The human embryo, but not its yolk sac, generates lympho-myeloid stem cells: Mapping multipotent hematopoietic cell fate in intraembryonic mesoderm

Immunity, Vol. 15, 487–495, September, 2001, Copyright ©2001 by Cell Press

The Human Embryo, but Not Its Yolk Sac, Generates Lympho-Myeloid Stem Cells: Mapping Multipotent Hematopoietic Cell Fate in Intraembryonic Mesoderm

Manuela Tavian,[1,3] Catherine Robin,[2,5] Laure Coulombel,[2,4] and Bruno Péault[1]
[1] INSERM U506
Hôpital Paul Brousse
94807 Villejuif Cedex
[2] INSERM U362
Institut Gustave Roussy
94805 Villejuif Cedex
France

Summary

We have traced emerging hematopoietic cells along human early ontogeny by culturing embryonic tissue rudiments in the presence of stromal cells that promote myeloid and B cell differentiation, and by assaying T cell potential in the NOD-SCID mouse thymus. Hematogenous potential was present inside the embryo as early as day 19 of development in the absence of detectable CD34$^+$ hematopoietic cells, and spanned both lymphoid and myeloid lineages from day 24 in the splanchnopleural mesoderm and derived aorta where CD34$^+$ progenitors appear at day 27. By contrast, hematopoietic cells arising in the third week yolk sac, as well as their progeny at later stages, were restricted to myelopoiesis and therefore are unlikely to contribute to definitive hematopoiesis in man.

Introduction

All blood cells are generated from a small cohort of pluripotent hematopoietic stem cells (HSC) through developmentally restricted progenitors which undergo lineage commitment and subsequent differentiation along a single pathway (for review, see Morrison et al., 1995).

It was long assumed that during development in higher vertebrates, the yolk sac (YS), which is the first blood-forming tissue, was also the unique provider of HSC, no intrinsic hematopoietic potential having been found in any other tissue inside the embryo (Moore and Metcalf, 1970). However, experiments in birds later demonstrated that definitive hematopoiesis stems from precursors emerging in the embryo proper (Dieterlen-Lièvre, 1975). In birds, intraembryonic hematopoietic activity is localized in a region neighboring the dorsal aorta, under and inside which blood cell progenitors have been identified (for review, see Dieterlen-Lièvre, 1994). The homologous territory in the mouse embryo, the aorta/gonad/mesonephros (AGM) region and its anlage, the paraaortic splanchnopleura (P-Sp), are endowed with hematopoietic potential (Godin et al., 1993; Medvinsky et al., 1993; Medvinsky and Dzierzak, 1998). Cells expressing markers of hematopoietic progenitors are indeed present in the mouse embryonic dorsal aorta (Garcia-Porrero et al., 1995, 1998; Wood et al., 1997; North et al., 1999; Petrenko et al., 1999).

In the human embryo, we have previously identified a dense population of blood cells adhering to the ventral side of the aortic endothelium that exhibits surface antigens and expresses genes that typify primitive progenitors (Tavian et al., 1996; Labastie et al., 1998; Watt et al., 2000). Hematopoietic cell clusters appear in the aorta at precisely 27 days of gestation, before the incipience of hepatic hematopoiesis. This timely emergence suggested a pivotal role for intraembryonic precursors in the colonization of the liver and therefore in the establishment of human definitive hematopoiesis (Tavian et al., 1999). Initiation of a hematopoietic cell differentiation program from human intraembryonic tissue has also been suggested by the expression, in the region underlying the dorsal aorta, of bone morphogenetic protein 4, known to induce ventral mesoderm (Marshall et al., 2000). Altogether, these results provided circumstantial evidence to indicate the existence of a blood-forming potential intrinsic to the human intraembryonic splanchnopleura. Indeed, ventral truncal territories dissected away from the human embryo and cultured on bone marrow stroma produced myeloid blood cells for 4–5 weeks (Tavian et al., 1999). However, the earliest embryo cultured in these experiments was 24 days old, that is, blood had been circulating for about 3 days and hence had possibly disseminated yolk sac-derived blood cell progenitors inside the embryo tissues.

In order to decipher the very origin of human HSC, we describe here the analysis of human intra- and extraembryonic tissues before and after they have been connected by the vascular tree. By virtue of a bone marrow stromal cell line permissive for simultaneous myeloid and B-lymphoid development (Berardi et al., 1997; Robin et al., 1999a) and in combination with xenogenic fetal thymus organ cultures (Robin et al., 1999b), we assessed the lineage commitment of intra- and extraembryonic hematopoietic cells. We demonstrate that the human yolk sac generates only progenitors with limited developmental potential, whereas multilineage lympho-myeloid progenitors emerge later and autonomously in the presumptive aortic territory.

Results

The aim of this study was to describe the emergence of hematopoietic cells inside the human embryo by assessing the presence in selected territories of progenitors establishing long-term (4–7 week) hematopoietic cultures in vitro. Analysis was focused on the truncal splanchnopleura and derived aorta, gonad, and mesonephros. Matching yolk sac was analyzed in parallel in each experiment. In a second phase, we compared intraembryonic hematopoietic progenitors with those derived from the yolk sac in terms of myeloid and lymphoid differentiation potentials.

[3] Correspondence: tavian@infobiogen.fr
[4] Present address: INSERM U421, Faculté de Médecine, Créteil, France.
[5] Present address: Department of Cell Biology and Genetics, Erasmus University, Rotterdam, The Netherlands.

