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The Placenta Is a Niche for Hematopoietic Stem Cells

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Summary

The hematopoietic system develops during embryogenesis at temporally and anatomically restricted sites. The anatomical origin of definitive HSCs is not fully resolved, and little is known about how the different fetal hematopoietic microenvironments direct HSC development. Here, we show that the mouse placenta functions as a hematopoietic organ that harbors a large pool of pluripotent HSCs during midgestation. The onset of HSC activity in the placenta parallels that of the AGM (aorta-gonad-mesonephros) region starting at E10.5–E11.0. However, the placental HSC pool expands until E12.5–E13.5 and contains >15-fold more HSCs than the AGM. The expansion of the $CD34^{+}c\text{-}kit^{+}$ HSC pool in the placenta occurs prior to and during the initial expansion of HSCs in the fetal liver. Importantly, the placental HSC pool is not explained by rare circulating HSCs, which appear later. These data support an important, but unappreciated, role for the placenta in establishing the mammalian definitive hematopoietic system.

Introduction

Blood cell production is sustained throughout the lifetime of an individual by hematopoietic stem cells (HSCs). HSCs are defined by their ability to engraft bone marrow (BM) of irradiated hosts, self-renew, and differentiate into all types of mature blood cells (Akashi and Weissman, 2001). During postnatal life, the bone marrow supports both self-renewal and differentiation of HSCs in specialized microenvironmental niches (Arai et al., 2004; Avecilla et al., 2004; Calvi et al., 2003; Nilsson et al., 2001; Tokoyoda et al., 2004; Zhang et al., 2003a), whereas embryonic hematopoiesis is compartmentalized into anatomical sites where specific stages of hematopoietic development and different lineage outputs are manifest.

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The hematopoietic system originates from the mesodermal germ layer, which gives rise to the hemangioblast, a bipotential precursor for blood and endothelium (Choi et al., 1998; Huber et al., 2004; Sabin, 1920). In murine embryos, commitment to the hematopoietic lineage is evident at embryonic days (E) 7.0–7.5 in the yolk sac, which first produces a transient population of primitive erythrocytes that express embryonic globins (Palis et al., 1999). Shortly thereafter, the yolk sac gives rise to a second wave of progenitors that consists of single- and multilineage myeloerythroid progenitor cells. However, the yolk sac microenvironment does not support differentiation of definitive progenitors, which instead exit via the vitelline veins to the fetal liver rudiment, where definitive erythroid differentiation is initiated. Paradoxically, HSCs, defined by the ability to reconstitute adult bone marrow hematopoiesis, are not yet found in these hematopoietic tissues, although embryonic blood cell production has been ongoing for ~3 days. The first definitive HSCs appear after E10.5 in low numbers in the AGM (aorta-gonad-mesonephros) region in the embryo proper and in the vitelline and umbilical arteries that connect the dorsal aorta with the yolk sac and the placenta, respectively (Cumano et al., 1996; de Bruijn et al., 2000; Godin et al., 1999; Medvinsky and Dzierzak, 1996; Muller et al., 1994). The AGM region develops from the para-aortic splanchnopleura and represents another independent site for de novo hematopoiesis. As HSCs in the AGM region are only found for a short time, and the number of HSCs and progenitors is low at all times, the AGM is not considered to function as a major site for expansion or differentiation of HSCs. Instead, accumulation of HSCs is seen shortly thereafter in the fetal liver, which also serves as the main site for hematopoietic differentiation during fetal life (Ema and Nakauchi, 2000) until hematopoiesis shifts to the bone marrow around birth. As no de novo formation of HSCs occurs in the fetal liver, myeloerythroid progenitors and HSCs from the yolk sac and the AGM region have been assumed to be responsible for fetal liver seeding (Houssaint, 1981; Johnson and Moore, 1975). However, the exceedingly rapid expansion of the fetal liver HSC pool, taken together with the length of the cell cycle, has raised the question of whether the few HSCs from the AGM region alone can account for the expansion of the fetal liver HSC pool, or whether the yolk sac, or yet another site, also contributes to this process (Kumaravelu et al., 2002) by supporting the genesis, maturation, or expansion of adult-type HSCs. Notably, as early as E9, the yolk sac contains immature HSCs, or pre-HSCs, that are capable of reconstitution of the hematopoietic system of newborn recipient mice (Yoder et al., 1997a; Yoder et al., 1997b). Whether these cells contribute to the adult, definitive hematopoietic system during normal development is unknown.

In avian embryos, de novo hematopoiesis occurs in an additional mesodermal appendage, the allantois (Caprioli et al., 1998; Caprioli et al., 2001). In mice, the allantois forms the umbilical cord and the mesodermal

components of the fetal placenta upon fusion with the chorion. The allantois develops from the epiblast during gastrulation and grows into the exocoelomic cavity as a mesodermal extension of the posterior primitive streak. The allantois fuses with the chorion at E8–E8.5 and forms a complex vascular network called the placental labyrinth, which consists of endothelial cell-lined fetal capillaries and trophoblast-lined maternal blood sinuses, and permits the exchange of gases and nutrients between maternal and fetal circulatory systems (Adamson et al., 2002; Downs, 2002; Downs and Gardner, 1995; Rossant and Cross, 2001). The placenta has not been recognized as a hematopoietic organ, although Melchers reported the presence of fetal B cell precursors in murine placenta 25 years ago by using coculture on rat thymic filler cells and plaque-forming cell assays (Melchers, 1979). Since these early studies, placental hematopoiesis has not been reassessed until Alvarez-Silva et al. showed by in vitro clonogenic assays that the placenta is a rich source for multipotential hematopoietic progenitors before they colonize the fetal liver. The results of these assays suggest that the placenta may function as a hematopoietic organ during development (Alvarez-Silva et al., 2003). However, hematopoietic progenitors in the mouse embryo form prior to and independently of HSCs that establish the definitive hematopoietic system. We therefore asked whether the placenta also supports the development of true HSCs that have the ability to self-renew and permanently reconstitute all lineages of the adult hematopoietic system.

