as *Gata-2* and *Runx1*) do not affect generation sensu stricto. In mammals, early YS-derived precursors gain multilineage long-term reconstituting activity on exposure to AGM-derived cell lines (109), suggesting that the intraembryonic environment positively regulates the potential of YS-derived precursors. However, this shift of activity has not been described for other AGM-derived cell lines (110), nor have the mechanisms involved been elucidated.

Supporting the concept of similar competence of the hematopoietic mesoderm is the observation that features characterizing HSCs (LTR ability and multilineage differentiation potentials) are gained by YS-derived

precursors on HoxB4 forced expression (111). That native YS and AGM precursors share an initial c-kit$^+$CD31$^+$CD41$^+$CD45$^-$ phenotype, despite their different outcomes, might also be relevant to this issue.

Together, these data give some weight to the hypothesis of a permissive function of environment on the differentiation and self-renewal potential of hematopoietic precursors. However, the amphibian transfer experiments seem to suggest that this competence is transient.

Numerous data on hematopoietic cell generation in the YS are available from experimental work performed in lower vertebrate models, as well as phenotypic and lineage relationship analyses in the murine model, mainly in embryonic stem (ES) cells and to a lesser extent during normal ontogeny. In contrast, little is known about HSC generation in the AGM. We thus review the available information for the YS below and point to converging and diverging mechanisms in the intraembryonic compartment.

From Mesoderm to Hematopoietic Cells: Function of the Endoderm

A common feature during extra- and intraembryonic hematopoietic precursor ontogeny is that hematopoietic cell generation occurs in a combination of mesoderm and endoderm germ layers called Sp, irrespective of the extra- or intraembryonic location (**Figure 2*a***). Nevertheless, in the YS Sp, mesoderm is combined to the visceral endoderm, which precedes gastrulation. In contrast, in the intraembryonic Sp (the presumptive AGM territory), mesoderm is tied to the definitive endoderm that derives from the epiblast during gastrulation (112, 113).

The visceral endoderm exerts two distinct effects on endothelial and/or hematopoietic development. At the onset of gastrulation, the visceral endoderm has an instructive effect on the whole epiblast to pattern the embryo and to specify the mesoderm and definitive endoderm toward its various fates, including hematopoietic and endothelial (114). These events depend on endodermal Wnt signaling, combined with BMP4 signaling from the extraembryonic ectoderm, among others. (For a review on normal and ES cell epiblast patterning, see Reference 115.) Once allocated to the YS, the extraembryonic mesoderm is then capable of autonomously producing hematopoietic and endothelial cells. This was established first in the avian model (116) and then in the murine model (114, 117) by experiments in which YS-mesoderm explants maintained in vitro in the absence of contaminating endodermal cells produced low levels of endothelial and hematopoietic cells. However, an adequate production of endothelial and hematopoietic cells requires the presence of soluble factors secreted by the endoderm because such production is only reached when these explants are combined with visceral endoderm (114, 116).

Researchers first investigated the commitment of the mesoderm toward a hematopoietic fate in the lower vertebrate models [amphibian (118, 119) and zebrafish (120, 121)] in which BMP4 signaling was identified as a key player. In the early murine embryo, the competence of the epiblast (the embryonic layer that gives rise through gastrulation to all the ectodermal and mesodermal derivatives, as well as the definitive endoderm) in producing hematopoietic cells, which is revealed on culture on the OP9 stromal line (122), is progressively restricted to the posterior fragment of the epiblast. As a consequence, the anterior epiblast, which is fated to produce neural tissue, is the first territory that loses the capacity to produce hematopoietic cells in such a condition. However, the addition of BMP4 to the culture allows this fragment to retain the ability to produce hematopoietic cells (123). Accordingly, BMP4 also promotes hematopoietic differentiation from murine (124) and human (125) ES cells. Park et al. (126) recently established in the murine ES model that BMP4 is required to induce mesodermal

precursors expressing the tyrosine kinase receptor flk-1 and the bHLH transcription factor Tal-1/SCL.

Gene invalidation in the mouse points to flk-1 and its ligand VEGF as essential for the initiation of endothelial and hematopoietic development. Flk-1 is expressed in the early mesoderm (127) and remains expressed afterward in a large array of mesoderm-derived cells types, including endothelial, hematopoietic, cardiac, and skeletal muscle cells (128). Flk-1 is strictly required for the generation of endothelial and hematopoietic cells in both the extra- and intraembryonic compartment (100, 129), owing to a defect in the migratory behavior of mesoderm cells (100), rather than an inability of Flk-$1^{-/-}$ cells to produce endothelial and hematopoietic cells (130, 131).