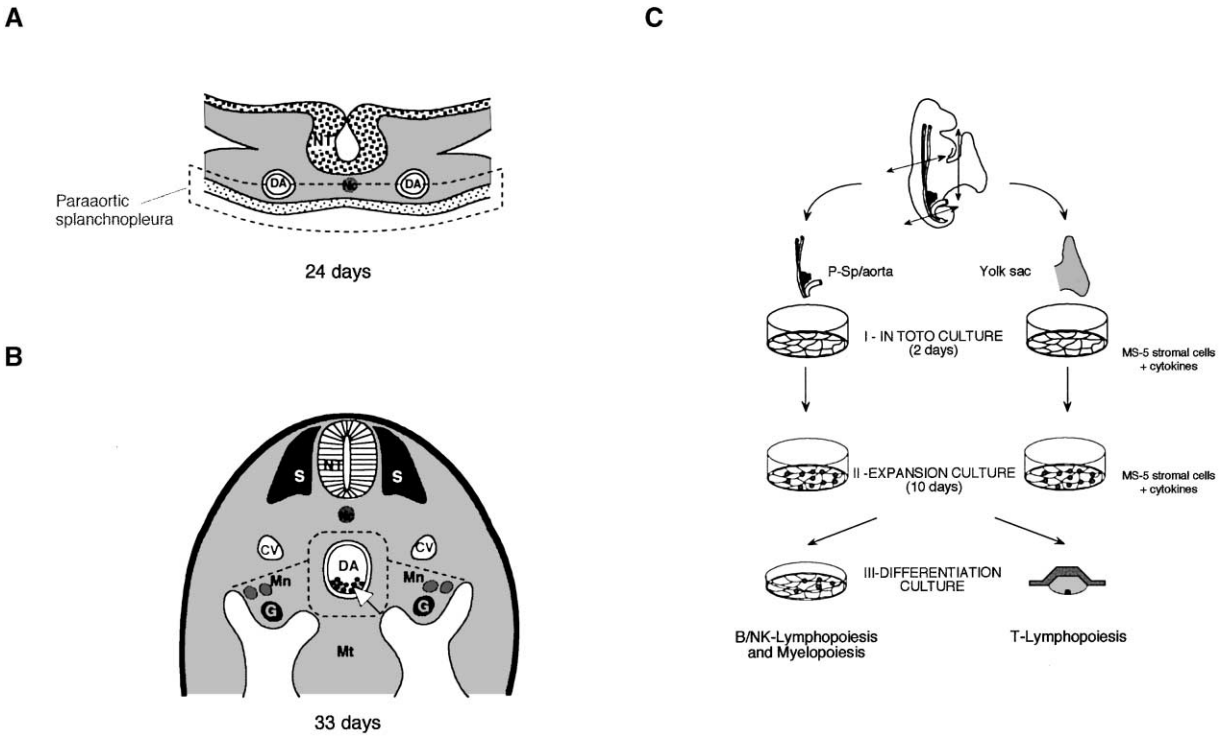

Figure 1. Human Embryo Dissection and Culture

(A and B) The paraaortic splanchnopleura and aorta in 24 and 33 day human embryos, respectively, were dissected along the dotted lines. (B) The AGM is dissected to separate the dorsal aorta (DA) and underlying mesoderm from the urogenital ridge (gonad, G; mesonephros, Mn; mesentery, Mt). Arrow shows intraarterial hematopoietic cell clusters. S, somites; Nc, notochord; NT, neural tube; CV, cardinal vein.

(C) In vitro culture conditions used to detect myeloid, NK, B, and T cell potentialities in early human embryos. In some instances, $CD34^+$ cells present in the dish at the end of the expansion culture (II) were sorted by flow cytometry prior to seeding into differentiation culture (III).

Hematopoietic Progenitors Are Detected in the Embryo Proper as Early as Day 19 of Development

Human embryos ranging from 19 to 48 days were analyzed. From 21 to 26 days, we dissected the whole paraaortic splanchnopleura (Figure 1A), including the dorsal aortae which, at these early stages of development, are not yet fused. From day 27 onward, the single dorsal aorta enclosed in a fine sheath of mesoderm (Figure 1B) was cut apart with microdissection scissors and microscalpels from its anterior bifurcation to its caudal end after the vitelline artery connection (Tavian et al., 1999). Explants were first seeded intact for 1–2 days on monolayers of confluent murine MS-5 stromal cells, which allow the survival, proliferation, and lympho-myeloid differentiation of human hematopoietic stem cells (Berardi et al., 1997; Robin et al., 1999a). Tissues were then dissociated by repeated pipetting inside the wells and cultured there for 7–10 additional days to amplify the hematopoietic cell compartment (see protocol in Figure 1C). Only those wells containing a layer of confluent hematopoietic cells were further processed, by harvesting and seeding the whole content of the well onto fresh MS-5 stromal layers (the content of one well was generally split into ten wells of a 96-well plate). Wells were checked for cell growth for 4–5 weeks and considered positive when containing, after 3 weeks, colonies of packed rounded cells exhibiting the typical morphology of hematopoietic cells. Results are shown in Table 1. In all instances, the hematopoietic identity of cells developing in the cocultures was confirmed by flow cytometry (see the following section). The two earliest specimens analyzed were at the 19th day of development, a presomite stage at which gastrulation is not completely achieved

Table 1. Blood Cell Formation in Human Intra- and Extraembryonic Territories Cultured in the Presence of MS-5 Stroma

Embryo age				
Carnegie stage	Days	Somites (pairs)	P-Sp/aorta#	Yolk sac
8	19	0	*2/2	2/2
9	21	1–3	1/1	1/1
10	22–23	4–12	5/5	5/5
11	24–25	13–20	4/6	6/6
12	***26–27***	21–29	11/14	12/12
13	***28–30***	30–35	5/7	5/5
14	***31–32***	§	4/4	4/4
15	***33–34***		4/4	4/4
16	***35–40***		2/2	ND
17–19	>40		0/2	ND

Results are presented as the number of cultures that sustained hematopoiesis out of the total cocultures performed at each stage.

* At this stage, somites are not yet formed and gastrulation is probably not completely achieved. The whole embryo was placed in culture.

The P-Sp and aorta were dissected from the embryo from stage 9 to stage 12 and from stage 13 onward, respectively.

§ From this stage on, the number of somites is no longer a reliable staging criterion.

The embryonic stages when hematopoietic cell clusters are present inside the aorta and vitelline artery are set in bold italics.

ND, not done; P-Sp, paraaortic splanchnopleura.

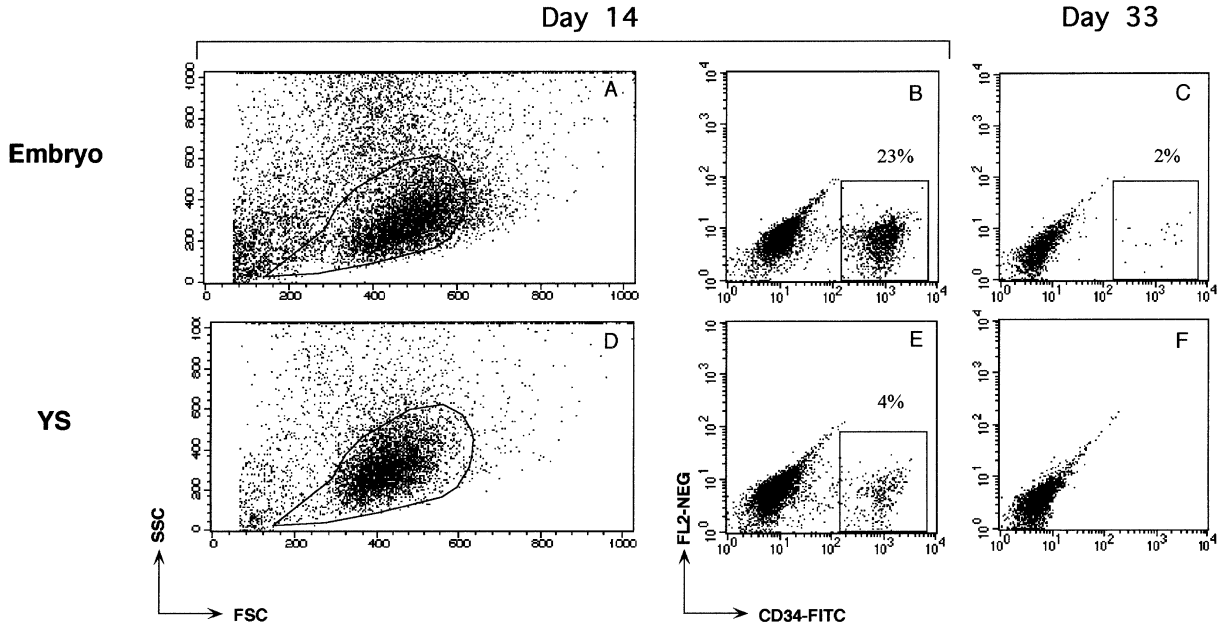

Figure 2. Generation in Culture of CD34-Positive Hematopoietic Progenitors from the Embryo and Yolk Sac at a Presomite Stage (Day 19)

Cells growing on MS-5 stroma from the embryo (B and C) and yolk sac (E and F) were stained with anti-CD34-FITC. Analyses were performed at days 14 (B and E) and 33 (C and F) of culture. Analyzed cells were first gated on forward/side scatter (A and D).

and the primitive streak can still be identified. The yolk sac and embryo were separated and cultivated intact in distinct wells for 10 days under the conditions described above, and then dissociated and cells from each well seeded into four wells on fresh stromal cells.