Results

We performed a spatial and temporal analysis of HSC activity during mouse embryonic development by using the long-term transplantation assay, which is the most stringent and reliable assay for HSCs. In this study, we also assessed HSC activity in the placenta, which has not been recognized as an organ that participates in HSC development. Collagenase-treated hematopoietic organs from E10.5–E18.5 conceptuses were transplanted in limiting dilutions, quantified as embryo equivalents (ee), into congenic, irradiated adult recipients together with competitor bone marrow cells (Table 1). Hematopoietic reconstitution by fetus-derived CD45.1/CD45.2 cells was analyzed by flow cytometry, and HSCs were quantified as repopulating units (RUs/ee).

The Onset of HSC Activity in the Placenta

Rare hematopoietic cells (0.1 RUs/ee) capable of contributing to peripheral blood of irradiated adult recipients over 10–12 weeks were found in the placenta already at E10.5 (33–36 sp, somite pairs). The temporal emergence of these hematopoietic cells was similar to what was seen in the caudal half of the embryo and the yolk sac (Figure 1A; Table 2). However, RUs in the entire conceptus were rare at this stage (0.3 RUs) and gave rise to very low (<1%–2%) hematopoietic reconstitution that frequently was lost over time. At E11.0 (37–40 sp), the conceptus contained, on average, one long-term repopulating unit (0.48 LT-RUs in placenta, Figure 1B; Table 2), and the first HSCs yielding a higher (>10%) level of hematopoietic reconstitution were seen in the AGM region and the placenta. No LT-RUs were yet found in the fetal liver rudiment or in circulating blood, although clonogenic progenitors were abundant (see below). By E11.5 (41–45 sp), the total number of LT-RUs in the conceptus had increased to ~11 (Figure 1C; Table 2). Consistent with previous studies (Kumaravelu et al., 2002), LT-HSCs were also found in the fetal liver and the yolk sac at this stage, and HSCs were fairly evenly distributed between the placenta and the known hematopoietic organs. The increase of HSC numbers was accompanied by a concomitant increase in reconstitution potential of fetal HSCs. The first engrafting cells also appeared in fetal blood, although the competitive reconstitution potential of circulating cells was low (<1%) (Figure 1C; Table 2). These results show that the placenta represents yet another site at which adult reconstituting HSCs reside during mouse development. The onset of HSC activity in the placenta coincides with that in the AGM and the yolk sac, and it precedes HSC seeding of the fetal liver. Importantly, placental HSCs appear before HSCs can be detected in circulating blood.

Pluripotency and Self-Renewal Ability of Placental HSCs

The multilineage potential of placental HSCs was verified by demonstrating the presence of fetus-derived CD45.1/CD45.2+ myeloid (Gr1/Mac1), B lymphoid (B220), and T-lymphoid progeny (CD4/CD8) in the peripheral blood of the recipient animals (Figure 2A). At E10.5, T-lymphoid lineage could not be confirmed for any fetal organs due to the low level of hematopoietic engraftment, whereas, from E11.0 onward, placental HSCs, similar to the HSCs derived from the AGM region, were able to give rise to multilineage long-term reconstitution for >5–10 months. Likewise, engraftment of placenta-derived hematopoietic cells had occurred in the bone marrow (Figure 2B), thymus, and spleen of the recipient animals (data not shown). Furthermore, placental HSCs were able to self-renew in the recipient animals, as demonstrated by secondary transplantation of 1:10 and 1:100 fractions of BM from primary recipients that had been transplanted with placenta (0.3–1 ee) and AGM- (1–3 ee) derived cells from E11.5 conceptuses (Figures 2B and 2C). These results show that HSCs located in the placenta possess functional properties of bona fide adult BM reconstituting hematopoietic stem cells.

Developmental Kinetics of Placental HSCs and Progenitors

To assess hematopoietic activity in the placenta beyond the time period when HSCs are generated in known hematopoietic sites, we defined the kinetics of HSCs and progenitors throughout mid- and late gestation. Remarkably, between E11.5 and E12.5, a marked expansion of HSCs occurred in the placenta and the fetal liver (20- and 28-fold, respectively), whereas no significant increase of the HSC pool was observed in the AGM region or the yolk sac (Figure 3A). Hematopoietic expansion in the placenta favored HSCs more than progenitors, as the clonogenic myeloerythroid progenitor pool in the placenta increased only 2-fold during this period (Figure 3B). The competitive repopulation