Among the endoderm-derived factors that may influence blood cell development, VEGF, which is expressed by the visceral endoderm (132), stands out as a major factor for blood island hematopoietic and endothelial cell development (133, 134). In *Drosophila*, VEGF is involved in blood cell guidance (135). In mammals, endoderm-derived VEGF may act as a cue to direct flk-1-positive mesodermal cells to the blood islands, but may also expand YS-erythroid cells (136). VEGF function in mesoderm-derived cell migration seems to apply in the intraembryonic compartment because, in *Xenopus* embryos, VEGF secreted by the hypochord (a transient structure of endodermal origin, present in amphibian and zebrafish embryos) may guide angioblast migration during the formation of the dorsal aorta (137). One may again hypothesize that such an attraction may also target the intraembryonic Sp, the mesodermal cells that give rise to intraembryonic HSCs, as well as the aorta angioblasts. Indeed, VEGF is expressed in the intraembryonic Sp endoderm when the paired aortae are already formed (i.e., at the time when HSCs emerge from the precirculatory Sp in organ culture) (138). Other factors (lmo2 and GATA-3), which display an endodermal expression restricted to the intraembryonic compartment (66), possibly play a role in intraembryonic hematopoietic development. Some actors involved in the steps leading from native mesoderm to hematopoietic cells in the extraembryonic compartment are thus present in the intraembryonic hemogenic site.

Invalidation of BMP4 in the mouse (103) leads to a variable phenotype (owing to contamination with maternal protein) from no mesoderm to reduced blood island formation (in the YS) and reduction in lateral plate mesoderm formation (which gives rise to the intraembryonic Sp from which the P-Sp/AGM develops). BMP4 is also expressed in the AGM (**Table 2**) in the area ventral to the aortic floor that contains the SAPs, both in murine (I. Godin & A. Manaia, unpublished results) and human (102) embryos, and may also be involved in intraembryonic HSC production.

Initiation of Hematopoietic and Endothelial Cell Production

Gene invalidation in the mouse allowed the identification of a set of genes (*Tal-1/SCL* and *lmo2*) that are equally required at the initial stage of hematopoietic cell production in both extra- and intraembryonic hemogenic sites. The bHLH transcription factor *Tal-1/SCL* and the transcription regulator *lmo2* are both expressed in the extraembryonic mesoderm immediately before YS blood islands are morphologically identifiable [*Tal-1/SCL* (93, 139, 140), *lmo2* (66, 141)] and thereafter are expressed in both endothelial and hematopoietic cells. The disruption of these two genes produces a similar phenotype, namely the absence of YS blood cells and a reduction in endothelial cells [*Tal-1/Scl* (142, 143), *lmo2* (144)]. Moreover, in complementation chimera, no contribution of invalidated ES cells to further stages of hematopoietic development (FL) was observed, meaning that these genes are also required for HSC generation in the AGM [*Tal-1/Scl* (98, 99), *lmo2* (97)].

In the zebrafish, disrupting the *Cloche* gene leads to a severe reduction in the amount of generated endothelial and hematopoietic

precursors, a defect partially rescued by a forced expression of *Tal-1/Scl*, thus placing this gene (whose mammalian homolog is still unknown) upstream of *Tal-1/Scl* (145). Moreover, *Tal-1/Scl* overexpression causes an overproduction of endothelial and hematopoietic precursors (lateral plate mesoderm), at the expense of the mesoderm normally fated to produce the somites (paraxial) and pronephros (intermediate) (146).

Whereas all the genes necessary for hematopoietic cell generation (and differentiation) in the YS are also required for HSC production in the P-Sp/AGM, the reverse is not true; the latter depends on a set of genes whose disruption does not interfere with YS-derived hematopoiesis. Among these genes, some (*Runx-1* and *GATA-2*) are first expressed in the extraembryonic mesoderm, then by YS endothelial and hematopoietic precursors (96, 141), as well as in the AGM (81, 96, 147). The invalidation of these genes leads to embryonic lethality between 9.5 and 11.5 dpc (at the stage extending from the starting hemogenic activity in the P-Sp/AGM and the onset of HSC expansion and differentiation activity in the FL) (101, 148–150).

Type of Precursors Produced: Hemangioblast? Hemogenic Endothelium? Other Alternatives?

The hierarchy tree leading from native mesoderm to hematopoietic precursors in the YS and AGM is currently unknown, although analysis of ES cell differentiation provides increasing information on extraembryonic mesoderm differentiation toward the endothelial and hematopoietic lineages, especially when they are compared with phenotype and potential analyses gathered during normal ontogeny.

Because of the close developmental relationship between the endothelial and hematopoietic lineages, the current models for hematopoietic precursor generation emphasize a direct ontogenetic link between the two lineages. (*a*) In the YS, the currently accepted model is that hematopoietic cell generation involves an intermediate and transient bipotent precursor endowed with the ability to give rise to both endothelial and hematopoietic cells within the blood island (**Figure 1*b***). (*b*) As for the intraembryonic compartment, most authors stand for an HSC generation process involving the transdifferentiation of the endothelial cells from the aortic floor, qualified as hemogenic endothelium (see **Figure 4**, below).