Hematopoietic potential was present in the embryo proper as early as day 19 (Table 1), as shown by the development of colonies of rounded cells over the stromal layer starting from day 7 of culture. The whole content of the wells was dissociated mechanically and cells were analyzed by flow cytometry. Hematopoietic cells could readily be distinguished from stromal cells and unrelated embryonic cells by virtue of morphological traits (side scatter/forward scatter gate; Figures 2A and 2D). That gate included CD34-positive cells, which peaked in percentage after 2 weeks (Figure 2B) and were still detectable in the wells after day 33 (Figure 2C). Since no hematopoietic $CD34^+$ cells are seen in the intact human embryo before day 27 (Tavian et al., 1999), these progenitors arose in the culture from earlier stem cells. At the same stage, the yolk sac was also hematopoietic, as expected since vitelline blood islands form from day 18.5 (Bloom and Bartelmez, 1940). In contrast to embryo cultures, yolk sac-derived $CD34^+$ cells were less numerous (Figure 2E), decreased rapidly in number, and disappeared by 4 weeks of culture (Figure 2F).

Intraembryonic hematogenous capacity was then regularly detected from 21 to 40 days of development, first in the paraaortic splanchnopleura, and then in the dissected aorta (Table 1). After 40 days of gestation, hematopoietic cells proliferating from cultured dissected aortae were less abundant and rapidly differentiated into large, scattered cells. Hematopoiesis established from these older tissues did not persist longer than 10–15 days (data not shown). These results show that hematopoietic progenitors, or their forerunners, emerge in the human P-Sp/aorta from at least 19 days of gestation.

In ten experiments in which the 24 to 34 day dissected P-Sp/aorta was analyzed in this setting, $CD34^+$ cells generated during the expansion phase of the culture (Figure 1C) were sorted by FACS prior to being further seeded into lympho-myeloid differentiation cultures. The number of $CD34^+$ cells sorted from one well ranged from 100 to 700. The outcome was the same as when the whole unsorted population was used (data not shown), indicating that the blood-forming activity observed in these experiments correlates with the emergence of $CD34^+$ cells.

The P-Sp/Aorta, but Not the Yolk Sac, Produces Both Lymphoid and Myeloid Cells

We set out to evaluate the lineage commitment of hematopoietic progenitors emerging in the human P-Sp/aorta and yolk sac, applying the protocol outlined in Figure 1C. In this setting, hematopoietic cells proliferate rapidly and are numerous enough to be analyzed after only 7 days of differentiation culture. At all stages studied from 19 to 40 days, cells from either the P-Sp/aorta or yolk sac can generate NK ($CD56^+$) and granulomonocytic ($CD15^+$) cells (Table 2; Figures 3A, 3B, and 3F). To further ascertain that natural killer cells differentiate in these conditions from extra- and intraembryonic hematopoietic progenitors, cells grown in the same conditions on MS-5 stroma from a 26 day P-Sp, a 32 day aorta and yolk sac, and a 36 day aorta were double stained with antibodies to CD56 and CD94, a c-type lectin broadly expressed on NK cells (Pende et al., 1997). In all instances, subsets of $CD56^+CD94^+$ cells were detected that accounted for 69%, 17%, 20%, and 34%, respectively of the whole CD56-expressing cell population (Figure 3C). Similarly, 24% of the $CD56^+$ cells developed in the same conditions from cord blood $CD34^+$ cells expressed CD94 (data not shown), confirming that CD94 expression is posterior to that of CD56 in the course of NK cell development (Carayol et al., 1998).

Table 2. Lymphoid, Myeloid, and NK Cell Development from the Human P-Sp/Aorta and Yolk Sac

Embryo age		Paraaortic splanchnopleura/aorta				Yolk sac			
Carnegie stage	Days	B $CD19^+$	M $CD15^+$	NK $CD56^+$	T $CD4^+$	B $CD19^+$	M $CD15^+$	NK $CD56^+$	T $CD4^+$
8	19	0/2	2/2	2/2	ND	0/2	2/2	2/2	ND
9	21	0/1	1/1	1/1	ND	0/1	1/1	1/1	ND
10	22–23	0/5	5/5	5/5	0/1	0/4	4/4	4/4	0/1
11	24–25	3/4	4/4	3/4	ND	0/4	4/4	3/4	ND
12	26–27	7/11	11/11	11/11	3/8	0/10	10/10	9/9	0/7
13	28–30	5/7	7/7	7/7	3/4	0/5	5/5	5/5	0/2
14	31–32	2/3	3/3	4/4	2/4	0/3	3/4	3/4	0/4
15	33–34	3/3	3/3	3/3	ND	0/2	2/2	2/2	ND
16	35–40	1/1	1/1	2/2	ND	ND	ND	ND	ND

ND, not done.

$CD19^+$ B cells also differentiate in vitro from the intraembryonic region, albeit from 24 days of gestation only (Table 2; Figures 3A, 3B, and 3F). B cells usually appear during the third week of coculture, significantly later than the other blood cell types, as previously observed for human HSC cultured on MS-5 cells (C.R. and L.C., unpublished data). The percentage of B cells increases over the next week to reach up to 34% of the cells analyzed (Figure 3B). B lymphocytes were often still present after 40 days of culture (data not shown). No such capacity to produce B cells was ever found in yolk sac cocultures (Table 2), from which CD19-positive cells were consistently very rare or absent at all stages of development tested, including after the onset of blood circulation between intra- and extraembryonic compartments (Figures 3B and 3F).

We then tested the T cell potential of extra- and intraembryonic progenitors in 17 independent experiments. The P-Sp/aorta and YS cultured intact for 10 days on MS-5 cells were dissociated and used to populate organ-cultured NOD-SCID mouse fetal thymuses (Figure 1C). Since the SCID mutation blocks T cell development, no removal of endogenous thymocytes was required (Robin et al., 1999b). Usually, five to ten thymic lobes were seeded per tissue analyzed, and each lobe was incubated with 50–100,000 total, unseparated donor cells. Positive control thymuses were incubated with 2,000–5,000 cord blood-derived $CD34^+$ sorted cells. In this setting, the ability to generate T lymphocytes was restricted to P-Sp/aorta progenitors. Human $CD4^+$ single-positive and $CD4^+CD8^+$ double-positive T lymphocytes were present in thymuses populated by human intraembryonic progenitors, while yolk sac-derived cells were always devoid of T cell potential (Figure 3D; Table 2). Under these conditions, the production of single-positive $CD4^-CD8^+$ T cells is compromised by the poor reactivity of mouse class I molecules with human cells (Blom et al., 1997). It was not determined whether human embryonic cells gave rise to other subsets of non-lymphoid hematopoietic cells inside the murine thymus environment. In the experiments carried out with P-Sp-derived cells, the proportion of human $CD4^+$ cells inside the chimeric thymus was variable but always significant (Figure 3E). Remarkably, within the same experiment, each individual lobe analyzed was populated with T cells. The results reported in Table 2 indicate that T-lymphoid potential emerges inside the embryo as early as day 26 of development, that is, shortly before the appearance of intraarterial hematopoietic cell clusters, which arise at the 27th day of gestation (Tavian et al., 1999).