Table 1. Donor Fetal Hematopoietic Organs

Day of Gestation	Fetal Organ	Cells/ee (10e3) ± SEM	Recipient Mice (n)	Doses Transplanted (ee)	Exp (n)
E9.5 (23–25 sp[a])	Placenta	130 ± 30	ND	ND	2
	Yolk sac	128 ± 20	ND	ND	2
	Caudal half	181 ± 79	ND	ND	2
	Decidua	532	ND	ND	1
E10.5 (33–36 sp)	Placenta	267 ± 66	33	3	5
	Yolk sac	134 ± 14	31	3	5
	Caudal half	245 ± 99	36	3	5
	Blood	116 ± 37	29	3	5
	Decidua	158 ± 29	5	3	2
	Rostral half	99 ± 5	8	3	2
E11.0 (37–40 sp)	Placenta	236 ± 28	27	0.3, 1	4
	Yolk sac	212 ± 22	22	0.3, 1, 3	4
	AGM	90 ± 15	22	0.3, 1, 3	4
	Caudal half (w/o AGM)	117	4	1	1
	Fetal liver	51 ± 3	19	0.3, 1, 3	4
	Blood	368 ± 146	18	0.3, 1, 9	4
	Cord	60	5	1	1
	Decidua	118	7	1	1
E11.5 (41–45 sp)	Placenta	458 ± 43	25	0.3, 1	3
	Yolk sac	293 ± 112	20	0.3, 1	3
	AGM	223 ± 74	19	0.3, 1	3
	Fetal liver	241 ± 91	15	0.3, 1	3
	Blood	182 ± 87	17	0.3, 1	3
	Cord	ND	5	1	1
E12.5	Placenta	1,528 ± 497	33	0.01, 0.1, 0.3	3
	Yolk sac	532 ± 122	15	0.1, 0.3	3
	AGM	368 ± 126	15	0.1, 0.3	3
	Fetal liver	1,254 ± 626	25	0.001, 0.01	3
	Blood	1,817 ± 403	21	0.3, 1	3
	Cord	ND	4	1	1
	Decidua	195	8	0.3	1
E13.5	Placenta	1,323 ± 576	39	0.01, 0.1	3
	Yolk sac	747 ± 396	24	0.01, 0.1	3
	AGM	365 ± 5	15	0.1, 0.3	3
	Fetal liver	4,192 ± 356	20	0.001	3
	Blood	1,624 ± 557	34	0.01, 0.1	3
	Decidua	260 ± 217	5	1	2
E15.5	Placenta	1,238 ± 210	26	0.3	4
	Yolk sac	558 ± 106	26	0.3	4
	Fetal liver	24,188 ± 2,314	12	0.001, 0.01, 0.1	4
	Blood	729 ± 173	30	0.1, 0.3	4
	Bone marrow	129 ± 67	12	1	2
E18.5	Placenta	1,842 ± 622	17	0.1, 0.3	3
	Yolk sac	1,442 ± 485	14	0.1, 0.3	3
	Fetal liver	242,515 ± 60,761	11	0.001, 0.01	3
	Blood	191 ± 81	20	0.1, 0.3	3
	Bone marrow	420 ± 232	14	0.1, 0.3	3

[a] sp, somite pairs.

ability of placental HSCs at this stage was high, resulting in sustained multilineage reconstitution that often increased over time, whereas fetal liver HSCs, although almost 2-fold higher in numbers at E12.5 (50 in the placenta and 93 in the fetal liver), were more heterogeneous in reconstitution potential early on, as shown by lower competitive repopulation ability of fetal liver HSCs at E12.5 (Table 2). At E13.5, the average competitive repopulation level of HSCs in the fetal liver was similar to placental HSCs (data not shown).

The size of the placental HSC pool was maintained through E13.5, but it diminished >10-fold by E15.5, while the HSC pool in the fetal liver continued to expand (Figure 3A). From E12.5 onward, a gradual increase of LT-RUs in fetal circulation occurred, exceeding in numbers those in the placenta by E15.5. Bone marrow seeding by clonogenic progenitors and rare cells that gave rise to transient in vivo reconstitution was evident at E15.5, whereas long-term reconstituting HSCs appeared in the BM by the end of gestation (Figure 3A). In summary, the developmental kinetics of HSCs and progenitors in the placenta is unique and does not reflect hematopoietic activity in any other fetal hematopoietic organ or in circulating blood.

Surface Phenotype of Placental HSCs and Progenitors

The immunophenotype of placental HSCs and progenitors was defined by assaying subpopulations of cells that had been sorted based on CD34 and c-kit expres-

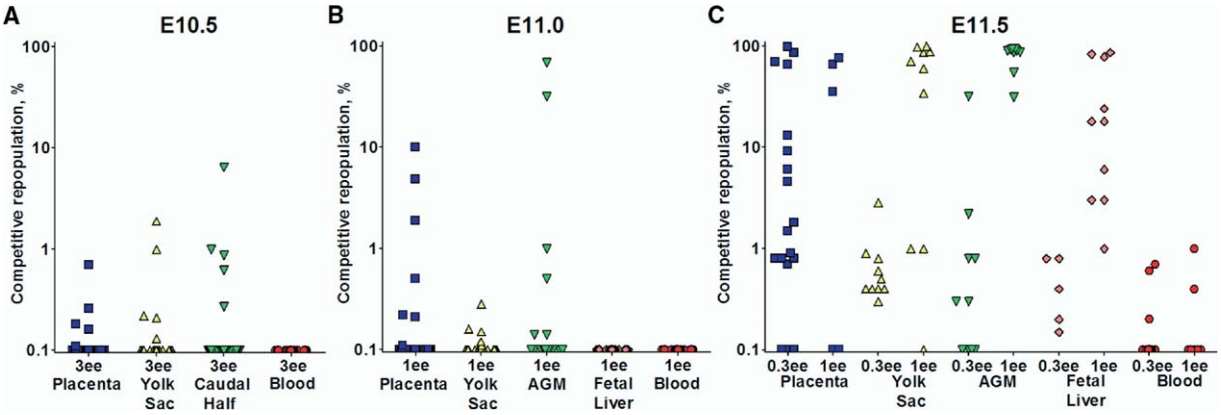

Figure 1. The Onset of HSC Development in the Mouse Embryo

(A–C) Reconstitution potential of fetal hematopoietic organs at (A) E10.5 (33–36 sp), (B) E11.0 (37–40 sp), and (C) E11.5 (41–45 sp). Symbols represent the competitive repopulation level of fetal CD45.1/CD45.2 cells, in logarithmic scale, at 10–12 weeks after transplantation, in individual recipient mice transplanted with the indicated embryo equivalents of each hematopoietic tissue.

sion. An equal dose in embryo equivalents of each sorted subpopulation (R1–R4, Figure 4A) was transplanted in order to determine which subpopulation contained placental HSCs. All HSC/RUs in E12.5 placentas were found within the c-kit^{hi}CD34^{+} population, which comprises up to 1%–2% of nucleated cells obtained from collagenase-treated placentas (Figures 4A and 4C). Likewise, most fetal liver HSCs were c-kit^{hi}CD34^{+} (Figures 4B and 4C). The majority (87%) of CFU-Cs in the placenta were also c-kit^{+}CD34^{+}, although some (13%) were found among c-kit^{+}CD34^{-} cells (Figure 4D). The proportions of c-kit^{hi}CD34^{+} and c-kit^{hi}CD34^{-} populations in the placenta contrast with the fetal liver, where the c-kit^{hi}CD34^{-} population was abundant, reflecting ongoing definitive erythropoiesis. Accompanying definitive erythropoiesis in midgestation fetal liver was further shown by the abundance of CFU-Es and CD71^{hi}ter119^{-} proerythroblasts (Zhang et al., 2003b), while these erythroid precursors were infrequent in the placenta (data not shown). These results suggest that the placenta provides a site of residence for c-kit^{hi} CD34^{+} HSCs, without promoting concomitant definitive erythroid differentiation.