Hemangioblast: mesoderm-derived bipotent precursor that gives rise to endothelial and hematopoietic cells

Extraembryonic compartment. Ranvier first put forward the hemangioblast model as early as 1874 (cited by Reference 151). He primarily considered the first step of the cytological evolution of YS blood islands, the hemangioblastic chord when cells in the chord do not yet display morphological differences (step 1 in **Figure 1*a***). The second model considered by early embryologists was that of the hemogenic endothelium, according to which already differentiated endothelial cells are able to give rise to hematopoietic precursors. The holders of this model (reviewed in 151) focused on the intermediate step of blood island evolution, when morphologically identifiable endothelial cells are still linked by a cell junction to the inner cells of the blood island during the lumen formation process (step 2 in **Figure 1*a***).

Two possible mechanisms may account for the simultaneous development of endothelial and hematopoietic cells from aggregates of seemingly identical cells. Either hemangioblastic aggregates result from hemangioblast expansion and differentiation and would thus be clonal in origin, or they result from selective adherence of lineage-unrelated mesodermal cells **(Figures 1*b*,*c***). To address this question, Tam's group (152) set out to allocate in the epiblast the cells that will ultimately give rise to YS endothelial and hematopoietic cells (152). The authors orthotopically grafted epiblast fragments taken at various gastrulation time points from *LacZ*- or *GFP*-expressing embryos to normal

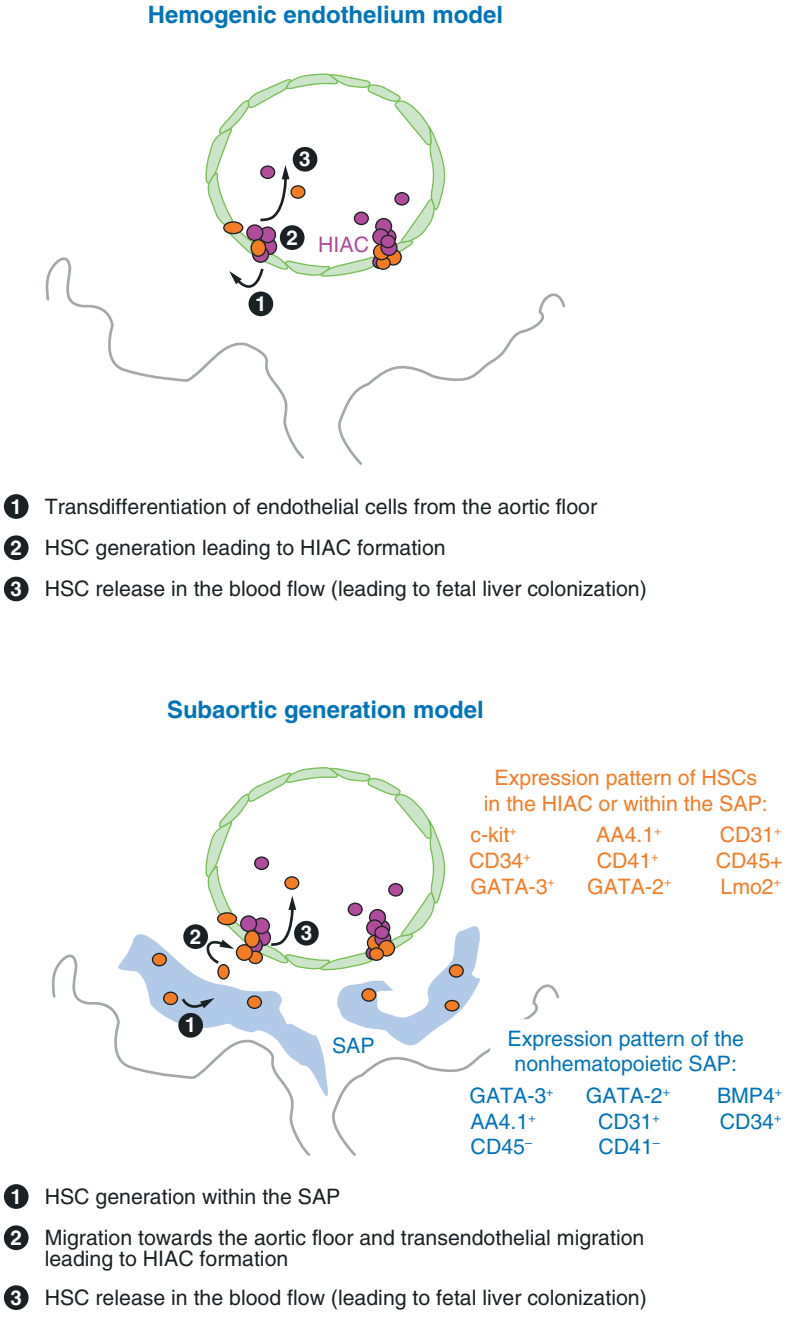

Figure 4

Schematic representation of the different models of HSC generation in the AGM region. The hemogenic endothelium and the subaortic generation models are shown. The surface markers and transcription factors expressed by the newly formed hematopoietic cells in the HIACs (*orange*) and the mesenchyme in the SAPs (*blue*) are shown.