Altogether, these results show that, even in the vascularized human embryo, lympho-myeloid stem cells are confined to intraembryonic tissues but are absent from the yolk sac, as illustrated by grouped analyses of a 31 day specimen (Figure 3F).

In the Human AGM, Only the Aorta Contains Hematopoietic Progenitors

The acronym AGM designates a large part of the embryo trunk that encloses, beside the dorsal aorta and surrounding mesoderm, the rudiments of the kidneys and gonads. In order to more accurately map hematopoietic territories in the human embryo, the mesonephros/gonads, aorta, and remainder of the embryo deprived of the liver were dissected separately and cultivated individually under the above described conditions. Six independent experiments carried out between 26 and 31 days of gestation show that in the human AGM, only the aorta encloses hematopoietic progenitors (Table 3; Figure 4), as assessed by development in culture of B, NK, and myeloid cells (Table 2).

Discussion

Recent years have seen the appreciation of the key role played by stem cells born inside the embryo in the settlement of definitive hematopoiesis. We have participated in this reconsideration of the very origin of mammalian blood cells by describing the emergence of cell clusters in the 5 week human embryo adhering to the ventral aortic endothelium and possessing phenotypic and genetic characteristics of primitive hematopoietic progenitors (Huyhn et al., 1995; Tavian et al., 1996; Labastie et al., 1998; Watt et al., 2000). The spatial and temporal distribution of these progenitors during embryogenesis suggests that they colonize the liver rudiment, implying their major role in the establishment of the human blood system (Tavian et al., 1999). Indeed, these groups of $CD34^+$ hematopoietic cells first come into existence on the 27th day of gestation, inside a segment of the aorta which is just dorsal to the liver rudiment, and only 2–3 days before hepatic colonization by $CD34^+$ progenitors (Tavian et al., 1999). At the stage of stem cell emergence in the truncal arteries, though,

the yolk sac has already been producing blood cells for about 10 days (Bloom and Bartelmez, 1940); some of these cells have disseminated inside the embryo, since blood circulates from day 21 (Tavian et al., 1999). To ascertain that the aorta-adherent $CD34^+$ hematopoietic cells do not derive from blood-borne progenitors but develop intrinsically to the vessel wall, the precirculation rudiment of the latter has been cultured in the presence of MS-5 stromal cells, a suitable support for human multilineage hematopoiesis.

The presumptive ventral half of the aorta, including endothelial cells and the surrounding mesoderm, is enclosed in the splanchnopleura, while dorsal aortic endothelial cells stem from somitic mesoderm (Pardanaud et al., 1996). As early as day 19 of development, 2 days before the onset of blood circulation, the embryo, dissected away from the yolk sac, gives rise to $CD34^+$ and then to differentiated hematopoietic cells in culture. The human embryo is therefore a site where hematopoietic stem cells are produced and not merely home and adhere. In line with these findings, molecular analysis of human and chicken embryos had previously shown in the aortic region the expression of genes that mark the onset of hematopoietic development, such as *SCL/tal-1* and *c-myb* (Vandenbunder et al., 1989; Labastie et al., 1998). Messengers for these genes were solely detected, on tissue sections, in the few hematopoietic cells that sprout from the ventral aortic endothelium, which may explain why Palis et al. (1999) failed to detect *SCL* and *c-myb* by PCR in the whole-mouse AGM. Yet, totipotential hematopoietic progenitors also arise in the mouse embryo proper prior to the onset of circulation (Cumano et al., 1996), and are therefore unrelated to yolk sac hematopoiesis. Thus, the human embryo contains ancestral blood cell progenitors, arising at least 8 days before $CD34^+$ hematopoietic cells can be detected on the aortic wall. The identity of these ancestors is not yet established, but they could be related to endothelial cell lineage. Indeed, preexisting endothelial cells are candidates, in the trunk of vertebrate embryos, for the generation of hematopoietic cells (Jaffredo et al., 1998; Nishikawa et al., 1998). Accordingly, the $CD34^+CD45^-$ endothelial cells we sorted by flow cytometry from the human embryonic AGM as well as from the yolk sac and embryonic liver included hematopoietic progenitors at a high frequency, suggesting a direct filiation of HSC from the vascular wall (E. Oberlin, personal communication). Endothelial development and HSC emergence would therefore be closely associated in both of the earliest sites of hematopoiesis, the yolk sac and the P-Sp/AGM. Although it is generally admitted that hematopoietic and vascular developments are segregated early in the yolk sac at the level of "hemangioblasts" (Choi et al., 1998), it was also hypothesized that even once differentiated, some endothelial cells can still produce blood elements (Sabin, 1920).

We have herein defined the lineage potential of extra- versus intraembryonic human blood cell progenitors. At the fourth week of human gestation, the paraaortic splanchnopleura and later, the aorta, can give rise to B, T, NK, and myeloid cells (Table 2). Despite the fact that blood has been circulating for several days at these stages, no such multilineage activity was ever detected in our culture conditions in the associated yolk sacs, which produced only myeloid and NK cells. Therefore, lympho-myeloid progenitors develop intrinsically in the human embryo. The mouse yolk sac was shown to harbor lympho-myeloid progenitors (Weissman et al., 1978) and B cell precursors (Tyan and Herzenberg, 1968; Ogawa et al., 1988; Palacios and Imhof, 1993; Huang et al., 1994; B.P., unpublished data) after 9 days of gestation, once connected to the embryo through blood vessels. In contrast, Cumano et al. (1996) and B.P. (unpublished data) failed to detect lymphoid potential in the mouse yolk sac taken before 8.5 days, the stage at which the yolk sac and embryo become connected through circulation. However, two reports have described the presence of B cell (Palacios and Imhof, 1993) and T cell precursors (Liu and Auerbach, 1991) in the yolk sac dissected before 8.5 days, suggesting that appropriate conditions may reveal multilineage hematopoietic potential in stem cells emerging outside the mouse embryo. In human ontogeny, we observed the most clear cut difference between the embryo and yolk sac in terms of hematopoietic potential. This may also reflect evolutionary differences between the human embryo and that of other vertebrates. The human yolk sac might represent, phylogenetically, the most extreme reduction of extraembryonic blood-forming activity. Conversely, embryonic intravascular stem cell clusters are larger in the human embryo than in chicken and mouse embryos. Even though the human yolk sac does not produce lympho-myeloid progenitors de novo, such stem cells originating in the aorta and carried by the blood stream should have been identified in our assays, as occurred in mouse embryo studies (Cumano et al., 1996). The observation that this was not the case may emphasize another difference between human and mouse hematopoiesis, the former being characterized by reduced mobility of aorta-derived multipotential progenitors. Along this line, we have previously observed that human circulating blood at these embryonic stages is devoid of long-term culture-initiating hematopoietic progenitors (Tavian et al., 1999).