Discussion

Midgestation Mouse Placenta Is a Previously Unrecognized Site of Residence for HSCs

We have reassessed the anatomical distribution of fetal hematopoietic stem cells and identified a previously unrecognized, to our knowledge, HSC niche in the murine conceptus. Our data show that, during midgestation, the mouse placenta harbors a large pool of HSCs that possess functional properties of true adult-type HSCs, i.e., the potential to provide high-level, long-term, multilineage reconstitution and to self-renew upon serial transplantation. The magnitude of HSC activity in the placenta is striking, as the placental HSC pool exceeds in numbers those in all other fetal hematopoietic organs other than the fetal liver. HSC activity, as defined by adult BM reconstitution ability, in the mouse placenta starts at E10.5–E11.0, which corresponds to 4–5 weeks of gestational age in the human embryo (Rugh, 1994). The onset of HSC activity in the placenta parallels that of the AGM region. HSC activity begins with the appearance of rare hematopoietic precursors that give rise to low-level, often transient reconstitution, whereas, by E11.5, hematopoietic reconstitution becomes robust. In contrast to the AGM and the yolk sac, the HSC pool in the placenta expands to ~50 HSC/RUs at E12.5–E13.5 and declines again during the third trimester. Thus, the kinetics of HSC activity in the placenta is unique and does not mimic hematopoietic activity in any other hematopoietic organ. Interestingly, the temporal kinetics of HSC activity in the placenta as defined by the long-term transplantation assay parallels that of B cell potential, as assessed by plaque-forming capacity (Melchers, 1979). In that study, B cell potential in the placenta was evident as early as E9.5, peaked at E12.5, and declined again toward the end of gestation, whereas B cell precursors in the fetal liver appeared later.

Anatomical Origin of Placental HSCs: Allantoic Mesoderm as a Putative Source for HSCs

The experimental approach used in this study allows the quantitation of the HSC pool that resides in each organ at each time point of analysis, but does neither reveal the origin of the HSCs nor their ultimate destination. However, the early appearance of HSCs in the placenta suggests that the allantoic mesoderm and its derivatives (the umbilical cord and the placenta) may be involved in HSC generation and/or maturation into adult-type HSCs (Figure 5). Indeed, previous studies have documented the formation of hematopoietic clusters and definitive HSCs in the umbilical artery; the formation of these clusters and HSCs coincides temporally with HSC generation in the dorsal aorta and the vitelline artery (de Bruijn et al., 2000). HSC activity in the umbilical cord was also assessed in this study in some experiments, demonstrating the presence of HSCs in the cord, albeit at lower numbers than in the

Table 2. The Onset of HSC Activity in the Mouse Embryo

Time Posttransplantation		3–4 Weeks			10–12 Weeks			>5 Months	
Day of Gestation	Organ	ER[a]/ Total	ST-RUs/ ee	Mean Rec.[b] ± SD (%)	ER/Total	LT-RUs/ ee	Mean Rec. ± SD (%)	ER >5 Months/ER at 10–12 Weeks[c]	Mean Rec. ± SD (%)
E10.5 (33–36 sp[d])	Placenta	5/33	0.096	0.32 ± 0.35	6/33	0.103	0.39 ± 0.43	3/4	0.18 ± 0.16
	Yolk sac	13/31	0.157	0.96 ± 1.57	8/31	0.107	0.45 ± 0.66	2/6	0.17 ± 0.07
	Caudal half	10/36	0.125	1.85 ± 4.07	9/36	0.107	1.05 ± 2.04	4/7	0.31 ± 0.11
	Blood	0/29	0	N/A	0/29	0	N/A	N/A	N/A
	Decidua	0/5	0	N/A	0/5	0	N/A	N/A	N/A
	Rostral half	0/8	0	N/A	0/8	0	N/A	N/A	N/A
	Total		0.378			0.317			
E11.0 (37–40 sp)	Placenta	14/27	0.733	2.8 ± 5.8	8/27	0.477	2.2 ± 3.5	7/8	2.5 ± 5.9
	Yolk sac	12/22	0.422	0.5 ± 0.7	7/22	0.133	0.2 ± 0.2	4/4	0.1 ± 0.07
	AGM	7/22	0.274	18.5 ± 21.5	9/22	0.306	20.7 ± 29.0	5/9	42.2 ± 35.0
	Caudal half (w/o AGM)	0/4	0	N/A	0/4	0	N/A	N/A	N/A
	Fetal liver	1/19	0.042	0.10	0/19	0	N/A	N/A	N/A
	Blood	3/18	0.1	0.14 ± 0.10	0/18	0	N/A	N/A	N/A
	Cord	0/5	0	N/A	0/5	0	N/A	N/A	N/A
	Decidua	0/7	0	N/A	0/7	0	N/A	N/A	N/A
	Total		1.571			0.966			
E11.5 (41–45 sp)	Placenta	20/25	2.5	25.9 ± 22.4	18/23	2.5	29.7 ± 35.8	12/12	28.9 ± 41.9
	Yolk sac	19/20	2.1	20.9 ± 27.0	19/20	2.1	28.7 ± 39.8	13/16	21.4 ± 35.7
	AGM	16/19	2.3	26.5 ± 26.0	15/19	2.0	50.1 ± 41.5	13/14	48.0 ± 43.6
	Fetal liver	15/15	3.3	20.3 ± 23.5	15/15	3.3	21.6 ± 32.4	11/13	12.7 ± 26.2
	Blood	8/17	1.8	1.8 ± 1.2	5/14	0.6	0.6 ± 0.3	3/5	1.8 ± 1.7
	Cord	1/5	0.2	26.2	1/5	0.2	84.6	1/1	92.6
	Total		11.4			10.9			
E12.5	Placenta	22/33	42.8	52.9 ± 36.0	23/33	50.0	55.5 ± 37.4	14/18	71.3 ± 32.1
	Yolk sac	11/15	3.1	46.4 ± 39.1	9/15	3.8	43.5 ± 43.1	6/9	55.9 ± 37.1
	AGM	4/15	2.7	32.7 ± 29.0	5/15	3.3	29.3 ± 30.0	2/2	71.0 ± 33.8
	Fetal liver	12/25	80.0	36.6 ± 35.5	14/25	93.3	9.9 ± 22.0	8/14	17.8 ± 28.7
	Blood	13/21	1.0	17.3 ± 20.0	15/20	1.3	14.8 ± 26.0	8/16	25.5 ± 36.3
	Cord	1/4	0.25	45.7	1/4	0.25	66.8	1/1	82.9
	Decidua	0/8	0	N/A	0/8	0	N/A	N/A	N/A
	Total		129.9			152.0			