embryos and allowed them to develop in vitro for a few days. Examination of the distribution and nature of labeled cells indicated that a simultaneous contribution to endothelial and hematopoietic cells constitutes a rare event. Cells aimed at giving rise to erythroid cells are produced first, whereas epiblast contribution to the majority of endothelial cells takes place later during gastrulation. Recently, Ueno et al. (152a) developed another approach to test the clonal origin of hemangioblastic aggregates, by following the contribution to YS blood islands of the progeny of ES cells labeled in three different colors, after injection in a wild-type blastocyst. Within individual blood islands, endothelial and hematopoietic components were multicolored, showing the polyclonal nature of hemangioblastic aggregates. These observations lead to the conclusion that precursors for the endothelial and hematopoietic lineages are independently recruited during the gastrulation stages analyzed but might be present at earlier stages. Thus, clonality for all components of a single blood island is thus inconsistent with these experimental data, and there is no direct evidence of a lineage relationship between the endothelial layer of the blood island and the primitive erythrocytes within.

Nevertheless, more relevant to developmental hematopoiesis than whether the hemangioblast exists is the overall analysis of mesodermal cell evolution in the ES model and in the YS that has been developed in recent years. These studies allowed the characterization of intermediate stages from native mesoderm to hemangioblastic mesoderm during ES development.

It is now accepted that the developmental steps occurring during the differentiation of ES cells and embryoid bodies recapitulate those taking place during normal ontogeny. During developmental hematopoiesis, this is particularly well documented for the events occurring during extraembryonic precursor generation and differentiation, based on the sequence of expression of various markers (e.g., transcription factors, growth factor receptors, adhesion molecules) correlated to in vitro analyses of precursors isolated from ES, embryoid bodies, or YS at various development stages.

Keller's group (153) identified, within differentiating embryoid bodies, the sequence of intermediate mesodermal precursors that generate hematopoietic and endothelial cells. The first colonies to appear after the initiation of expression of the mesodermal marker *brachyury*, called transitional (153), correspond to the developmental stage immediately preceding the determination of the mesoderm toward the hemangioblastic lineage. These transitional colonies produce cardiomyocytes as well as hematopoietic and endothelial cells. The next step in the sequence involves the specification among *brachyury*-expressing mesodermal cells into a flk^- subpopulation that leads to cardiomyocytes (154), whereas $brachyury^+/flk\text{-}1^+$, which is present in the posterior part of the primitive streak of $brachyury^{GFP/+}$ embryos (155), contains blast colony–forming cells (156–158). Blast colony–forming cells, which no longer express *brachyury*, are considered the in vitro ES-derivative equivalent to hemangioblasts. Their development depends on VEGF and gives rise to endothelial and hematopoietic cells (157, 158). In a complementary approach, Nishikawa's group (159) set out to correlate the phenotype and in vitro potential of ES cells at various differentiation stages, at both the population and single-cell level. $Flk\text{-}1^+$ single ES cells gave rise to endothelial and hematopoietic cells, as well as to smooth muscle cells (lateral plate mesoderm derivatives) (159), so their differentiation potential is larger than the bipotent fate attributed to the hemangioblast. Indeed, the same group recently showed that $Flk\text{-}1^+$ ES cells also produce paraxial mesoderm derivatives (myocytes, chondrocytes, osteocytes) in addition to lateral plate mesoderm derivatives (160). Within the $Flk\text{-}1^+$ subpopulation, mesodermal precursors endowed with hemangioblastic potential are mostly composed in the $Flk1^+VE\text{-}cadherin^+CD45^-$

subset, sorted either from differentiating ES cells or from the YS and embryo body of 9.5-dpc embryos. However, some hemangioblastic progeny is also obtained from the Flk1$^+$VE-cadherin$^-$CD45$^-$ subset (55, 161). In both subpopulations sorted from ES cell cultures or from the YS and caudal part of 9.5-dpc embryos, the ability to produce an erythromyeloid progeny strictly correlates with the expression of α4-integrin (162).

Although both Keller's and Nishikawa's group have shown in a rigorous single-cell experimental setup the existence of a common precursor for hematopoietic and endothelial cells and established the conditions that allow them to develop in vitro, other differentiation potentials have not been investigated, and the strict bilineage nature of these cells awaits confirmation. The hypothesis of a hemangioblastic origin of blood cells in the YS is thus widely accepted but is still waiting for a direct demonstration.