Day 24 of development marks the appearance of lymphoid potential inside the human embryo, whereas the capacity to produce myeloid cells existed there at least 5 days earlier. The absence of B cells in cultures from earlier embryos from 19 to 23 days of gestation where we observed the development of NK and myeloid cells is unlikely to be explained by quantitative considerations, as culture on MS-5 stroma is a very sensitive assay that can drive multilineage development of single human HSC (Robin et al., 1999a). The 24 day limit may rather reflect a key developmental switch, possibly provided by the neighboring endoderm and conferring to early progenitors the ability to engage in lymphoid differentiation programs.

In all experiments reported herein, B cell and T cell developmental potentials were coupled. In striking contrast, NK cell potential was dissociated from B and T cell activity in the pre-24 day embryo and at all stages in the yolk sac, which produced $CD15^+$ and $CD56^+$ cells but no T or B cells. We have no functional characterization of the $CD56^+$ cells that grew in our cultures. However, Carayol et al. (1998) have described preferential development of human HSC cultured on MS-5 stroma in $CD56^+$ cells which were endowed with natural killer cell function. Our demonstration of CD94 expression at the surface of a significant proportion of $CD56^+$ cells differentiated from both extra-

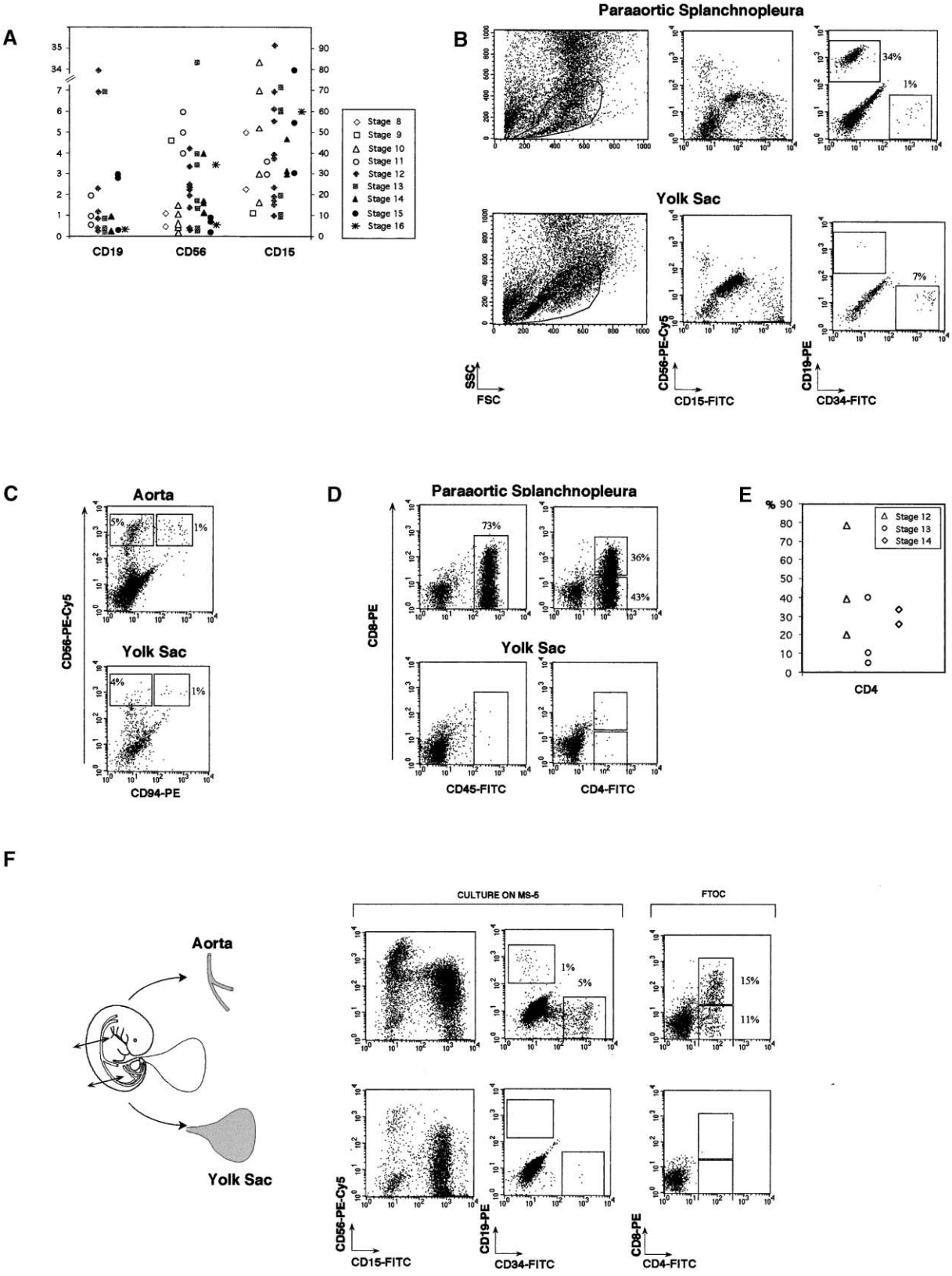

Figure 3. Development of Myeloid, NK, B, and T Cells from the Human P-Sp/Aorta and Yolk Sac

(A) Percent CD19$^+$ B cells (left scale), CD56$^+$ NK cells, and CD15$^+$ myeloid cells (right scale) produced by the human P-Sp/aorta dissected at successive stages of development and cultured for several days on MS-5 stromal cells. Negative values have not been included (see Table 2).
(B) Myeloid, B, and NK cell development from 27 day YS and P-Sp. Cells cultured for 19 days on MS-5 stromal cells were stained with anti-CD15-FITC, anti-CD19-PE, and anti-CD56-PE-CY and analyzed inside the morphology gates indicated.

Table 3. Localization of Hematopoietic Potentials inside the Human AGM

Embryo age		AGM		
Carnegie stage	Days	P-Sp/aorta	Mn + G	So + NT
12	26–27	1/1	0/1	0/1
13	28–30	3/4	0/4	0/4
14	31	1/1	0/1	1/1

Mn, mesonephros; G, gonad; So, somatopleura; NT, neural tube.

and intraembryonic tissues has also further documented the belonging of these cells to the NK cell lineage. Although NK cells derive in the adult mouse bone marrow from a clonogenic common lymphoid progenitor (CLP) that differentiates into T, B, and NK lineages but not into myeloid lineages (Kondo et al., 1997), the existence of CLP in embryonic and fetal tissues has not yet been documented (Akashi et al., 2000). Sanchez et al. (1994) did identify a common progenitor for T lymphocytes and NK cells in human prenatal life, but this cell was only described in the second trimester thymus, that is, at a relatively late stage, when the thymus has achieved its definitive structure and organization, and the yolk sac and blood-forming AGM have long disappeared. On the other hand, the existence of NK/myeloid progenitors that could explain our observations has not been documented yet. A population of $CD34^+CD33^+$ cells has been identified in the human postnatal thymus that gives rise in culture to both NK and dendritic cells (Marquez et al., 1998). However, the ability of these cells to give rise to other myeloid cells was not examined by the authors, who concluded that both NK and dendritic cells inside the thymus were branching off the lymphoid cell lineage.