[a] ER, engrafted recipients.
[b] Rec., Reconstitution.
[c] Only mice that survived over 5 months were included.
[d] sp, somite pairs.

placenta (Table 2). Whether de novo hemogenic activity extends into the placenta remains to be studied.

Previous studies have suggested that the allantoic vasculature forms de novo by vasculogenesis rather than by angiogenic sprouting of the intraembryonic vasculature. Allantoic vasculogenesis is initiated at the distal tip of the allantois that contains the oldest mesoderm, and it progresses in two directions, distally to fuse with the chorion and to form the placenta labyrinth, and proximally to connect with the dorsal aorta (Downs et al., 2004). Grafting experiments that used genetically marked allantoises implicate similar developmental potential for allantoic and lateral plate mesoderm, as mesoderm from the distal and proximal regions of the allantois was capable of forming not only vasculature in the placenta and the umbilical cord, but also endothelium and underlying mesenchyme of the dorsal aorta, a site at which AGM HSCs are formed (Downs and Harmann, 1997). In contrast, allantoic mesoderm did not contribute to paraxial (presomitic) mesoderm. Thus, it is likely that allantoic mesoderm gives rise to HSCs in the umbilical artery and possibly also in the placenta, and may therefore represent another independent focus for de novo hematopoiesis, in addition to the yolk sac and the embryo proper. This hypothesis is supported by the expression of markers that are associated with commitment of mesoderm to hematopoietic fate such as *brachyury*, *Flk1/VEGF-R2*, and *SCL/tal1* (Chung et al., 2002) in the allantoises of E7.5–E8.0 embryos (Drake and Fleming, 2000; Palis et al., 1999) and *runx1/AML1*, a marker for HSC genesis, in the distal tip of E8.5 allantois (North et al., 1999). A possible erythropoietic role for murine allantois was addressed in one study by culturing prefusion allantoises from head-fold-stage embryos in vitro in explant cultures and analyzing for the presence of benzidine-positive, hemoglobinized red cells (Downs et al., 1998). However, no signs of ongoing erythropoiesis in the explants were found.

Anatomical Origin of Placental HSCs: Placenta as a Niche for HSCs that Are Generated Elsewhere

As the placenta is a highly vascular organ that is continuously perfused with circulating blood cells, it was cru-

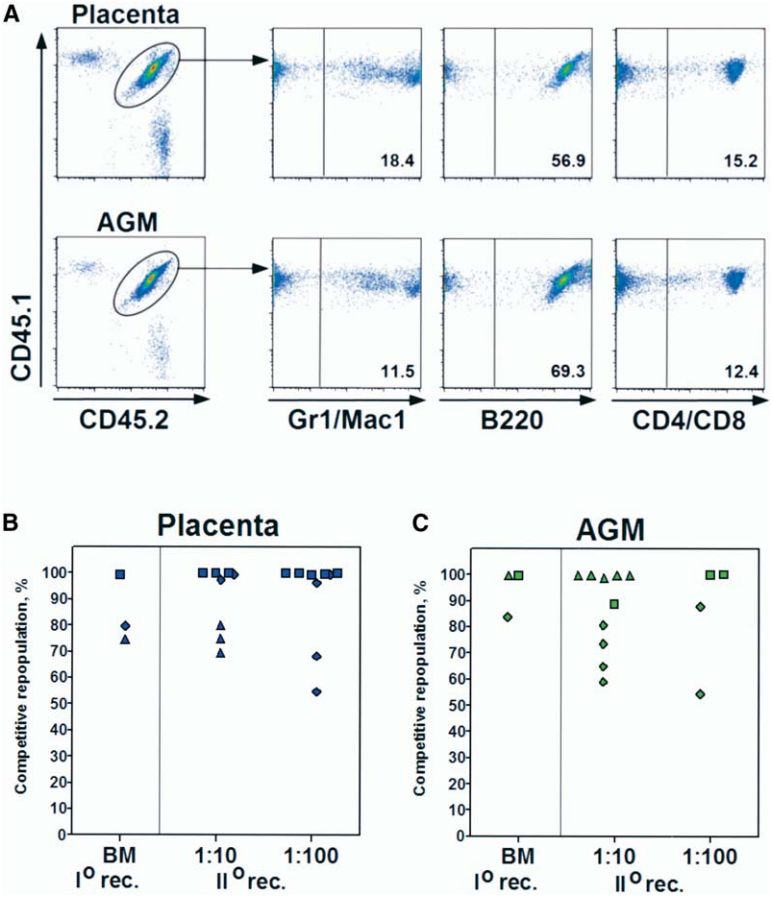

Figure 2. Multipotency and Self-Renewal Ability of Placental HSCs

(A) Multilineage reconstitution ability of E11.5 placenta and AGM HSCs as assessed by the presence of fetus-derived myeloid, B-lymphoid, and T-lymphoid cells in the recipients' peripheral blood >5 months after transplantation (representative animals are shown).