As mentioned above, the first differentiated hematopoietic cells appear in the YS blood islands at the neural plate stage (7.0 dpc) (141). The best-characterized population at this stage is the first wave of primitive erythrocytes. The other cell types present in the early YS are primitive megakaryocytes prone to an accelerated production of platelets and a reduced ploidy compared with adult type megakaryocytes (163) and macrophages (90, 141). Interestingly, these various cell types first derive from monopotent precursors, the production of pluripotent (erythromyeloid) precursors representing a secondary event that occurs at approximately 8 dpc (90, 141), so in the YS, the mature hematopoietic cells appear before immature precursors in an inverted pathway compared with later stages of ontogeny and adulthood.

Intraembryonic compartment. As stated above, a major difference in extra- and intraembryonic mesoderm differentiation is that the YS Spl mesoderm gives rise simultaneously to endothelial and hematopoietic derivatives, whereas in the intraembryonic compartment, vascular development occurs without concomitant hematopoietic cell production. Indeed, recent analyses performed during gastrulation indicate that precursors aimed to give rise to the intraembryonic vasculature, which do not display hematopoietic potential, are recruited independently from those that will produce extraembryonic endothelial and hematopoietic cells (71). As a consequence, the hypothesis of a common hemangioblastic precursor in the intraembryonic Sp does not appear likely. However, the localization of intraembryonic HSCs in the aortic region points again to possible lineage relationships between endothelial and hematopoietic cells.

In the early nineteenth century, researchers believed that the intra-aortic clusters arising in the aorta derived from the underlying endothelium but differed from the hemogenic endothelium of the YS (step 2 in **Figure 1*a***) by the type of produced cells, "which would be more immature in the aorta and would only secondarily give rise to red cells" (reviewed in 151, but see also 164). The theory of an endothelial origin of AGM HSCs was revived with the rediscovery of the aortic clusters, which followed the identification of intraembryonic HSCs. In support of this model were the concordant results obtained by various groups aiming to characterize the phenotype of AGM HSCs. Most of the used markers—VE-cadherin, the angiopoietin-1 receptor Tie-2/Tek, CD31, Runx (165–167)—except Sca-1, which is expressed late during ontogeny, are expressed by native mesodermal cells and remain expressed by mesodermal cells' endothelial and hematopoietic derivatives, including cells from the intra-aortic clusters. To discriminate endothelial and hematopoietic cells, the authors looked for the expression of the pan-leukocyte marker CD45 and the ability to uptake acetylated low-density lipoproteins (AcLDLs) as a marker for endothelial cells. In all instances, HSCs were identified in the fraction negative for CD45 expression and, when tested, positive for AcLDL uptake,

leading the authors (107, 161, 168–171) logically to conclude that the endothelial cells were able to give rise to intraembryonic HSCs through a transient transdifferentiation process (**Figure 4**).

An alternative to this hemogenic endothelium pathway is that native AGM HSCs would lack CD45 and that more cell types than initially thought would be capable of AcLDL uptake during ontogeny. Indeed, a recent study aiming to clarify the phenotype of native HSCs and gain some insight into their in situ localization demonstrated that HSCs expressed, in addition to the CD45$^-$ *c-kit*$^+$ CD31$^+$, the CD41 antigen, which was expressed only by hematopoietic cells (81). These AGM HSCs express a set of transcription factors shared by hematopoietic and endothelial cells (Lmo2 and GATA-2), but also GATA-3, which is not expressed by endothelial cells. Cells expressing these transcription factors and cell surface markers were further allocated in situ both within HIACs and SAPs, indicating that they do not colocalize with endothelial cells. These HSCs display AcLDL uptake capacity, an ability shared by most AGM cells at this stage (81). An alternative model that takes into account the extended phenotype of native HSCs and their in situ localization could be drawn from this model (**Figure 4**). According to this model, HSCs would be generated within SAPs, whose nature and function remain to be elucidated. HSCs would migrate toward the aortic floor and reach the blood flow to colonize the FL. This translocation across the ventral endothelium of the aorta would lead to the appearance of HIACs, which are detected only when the number of AGM HSCs reaches a peak at 10.5 dpc (32).

FATE OF HEMATOPOIETIC CELLS

Environment

As mentioned above, the particular location of HSCs in the BM and their interactions with the surrounding stroma are difficult to investigate by conventional imaging techniques. As a consequence, the nature of the cellular interactions in the BM that might determine whether an HSC is resting or entering a cell cycle and whether, on division, differentiation or self-renewal occurs remains poorly understood. Recently, direct visualization of the presumptive HSCs in the BM and their interactive cellular partners has been achieved.