Other categories of progenitors may exist only at early stages of ontogeny such as the B, T, and macrophage common progenitor identified in mouse fetal liver by Lacaud et al. (1998). The existence of these progenitors may also account for the dramatic B cell and macrophage development observed upon culture of early mouse fetal thymocytes on bone marrow stroma (Péault et al., 1994).

Finally, as another explanation for the development of NK cells from the yolk sac in the absence of lymphoid cells, common T, B, and NK progenitors would also be present outside the embryo but at an extremely low frequency, and MS-5 would dramatically favor NK cell development at the expense of B cell differentiation. While we have not formally excluded this possibility yet by performing clonal assays, it is less likely, since although in some instances NK cells seem to quantitatively dominate B cells on MS-5 stroma (see Figure 3E), other experiments

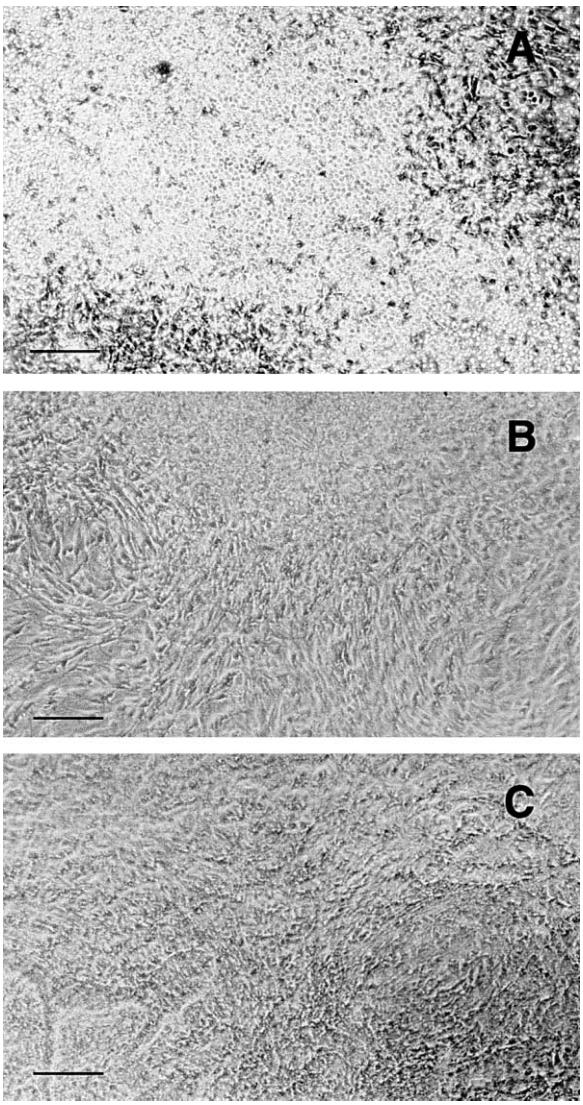

Figure 4. Coculture over a Layer of MS-5 Stromal Cells of Territories Dissected from a 30 Day Human Embryo

No hematopoietic cells developed from the separated urogenital ridge (C) or somatopleura and neural tube (B). Only the aorta gave rise to hematopoietic cell colonies adhering to the stromal cell layer (A). The scale bars indicate 100 μm.

pointed out inverse ratios (Figure 3B). Simultaneous B cell and NK cell development of single progenitors seeded on MS-5 has also shown that both lineages are, on average, equally supported by this stromal cell line (Robin et al., 1999a). Hence, the consistent absence of B lymphocytes

(C) Further identification of $CD56^+$ cells as NK cells developed in culture from the YS and aorta. Cells developed on MS-5 stroma from 32 day YS and aorta were double stained with anti-CD56-PE-Cy5 and anti-CD94-PE and analyzed inside the same morphology gate as in (B).
(D) T cell potential of cells derived from 26 day YS and P-Sp. Cell suspensions prepared from NOD-SCID mouse embryonic thymuses cultured organotypically 20 days earlier in the presence of cells developed on MS-5 stroma from the P-Sp and YS were stained with anti-CD8-PE and anti-CD4-FITC, or with anti-CD45-FITC and anti-CD8-PE. Human $CD45^+$ cells were gated on forward and side scatters.
(E) Percent CD4-positive cells (including single- and double-positive cells) present in NOD-SCID mouse embryonic thymuses cultured for 22 days in the presence of human intraembryonic hematopoietic progenitors. Negative values have not been included (see Table 2).
(F) Representative FACS staining profiles of myeloid, NK, B, and T cells developed from the yolk sac and aorta of the same 31 day human embryo. After expansion in bulk culture on MS-5 stromal layers and dissociation, cells were split and transferred to culture conditions promoting either myeloid, B, and NK or T cell differentiation, as illustrated in Figure 1C. Analyses were performed under the same conditions as in (B) and (D).

in our yolk sac MS-5 cocultures is probably not to be explained by a competitive advantage of NK cells. In addition, yolk sac-derived cells never repopulated the thymus either, whereas T cell development from embryonic tissues was spectacular (see Figures 3D and 3F).

We conclude, therefore, that rare pre-HSC emerge in the human paraaortic mesoderm and are at the origin of several hundred CD34-positive early hematopoietic progenitors that are seen on the aortic endothelium from day 27 of gestation. These ancestral blood-forming cells remain confined to the aorta and connected vitelline artery, as neighboring kidney and gonad rudiments were always devoid of hematopoietic activity in our culture system, confirming other recent observations in mice (Godin et al., 1999; de Bruijn et al., 2000). We also show here that the aorta, dissected from the embryo after day 40 and emptied of circulating blood, no longer gives rise to long-term hematopoiesis in vitro. Our present data provide direct evidence for the existence inside the human embryo of a discrete region that produces multipotential hematopoietic stem cells, which are likely at the origin of the whole human blood system. By reason of its quantitatively high stem cell-producing activity, this well-circumscribed intraembryonic hemogenic site represents a suitable model to decipher the role of microenvironmental cells in inducing lympho-myeloid fates.

Experimental Procedures

Human Tissues

Forty-seven human embryos ranging from 19 to 48 days of development were obtained immediately after voluntary terminations of pregnancy induced with the RU 486 antiprogestative compound. In all cases, informed consent to the use of the embryo in research was obtained from the patient, and the embryo was collected according to the guidelines and with the approval of both our national (CCNE) and institutional (COPE) ethics committees. Developmental age was estimated based on several anatomical criteria, as previously described in Tavian et al. (1999) according to Carnegie stages (O'Rahilly and Müller, 1987).