(B and C) Self-renewal ability of (B) E11.5 placenta and (C) AGM HSCs upon serial transplantation of BM from primary recipients. Peripheral blood of secondary recipients was analyzed 3 months after transplantation, and reconstitution of individual secondary recipients is shown as the competitive repopulation level, in linear scale. Comparable symbols are used for each donor and secondary recipient.

cial to compare the frequency of HSCs and progenitors in the placenta and blood to assess the possibility that placental HSCs would simply represent circulating HSCs. Importantly, HSCs in the placenta appeared earlier and in higher frequency than HSCs in circulating blood, suggesting that placental HSCs represent a unique stem cell population that is distinct from rare circulating HSCs. However, as circulation has already been established by the time that adult-type HSCs are found in the placenta, these data do not exclude seeding of immature HSC precursors from other hematopoietic sites for maturation into the placenta, or sequestration of rare circulating HSCs in placental HSC niches.

The placenta receives its blood supply from the dor-

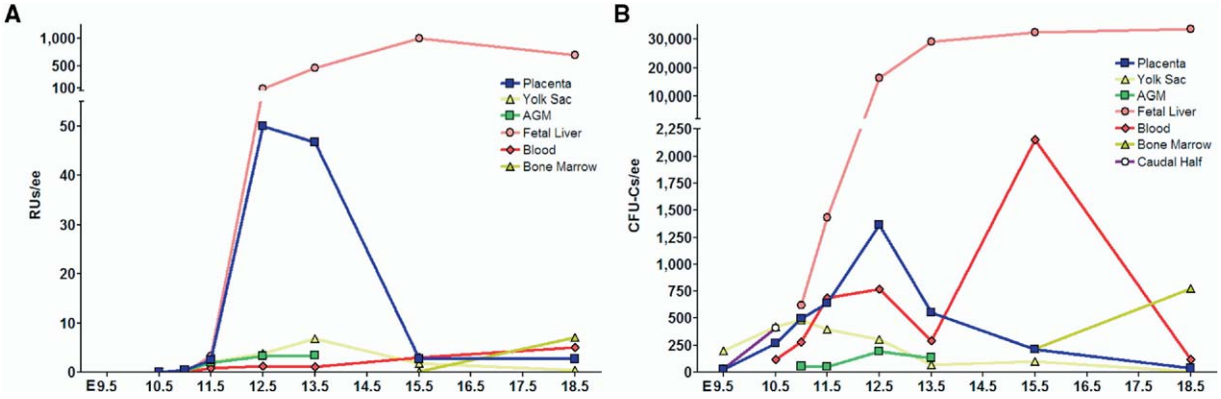

Figure 3. Developmental Kinetics of Placental HSCs and Clonogenic Progenitors

(A) Number of total LT-RUs in fetal hematopoietic sites representing ability to repopulate peripheral blood >10–12 weeks after transplantation.
(B) Number of total in vitro clonogenic progenitors (CFU-Cs) in fetal hematopoietic sites. The graphs represent data from a minimum of three individual transplantation experiments at each gestational age (see Table 1).

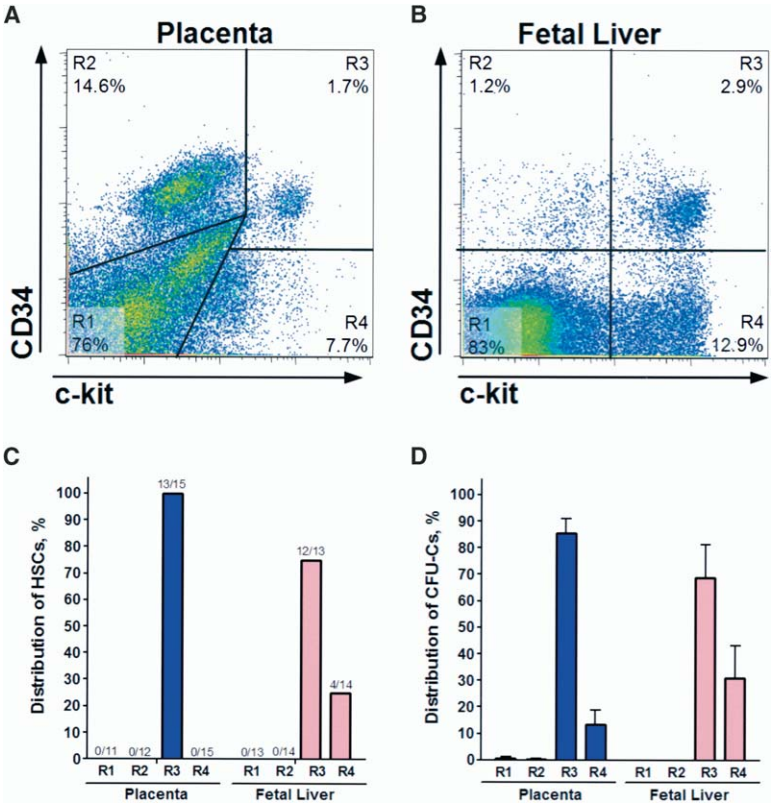

Figure 4. Immunophenotype of Placenta and Fetal Liver HSCs and Clonogenic Progenitors

(A and B) Surface phenotype analysis of HSCs and clonogenic progenitors in (A) E12.5 placenta and (B) fetal liver by flow cytometry for expression of CD34 and c-kit. The numbers on top of each quadrant indicate the frequency of each cell population.