Suda's (172) group has concentrated on the role of the molecule angiopoietin-1 (Ang-1) in this interaction. Although expressed by both hematopoietic and what have been identified as osteoblastic cells, Ang-1 has been shown to be responsible for the maintenance of HSCs in a resting multipotent stage of undifferentiation. In parallel studies, Scadden and collaborators (173) showed that in a transgenic mouse, in which the number of osteoblasts is increased by the expression of a constitutively active parathyroid hormone receptor, not only is the bone mass increased, but so is the number of HSCs. The mediator for this increase has been identified as the Notch ligand Jagged1 (173, 174). Interestingly, although HSCs appear increased, stromal lines derived from these mice are unable to transfer the phenotype and appear grossly abnormal in their capacity to induce BM formation in ectopic explants (175).

Along the same line, mice with a conditionally inactivated BMP receptor exhibit higher numbers of osteoblastic cells that express N-cadherin. As a consequence, higher numbers of HSCs are present in the BM. The Wnt signaling mediator β-catenin was shown to be expressed by HSCs as the interaction occurs (176, 177). In this case, as in the abovementioned studies, HSCs were located close to the bone surface and mainly in trabecular bone.

Taken together, these three studies indicate that resting HSCs are located in BM in close contact with osteoblastic cells, and the number of HSCs is limited by the number of osteoblasts, so an increase in the number of osteoblasts leads to an increase in the number of

HSCs. The molecular partners that are functionally determinant in this interaction are still a matter of speculation. β-catenin conditional inactivated mice have normal numbers of HSCs in the BM, indicating that this signaling pathway is not essential for maintaining HSCs (178), but β-catenin overexpression leads to a block in HSC differentiation, resulting in the accumulation of HSCs (178a,b).

More recently, Morrison's group described an alternative combination of surface markers that allow the purification of HSCs in BM. The expression pattern of SLAM family members allowed them to distinguish between long- and short-term reconstituting cells. Thus, HSCs express a unique combination of $CD150^+$, $CD48^-$, and $CD224^-$ (77). BM cells isolated according to this protocol exhibit a frequency of LTR close to 1:3, similar to previously published enrichment procedures. Using this simplified identification scheme, the authors analyzed the anatomical location of HSCs in the BM and spleen and showed that HSCs are in close contact to the bone surface (as previously mentioned) but also that a large fraction of these cells were in contact with the endothelium of sinusoids. In summary, HSCs in the BM appear to be in contact with either mesenchymal cells in the bone surface or with endothelial cells in the sinusoids.

Little is known about the hematopoietic environmental properties in the FL. The large expansion of HSCs in the FL between 12 and 15 dpc makes this environment particularly interesting in understanding HSC amplification. However, information on the precise localization of HSCs in the FL is almost nonexistent. In the FL, the hematopoietic niche is obviously different from that in BM, and no differentiated osteoblasts are present in this site. However, myelo-supportive stroma, able in vitro to differentiate into mesenchymal components (osteoblast, chondrocytes, and adipocytes), have been isolated from this organ (179, 180). Therefore, in the FL the amplification of the HSC pool relies on this myelo-supportive stroma, on endothelial cells, or on an unidentified cell type (or pathway). The elucidation of the function of each of these cell types will be fundamental to understanding the processes that lead to hematopoiesis.

Almost paradoxically, more studies are available in the expression of particular molecular patterns in the close vicinity of the emerging HSCs in the AGM. Although many were initially done to recognize the HSCs themselves, and from there to deduce the mechanisms of HSC generation, these studies are valuable in that they identified a number of molecules that are expressed by neighboring cells. Manaia et al. (66) identified cellular clusters that were localized in the ventral aspect of the dorsal aorta, subjacent to the endothelial layer. These structures, the SAPs, coincide in time with the presence of intra-aortic hematopoietic clusters and with the detection of hematopoietic multipotent cells in this region. Cells within the SAPs express hematopoietic markers. The first described were GATA-2, GATA-3, and AA4.1 (66, 81). The number of cells detected in the SAPs exceeds the number of hematopoietic cells detected in this region; thus, another unidentified cellular component coexists in the Sp-AGM region in close association with the emerging HSCs. Later, cells within the SAPs were shown to express CD31 and CD34, both coexpressed by endothelial and hematopoietic cells (81; A. Cumano & I. Godin, unpublished data). These observations were also later done in human embryos in which GATA-3, BMP4, and tenascin-C were detected in this region (67, 68, 181) (**Table 2**). Although most markers are coexpressed by endothelial and hematopoietic cells, GATA-3 and BMP4 stand out as conspicuously absent from endothelial cells. Thus, although the presence of endothelial cells in SAPs cannot be ruled out, another unidentified cell type coexists in this region.