Whole-Tissue Culture

The splanchnopleura, truncal aorta, or yolk sac were sterilely dissected under the microscope and seeded undissociated in a 24-well plate containing MS-5 stromal cells. Explants were cultivated in toto for 24–48 hr, dissociated mechanically through a 26-gauge needle, and cultivated for 7–10 additional days on MS-5 stroma in RPMI (Gibco) supplemented with 10% heat-inactivated human serum (Stem Cell Technologies), 5% fetal calf serum (FCS), and the following human recombinant cytokines: 50 ng/ml SCF (Amgen), 50 ng/ml Flt3 ligand (Diaclone), 50 ng/ml pegylated megakaryocyte growth and differentiation factor (MGDF; Amgen), 5 ng/ml IL2 (Diaclone), 10 ng/ml IL15 (Diaclone), 10 ng/ml IL3 (Amgen), and 20 ng/ml IL7 (Diaclone).

Simultaneous B Cell, NK Cell, and Granulomonocytic Differentiation In Vitro

Cells cocultured on MS-5 were harvested from each well and seeded on a 96-well plate containing layers of MS-5 cells preestablished under the same culture conditions as described above. Half of the medium was replaced weekly. Plates were scored visually under the inverted microscope from week 1 through week 5 for the presence of hematopoietic cells, and only wells where significant proliferation had occurred were selected. Cells were subsequently collected by pipetting and labeled with the following MoAbs: CD19-PE (Becton Dickinson), CD15-FITC, CD56-PE-Cy5, CD94-PE, and CD34-FITC (Immunotech). Background staining levels were measured using isotype-matched controls. Analysis was performed on a FACScan (Becton Dickinson) using CellQuest software. In some instances, $CD34^+$ cells present in the coculture were sorted on a FACS Vantage cell sorter (Becton Dickinson). In 17 of these experiments, half of the cells were used for T-lymphoid potential analysis as described below.

T Cell Development in Fetal Thymus Organ Culture (FTOC)

FTOCs were performed following standard procedures adapted by Robin et al. (1999b) using NOD-SCID mouse embryonic (day 14) thymic lobes. Briefly, 25 μl hanging drops of complete medium (RPMI 1640 supplemented with 10% heat-inactivated human serum, 5% FCS, 5 ng/ml rh-IL2, 20 ng/ml rh-IL7, and 50 ng/ml rh-SCF) containing 50–100,000 cells harvested from amplification cultures were prepared in Terasaki plates. One fetal thymus lobe was added to each drop, and plates were incubated for 48 hr. Thymus lobes were then transferred onto floating filters (Isopore membrane, 25 mm diameter, 0.8 μm pore size; Millipore) in medium without cytokines and cultured there for a further 18–22 days. Single-cell suspensions were obtained by dissociating cultured thymus lobes with two needles. Each lobe was treated separately and the resulting cells were stained with anti-human CD4-FITC, anti-CD8-PE, and anti-CD45-FITC (Immunotech).

Acknowledgments

We are indebted to I. Dagousset and C. Carriere for their help with human embryo procurement. We would like to thank F. Colucci for critical reading of the manuscript. M.T. was a recipient of fellowships from Fondation pour la Recherche Médicale and Stem Cells Inc.

Received April 5, 2001; revised July 27, 2001.

References

Akashi, K., Reya, T., Dalma-Weiszhausz, D., and Weissman, I.L. (2000). Lymphoid precursors. Curr. Opin. Immunol. *12*, 144–150.

Berardi, A.C., Meffre, E., Pflumio, F., Katz, A., Vainchenker, W., Schiff, C., and Coulombel, L. (1997). Individual $CD34+CD38lowCD19-CD10-$ progenitor cells from human cord blood generate B lymphocytes and granulocytes. Blood *89*, 3554–3564.

Blom, B., Res, P., Noteboom, E., Weijer, K., and Spits, H. (1997). Prethymic $CD34+$ progenitors capable of developing into T cells are not committed to the T cell lineage. J. Immunol. *158*, 3571–3577.

Bloom, W., and Bartelmez, G.W. (1940). Hematopoiesis in young human embryos. Am. J. Anat., *67*, 21–53.

Carayol, G., Robin, C., Bourhis, J.H., Bennaceur-Griscelli, A., Chouaib, S., Coulombel, L., and Caignard, A. (1998). NK cells differentiated from bone marrow, cord blood and peripheral blood stem cells exhibit similar phenotype and functions. Eur. J. Immunol. *28*, 1991–2002.

Choi, K., Kennedy, M., Kazarov, A., Papadimitriou, J.C., and Keller, G. (1998). A common precursor for hematopoietic and endothelial cells. Development *125*, 725–732.

Cumano, A., Dieterlen-Lièvre, F., and Godin, I. (1996). Lymphoid potential, probed before circulation in mouse, is restricted to caudal intraembryonic splanchnopleura. Cell *86*, 907–916.

de Bruijn, M.F., Speck, N.A., Peeters, M.C., and Dzierzak, E. (2000). Definitive hematopoietic stem cells first develop within the major arterial regions of the mouse embryo. EMBO J. *19*, 2465–2474.

Dieterlen-Lièvre, F. (1975). On the origin of haemopoietic stem cells in the avian embryo: an experimental approach. J. Embryol. Exp. Morphol. *33*, 607–619.

Dieterlen-Lièvre, F. (1994). Hemopoiesis during avian ontogeny. Poultry Sci. Rev. *5*, 273–305.

Garcia-Porrero, J.A., Godin, I.E., and Dieterlen-Lièvre, F. (1995). Potential intraembryonic hemogenic sites at pre-liver stages in the mouse. Anat. Embryol. *192*, 425–435.

Garcia-Porrero, J.A., Manaia, A., Jimeno, J., Lasky, L.L., Dieterlen-Lièvre, F., and Godin, I.E. (1998). Antigenic profiles of endothelial and hemopoietic lineages in murine intraembryonic hemogenic sites. Dev. Comp. Immunol. *22*, 303–319.

Godin, I.E., Garcia-Porrero, J.A., Coutinho, A., Dieterlen-Lièvre, F.,

and Marcos, M.A. (1993). Para-aortic splanchnopleura from early mouse embryos contains B1a cell progenitors. Nature *364*, 67–69.

Godin, I., Garcia-Porrero, J.A., Dieterlen-Lièvre, F., and Cumano, A. (1999). Stem cell emergence and hemopoietic activity are incompatible in mouse intraembryonic sites. J. Exp. Med. *190*, 43–52.

Huang, H., Zettergren, L.D., and Auerbach, R. (1994). In vitro differentiation of B cells and myeloid cells from the early mouse embryo and its extraembryonic yolk sac. Exp. Hematol. *22*, 19–25.

Huyhn, A., Dommergues, M., Izac, B., Croisille, L., Katz, A., Vainchenker, W., and Coulombel, L. (1995). Characterization of hematopoietic progenitors from human yolk sacs and embryos. Blood *86*, 4474–4485.

Jaffredo, T., Gautier, R., Eichmann, A., and Dieterlen-Lièvre, F. (1998). Intraaortic hemopoietic cells are derived from endothelial cells during ontogeny. Development *125*, 4575–4583.

Kondo, M., Weissman, I.L., and Akashi, K. (1997). Identification of clonogenic common lymphoid progenitors in mouse bone marrow. Cell *91*, 661–672.

Labastie, M.C., Cortés, F., Roméo, P.H., Dulac, C., and Péault, B. (1998). Molecular identity of hematopoietic precursor cells emerging in the human embryo. Blood *92*, 3624–3635.