(C and D) Distribution of (C) HSCs and (D) clonogenic progenitors between the four isolated populations indicated in (A) and (B) (n = 3 experiments). The numbers in (C) indicate the number of engrafted mice/total recipients transplanted with 0.05–0.3 ee from each population. Error bars in (D) indicate SD. R1, $CD34^{-}c\text{-}kit^{-}$; R2, $CD34^{+}c\text{-}kit^{-}$; R3, $CD34^{+}c\text{-}kit^{+}$; R4 $CD34^{-}c\text{-}kit^{+}$.

sal aorta via the umbilical artery, after which oxygenated blood drains via the umbilical vein to the fetal liver (Figure 5). In comparison, the vitelline vessels and the yolk sac are not directly connected to the placental circulation, but form another vascular circuit between the dorsal aorta (upstream of the umbilical artery) and the fetal liver. Interestingly, the AGM region does not harbor large quantities of readily transplantable HSCs at any time of development, in spite of the extensive potential of AGM HSCs to expand in explant cultures (Kumaravelu et al., 2002), suggesting that AGM HSCs are expanded elsewhere in the conceptus. It has been suggested that developing HSCs bud through the endothelial wall of the dorsal aorta and umbilical and vitelline arteries into the circulation (North et al., 1999), through which they seed hematopoietic organs. As the placenta is downstream of the dorsal aorta in fetal circulation, AGM HSCs may circulate through the placenta prior to colonizing the fetal liver. It is possible that the placenta provides a temporary niche where nascent HSCs, or their precursors, from the dorsal aorta and the umbilical artery mature and expand.

The Placenta as a Prehepatic HSC Niche that Supports HSCs without Promoting Myeloerythroid Differentiation

The placenta and the fetal liver were the only two organs that accumulated a large pool of readily transplantable HSCs during midgestation. The $c\text{-}kit^{hi}CD34^{+}$ surface phenotype of placental HSCs is comparable to that of fetal liver and AGM HSCs (Figures 4B and 4C) (Sanchez et al., 1996). A distinctive feature for $CD34^{+}$ fetal HSCs is active cycling, which results in the expansion of the HSC pool during development. In contrast, HSCs in the adult BM are $CD34^{+}$ and $CD34^{-}$, the quiescent HSCs being in the $CD34^{-}$ fraction (Dao and Nolta, 2000; Ogawa et al., 2001; Zanjani et al., 1999). Analysis of placental HSC and clonogenic progenitor frequencies by phenotype and functional assays provided evidence that the placental microenvironment supports pluripotent HSCs without promoting their differentiation along the myeloerythroid lineage. In contrast, the fetal liver, in addition to harboring a similar $c\text{-}kit^{hi}CD34^{+}$ population enriched for HSCs as the placenta, was colonized with a large number of progenitor cells that were committed for definitive erythroid differentiation (i.e., CFU-Es and $CD71^{+}ter119^{-}$ proerythroblasts). These data implicate inherent differences between fetal liver and placental HSC/progenitor populations and hematopoietic microenvironments, where the fetal liver, but not the placenta, supports active myeloerythroid differentiation during midgestation. These results are in accordance with the data from Alvarez-Silva et al., who showed that the hematopoietic developmental profile of placental and fetal liver cells in methylcellulose was very different, as the placenta harbored multipotential, high-proliferative potential progenitors, whereas the fetal liver was rich in unilineage progenitors (Alvarez-Silva et al., 2003).

The initially nonhematopoietic fetal liver rudiment is colonized, first by hematopoietic progenitors and a few days later by stem cells, when the vascular connections

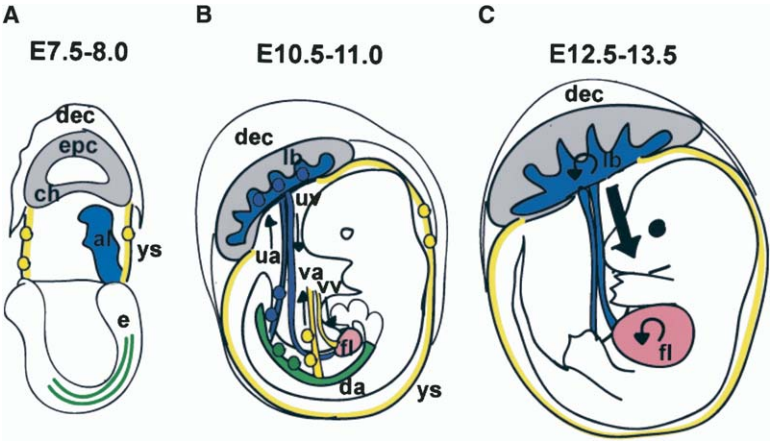

Figure 5. Revised Model of Fetal HSC Development from Hematopoietic Commitment to HSC Genesis and Expansion

(A) Allantois develops from the primitive streak as a mesodermal extension in the exocoelomic cavity and fuses with the chorion. The first signs of hematopoietic activity are seen as yolk sac blood islands.

(B) Allantoic mesoderm has formed the umbilical vasculature and placenta labyrinth. Colored circles in the large vessels, the yolk sac, and the placenta illustrate putative sites for de novo generation of progenitors and HSCs. Hematopoietic seeding of the fetal liver has been initiated via the vitelline and umbilical veins from the yolk sac and the placenta, respectively. The arrows indicate the direction of circulation and possible routes for seeding of HSCs and progenitors. Placenta may serve as another site for HSC genesis and as a niche for HSCs derived from the dorsal aorta and the umbilical artery.

(C) The HSC pool in placenta expands during midgestation, suggesting maintenance/self-renewal of HSCs in the placenta microenvironment. The decline of the placental HSC pool toward the end of gestation may reflect mobilization of placental HSCs to intraembryonic sites. dec, decidua; epc, ectoplacental cone; ch, chorion; al, allantois; ys, yolk sac; e, embryo proper; lb, placenta labyrinth; da, dorsal aorta; ua, umbilical artery; uv, umbilical vein; va, vitelline artery; vv, vitelline vein; fl, fetal liver.

to the yolk sac and the placenta are established. The first task of fetal liver hematopoiesis is to produce definitive red cells from yolk sac-derived progenitors. At the same time, a pool of pluripotent HSCs accumulates in the placenta. It is possible that the initial maturation and expansion of definitive HSCs occurs in a prehepatic HSC niche in the placenta, separated from signals that promote myeloerythroid differentiation.