After the presence of stromal cells in this region was first suggested, it was further evaluated (110, 180, 182). Although stromal cell lines have been developed from total AGM

cell suspensions, their location in vivo in relation to the hematopoietic cells has not been determined. In addition, an ex vivo evaluation of the number of osteoblastic progenitors in this region calculated an average of one to two progenitors per embryo (180). This reduced representation of osteoblast precursors does not favor the hypothesis of a physiological role of these cells in the generation and/or expansion of HSCs, as they are unlikely to contact a large number of these cells.

The Hematopoietic Environment

Stem cells, irrespective of where they are specified, will likely be nonfunctional unless they home to the specific location where they can expand and differentiate. The HSCs are mostly found in adult BM, where in close contact with the surrounding stroma they will either maintain their stem cell nature or differentiate into a mature cell type. The stromal cell compartment in the BM is ill defined and includes endothelial cells that surround the sinusoids and mesenchymal cells that can be either interspersed in the marrow, in close contact the bone surface, or with the endothelial cells. These are two well-defined lineages with specific functions. Mesenchymal cells were defined in the 1970s as an independent lineage that comprises osteoblasts, chondrocytes, adipocytes, and fibroblast-like cells that secrete cytokines important for the expansion of differentiated hematopoietic precursors, the myelo-supportive stroma (183). These different cell types coexist in the BM and can be generated in clonal in vivo assays from a unique progenitor, the mesenchymal stem cell. Cells with such properties can be found in the BM of adult mice and humans. One of the main problems when analyzing the stromal cell compartment in a sample of BM is the difficulty in identifying these different cell types by surface markers. In the mouse, these cells are also not highly proliferative in vitro. Researchers have therefore relied on the establishment of stable lines that have been the product either of long-term culture and selection or of active transformation (T SV40). We must keep in mind that these stromal cell lines might or might not preserve the phenotypic characteristics and function performed in vivo. They have nevertheless been extremely important in improving the in vitro differentiation tests in that they are unique in supplying the differentiation and/or survival signals required for the analysis of hematopoietic single-cell differentiation potential. However, in spite of some isolated reports, no maintenance of LTR activity of cultured HSCs has been consistently obtained under these conditions (184). Thus, the signals delivered in such in vitro culture systems do not favor the self-renewal of HSCs.

The technical limitations in the analysis of the BM hematopoietic environment stated above largely justify the slow progress in the characterization of the in vivo function of these cells. Only recently have major advances in imaging technology allowed initial studies that attempt to characterize in situ the microanatomy of the interactions between HSCs and surrounding cells (this complex of interactions has been generally named the niche). Another even less well-characterized cell type in close association with capillary vessels, the pericytes, might or might not contribute to hematopoiesis. Because, in the absence of specific cell markers, their lineage assignment is the position they occupy in close association with endothelial cells in capillaries, they may or may not be a homogeneous population. However, within the general designation of pericyte, there is a cell type distinct from the endothelial lineages, which is important in providing the integrity of the capillaries and whose contribution to the niche is ill defined.

HSCs in the Sp/AGM, as they are generated, likely do not proceed to in situ differentiation. Experiments aiming at detecting intermediate hematopoietic precursors, such as those identified in the YS and later in FL and BM, revealed that the vast majority of hematopoietic cells in this region remain

multipotent (32). The few erythroid- and myeloid-restricted precursors found between 9–12 dpc are also found in the circulating blood and thus are assumed to be contaminants of the cell preparation. Moreover, multipotent hematopoietic progenitors are found in the circulating blood as soon as 10 dpc when HSC generation has not attained a maximum (74). This observation suggests that HSCs rapidly enter circulation, from which they can reach the different hematopoietic organs. As mentioned above, compelling evidence supports the notion that the FL, thymus, or BM is colonized by exogenous HSCs rather than supporting in situ HSC generation from some unidentified undifferentiated mesodermal cell. Thus, the P-Sp/AGM can be considered as a site of HSC generation but not a hematopoietic site because the conditions for supporting hematopoietic differentiation are not active.

Recently, it was shown that interleukin-3 may play a role in the amplification/maintenance of HSCs in the AGM as a Runx-1 effector (185). This surprising observation highlights that hematopoietic cytokines may operate at early stages of HSC development. It nevertheless raises questions about the timing and cell types involved in their synthesis in the embryo.