Lacaud, G., Carlsson, L., and Keller, G. (1998). Identification of a fetal hematopoietic precursor with B cell, T cell, and macrophage potential. Immunity *9*, 827–838.

Liu, C.P., and Auerbach, R. (1991). In vitro development of murine T cells from prethymic and preliver embryonic yolk sac hematopoietic stem cells. Development *113*, 1315–1323.

Marquez, C., Trigueros, C., Franco, J.M., Ramiro, A.R., Carrasco, Y.R., Lopez-Botet, M., and Toribio, M.L. (1998). Identification of a common developmental pathway for thymic natural killer cells and dendritic cells. Blood *91*, 2760–2771.

Marshall, C.J., Kinnon, C., and Thrasher, A.J. (2000). Polarized expression of bone morphogenetic protein-4 in the human aorta-gonad-mesonephros region. Blood *96*, 1591–1593.

Medvinsky, A.L., and Dzierzak, E.A. (1998). Development of the definitive hematopoietic hierarchy in the mouse. Dev. Comp. Immunol. *22*, 289–301.

Medvinsky, A.L., Samoylina, N.L., Muller, A.M., and Dzierzak, E.A. (1993). An early pre-liver intraembryonic source of CFU-S in the developing mouse. Nature *364*, 64–67.

Moore, M.A., and Metcalf, D. (1970). Ontogeny of the haemopoietic system: yolk sac origin of in vivo and in vitro colony forming cells in the developing mouse embryo. Br. J. Haematol. *18*, 279–296.

Morrison, S.J., Uchida, N., and Weissman, I.L. (1995). The biology of hematopoietic stem cells. Annu. Rev. Cell Dev. Biol. *11*, 35–71.

Nishikawa, S.I., Nishikawa, S., Kawamoto, H., Yoshida, H., Kizumoto, M., Kataoka, H., and Katsura, Y. (1998). In vitro generation of lymphohematopoietic cells from endothelial cells purified from murine embryos. Immunity *8*, 761–769.

North, T., Gu, T.L., Stacy, T., Wang, Q., Howard, L., Binder, M., Marin-Padilla, M., and Speck, N.A. (1999). Cbfa2 is required for the formation of intra-aortic hematopoietic clusters. Development *126*, 2563–2575.

Ogawa, M., Nishikawa, S., Ikuta, K., Yamamura, F., Naito, M., Takahashi, K., and Nishikawa, S. (1988). B cell ontogeny in murine embryo studied by a culture system with the monolayer of a stromal cell clone, ST2: B cell progenitor develops first in the embryonal body rather than in the yolk sac. EMBO J. *7*, 1337–1343.

O'Rahilly, R., and Müller, F. (1987). Developmental Stages in Human Embryos. (Washington: Carnegie Institution of Washington).

Palacios, R., and Imhof, B.A. (1993). At day 8–8.5 of mouse development the yolk sac, not the embryo proper, has lymphoid precursor potential in vivo and in vitro. Proc. Natl. Acad. Sci. USA *90*, 6581–6585.

Palis, J., Robertson, S., Kennedy, M., Wall, C., and Keller, G. (1999). Development of erythroid and myeloid progenitors in the yolk sac and embryo proper of the mouse. Development *126*, 5073–5084.

Pardanaud, L., Luton, D., Prigent, M., Bourcheix, L.M., Catala, M., and Dieterlen-Lièvre, F. (1996). Two distinct endothelial lineages in ontogeny, one of them related to hemopoiesis. Development *122*, 1363–1371.

Péault, B., Khazaal, I., and Weissman, I.L. (1994). In vitro development of B cells and macrophages from early mouse fetal thymocytes. Eur. J. Immunol. *24*, 781–784.

Pende, D., Sivori, S., Accame, L., Pareti, L., Falco, M., Geraghty, D., Le Bouteiller, P., Moretta, L., and Moretta, A. (1997). HLA-G recognition by human natural killer cells. Involvement of CD94 both as inhibitory and as activating receptor complex. Eur. J. Immunol. *27*, 1875–1880.

Petrenko, O., Beavis, A., Klaine, M., Kittappa, R., Godin, I., and Lemischka, I.R. (1999). The molecular characterization of the fetal stem cell marker AA4. Immunity *10*, 691–700.

Robin, C., Pflumio, F., Vainchenker, W., and Coulombel, L. (1999a). Identification of lymphomyeloid primitive progenitor cells in fresh human cord blood and in the marrow of nonobese diabetic-severe combined immunodeficient (NOD-SCID) mice transplanted with human CD34(+) cord blood cells. J. Exp. Med. *189*, 1601–1610.

Robin, C., Bennaceur-Griscelli, A., Louache, F., Vainchenker, W., and Coulombel, L. (1999b). Identification of human T-lymphoid progenitor cells in CD34+ CD38low and CD34+ CD38+ subsets of human cord blood and bone marrow cells using NOD-SCID fetal thymus organ cultures. Br. J. Haematol. *104*, 809–819.

Sabin, F.R. (1920). Studies on the origin of blood vessels and of red blood corpuscles as seen in the living blastoderm of chicks during the second day of incubation. Contrib. Embryol. *9*, 213–262.

Sanchez, M.J., Muench, M.O., Roncarolo, M.G., Lanier, L.L., and Phillips, J.H. (1994). Identification of a common T/natural killer cell progenitor in human fetal thymus. J. Exp. Med. *180*, 569–576.

Tavian, M., Coulombel, L., Luton, D., Clemente, H.S., Dieterlen-Lièvre, F., and Péault, B. (1996). Aorta-associated CD34+ hematopoietic cells in the early human embryo. Blood *87*, 67–72.

Tavian, M., Hallais, M.F., and Péault, B. (1999). Emergence of intraembryonic hematopoietic precursors in the pre-liver human embryo. Development *126*, 793–803.

Tyan, M.L., and Herzenberg, L.A. (1968). Studies on the ontogeny of the mouse immune system. II. Immunoglobulin-producing cells. J. Immunol. *101*, 446–450.

Vandenbunder, B., Pardanaud, L., Jaffredo, T., Mirabel, M.A., and Stehelin, D. (1989). Complementary patterns of expression of c-ets 1, c-myb and c-myc in the blood-forming system of the chick embryo. Development *107*, 265–274.

Watt, S.M., Butler, L.H., Tavian, M., Buhring, H.J., Rappold, I., Simmons, P.J., Zannettino, A.C., Buck, D., Fuchs, A., Doyonnas, R., et al. (2000). Functionally defined CD164 epitopes are expressed on CD34(+) cells throughout ontogeny but display distinct distribution patterns in adult hematopoietic and nonhematopoietic tissues. Blood *95*, 3113–3124.

Weissman, I.L., Papaioannou, V., and Gardner, R. (1978). Fetal hematopoietic origins of the adult hematolymphoid system. In Cold Spring Harbor Conferences on Cell Proliferation, Volume 5, Differentiation of Normal and Neoplastic Hematopoietic Cells, P.A. Mark and J.E. Till, eds. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press), pp. 33–47.

Wood, H.B., May, G., Healy, L., Enver, T., and Morriss-Kay, G.M. (1997). CD34 expression patterns during early mouse development are related to modes of blood vessel formation and reveal additional sites of hematopoiesis. Blood *90*, 2300–2311.