The Fetal Liver

Hematopoietic cells colonize the FL, starting at 10 dpc, and in situ hematopoietic activity is evident thereafter. Considering the limited number of HSCs generated in the P-Sp/AGM (500–1000 cells) (32) and the extent of hematopoietic activity observed at early stages in FL, it is likely that the majority of cells that initially colonize the FL are progenitors of YS origin that rapidly differentiate to enucleated erythrocytes and myeloid cells. Thus, within 24 h, between 10 and 11 dpc, the frequency of primitive erythrocytes decreases from 80% to 10% of the circulating cells (A. Cumano & I. Godin, unpublished observation). These enucleated erythrocytes probably reach terminal differentiation in the FL by exposure to erythropoietin. Unlike the BM, FL provides a unique set of environmental conditions that allow the expansion of HSCs. Until 15 dpc, HSCs expand in numbers and acquire the final characteristic surface markers that define them in the adult BM (78). In addition to the expansion of HSCs, the FL supports the differentiation of erythrocytes, myeloid cells, and lymphocytes. Although some hierarchical lineage differentiation is already established in the FL and common lymphoid progenitors have been identified, the differentiation potential of these cells appears less strict than that in BM. Thus, the common lymphoid progenitors show some restricted monocyte/macrophage potential; B/macrophage-restricted progenitors have been isolated; and growth requirements, at least within the B lineage, seem to vary from those in BM (186, 187).

The bases for these differences have not been defined, but environmental conditions that are obviously different from those in BM might play a role in this phenomenon. Thus, although stromal cells have been isolated from the FL and they exhibit similar properties to those in BM, the complex cell interactions established in this location that constitute the niche for hematopoiesis might differ considerably. In the absence of markers that allow the direct identification of the mesenchymal compartment, the direct visualization of the interaction between the hematopoietic and stromal compartment in the FL is virtually undocumented.

The origin of HSCs that colonize the FL and undergo expansion and differentiation has also been a matter of debate. The discovery of HSCs in the AGM region raised the possibility that they would migrate to the FL and from there to the BM. In the chicken chimeric system, investigators convincingly showed that intraembryonic hematopoietic cells, in contrast to their YS counterparts, contribute almost exclusively to adult hematopoiesis. In

the mouse, lineage-tracing experiments have been more difficult to perform. A transgenic mouse that expresses Cre-recombinase under the control of the CD41 promoter was crossed to the Rosa-26 loxp mouse. CD41 was expressed in HSCs in the AGM region. Surprisingly, although the contribution of recombined hematopoietic cells to fetal hematopoiesis was high, adult BM HSCs did not show a significant percentage of rearrangements. These results argue that subsequent waves of HSCs, generated after the extinguishment of AGM production, are the main contributors to adult hematopoiesis (84). A subsequent work used the SCL promoter to drive CreERT and to induce, on tamoxifen administration, Cre-mediated deletion in Cre reporter mice. The results show that induction around 10 dpc marked most hematopoietic cells in mid-gestation, and these cells effectively contributed to adult BM hematopoiesis. This experiment shows, in contrast to the previous one, that production of new HSCs after 10–11 dpc is reduced, and if it occurs, it does not significantly contribute to adult hematopoiesis (188). Both experiments are not conclusive; therefore, direct evidence for the lifelong contribution of AGM HSCs to adult BM hematopoiesis is still missing. SCL is expressed in endothelial cells and HSCs, and both experiments rely on the correct expression of the transgene. Moreover, the SCL-mediated deletion in HSCs from the AGM was not directly assessed, and YS-derived hematopoietic cells also express SCL. Therefore, although it is generally accepted that HSCs are not generated in the FL or BM, direct evidence is still inconclusive, and, in addition to the P-Sp/AGM, later waves of YS HSCs could contribute to adult hematopoiesis, as suggested Medvinsky and colleagues (189).

The Fetal Spleen

The fetal spleen is a transient hematopoietic organ starting between 13–14 dpc until the first weeks of postnatal life. HSCs circulating from the FL home to the spleen at 13–14 dpc, and hematopoietic differentiation occurs there (32). HSCs do not expand significantly in the fetal spleen in contrast to the FL (190). In the absence of a robust maintenance of an HSC pool in this site, hematopoietic differentiation relies on the presence of intermediate precursors that either derive from differentiation of HSCs or home directly from the FL to the fetal spleen. Particular lineages of stromal cells might condition fetal spleen hematopoiesis and restrict or favor the differentiation of particular hematopoietic components (190).

CONCLUSION

Compelling evidence that HSCs originate during embryonic development and successively colonize the FL, spleen, and BM suggests that they are generated in a restricted period of time and in precise locations. Here, we discuss experiments that point to the P-Sp/AGM as the initial site of HSC production, although the YS and maybe the placenta contribute at later stages to this cell pool. Although gene-inactivation experiments have revealed a number of tyrosine kinase receptors and transcription factors required for adequate HSC generation, the exact progenitor cell in which they act and the functional activity they exert are still elusive. The past few years have seen an increasing interest in the hematopoiesis field in dissecting the complex cellular interactions that take place in the BM between HSCs and the surrounding mesenchymal compartment (i.e., the niche). The comparative analysis of the mesenchymal compartment in the P-Sp/AGM, but also in the YS and in FL, will indicate the major developmental pathways required for HSC formation and expansion. The understanding of these pathways will allow, in the future, the manipulation of undifferentiated mesoderm cells or even of ES cells for large-scale HSC production in vitro.

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