The microenvironmental cues that support HSCs in the placenta are yet to be defined. Normal placental development is critical for life, as shown by a large variety of mouse mutants that exhibit different degrees of placental abnormalities that often underlie embryonic lethality. These defects can arise from the allantoic, trophoblast, or maternal components of the placenta (Rossant and Cross, 2001). Factors that support hematopoiesis may be derived from the vasculogenic milieu that drives the formation of the placenta labyrinth, as HSC development and vasculogenesis share many common regulators. Interestingly, different cell types derived from the trophoblast lineage (trophoblast giant cells, spongiotrophoblasts, and syncytiotrophoblasts) express a variety of growth factors and signaling molecules such as PlGF, PDGF, IGF-2, and LIF, which have been implicated to play a role in HSC biology in other contexts (Constancia et al., 2002; Hattori et al., 2002; Kaminski et al., 2001; Leveen et al., 1994; Takahashi et al., 2003; Zhang and Lodish, 2004). Furthermore, one of the unique features of the placenta is the intimate contact with the maternal circulation that supplies the growing embryo with oxygen and nutrients and is involved in immune protection of the fetus. Understanding how the placenta supports HSC formation, maturation, and/or expansion may ultimately give important clues about how stemness in the hematopoietic system is established and regulated, and may aid in developing better strategies for HSC expansion. Of note, one recent study has reported the ability of a human placenta-derived mesenchymal progenitor cell line to support expansion of cord blood $CD34^+$ cells (Zhang et al., 2004).

Placental HSCs as a Source of Definitive, Adult-Type HSCs

Placental HSC activity declines toward the end of gestation, possibly reflecting mobilization of placental HSCs to the fetal liver (Figure 5) and other developing hematopoietic organs within the embryo, such as thymus, spleen, and bone marrow. Indeed, during the time when the placental HSC pool was diminishing, the fetal liver HSC pool was continuously expanding and HSC seeding had been initiated in the fetal spleen (Christensen et al., 2004). While mobilization of placental HSCs and colonization of intraembryonic hematopoietic organs is an attractive hypothesis, these data do not exclude differentiation along the lymphoid pathway, or cell death, as explanations for the decline of HSC activity in the placenta.

Interestingly, no major influx of fetal HSCs into the circulation was observed at any time. Rather, a low basal level of long-term reconstituting HSCs was found in circulating blood from E12.5 onward, slightly increasing during the third trimester. These results are consistent with previous studies and support the notion that most HSCs preferably reside in HSC niches, while only a small number circulate in the bloodstream (Abkowitz et al., 2003; Christensen et al., 2004; McKinney-Freeman and Goodell, 2004). Umbilical cord blood in humans and other mammals contains an accessible source for HSCs at term, although the major limitation of the use of human cord blood stem cells in transplantation is their low abundance, being insufficient to provide adequate hematopoietic recovery for adults (Cairo and Wagner, 1997). Whether cord blood cells represent randomly circulating HSCs or a more concentrated HSC pool from the placenta and the umbilical cord is not fully defined, although studies comparing progenitor frequency in human cord blood and circulating

blood in newborn infants have implied that the composition of the two samples is similar (Zhang et al., 2002). In order to put the magnitude of placental HSC activity in perspective, it is worth noting that, in the mouse embryo, the quantity of HSCs in the placenta during midgestation (corresponding to 1–2 months of developmental age in human embryos) far exceeds that present at full term in the placenta, cord blood, and circulating blood in toto. Whether the human placenta also functions as a hematopoietic organ during development remains to be studied.

These results show that the placenta provides a previously unappreciated source for pluripotent HSCs during mouse development. While further studies are needed to define the precise origin of placental HSCs and the function of the placenta microenvironment as an HSC-supportive niche, the unique kinetics and magnitude of HSC activity in the placenta imply yet another important function for the placenta in the mammalian embryo.

Experimental Procedures

Animals and Tissues

C57BL/6 (CD45.2) and C57BL/6.SJL (CD45.1) mice (Charles River Laboratories or Taconic) were used to obtain CD45.1/CD45.2 F1 embryos, thus allowing the identification of maternal and fetal hematopoietic cells. Mice were kept at the Animal Research facilities at Children's Hospital and Dana-Farber Cancer Institute, Boston, according to institutional guidelines. The day of copulation plug was regarded as E0.5, and embryos between E9.5 and E11.5 were somite staged. The placenta, separated from the decidua, and fetal hematopoietic tissues were dissected, and embryos were exsanguinated for collection of fetal blood. At E9.5–E10.5, the caudal half of the embryo comprising the pSP (para-aortic splanchnopleura)/AGM region and the fetal liver rudiment was analyzed in one preparation. Care was taken to remove the umbilical and vitelline arteries from the placenta, the AGM, and the yolk sac. The umbilical cord, decidua, and body remnants of the caudal half of the embryo after dissection of AGM, and the rostral half of the embryo, were analyzed in some experiments. Tissues were kept on ice, washed in PBS, dissociated mechanically through a 16G needle, and treated with 0.12% collagenase type I (Sigma) in PBS with 10% FCS for 1.5 hr in 37°C, followed by passages through 18–25G needles. Some experiments were performed in parallel with trypsin treatment, which, in general, resulted in lower cell count and HSC/progenitor yield (data not shown). Single cell suspensions were filtered through 50 μm filters (Dako) and subjected to cell-counting (Crystal violet, Sigma) and functional assays.

Methylcellulose Colony-Forming Assay

Clonogenic progenitors were assayed in 1% methylcellulose (MethocultTM, 3234, Stem Cell Technologies) supplemented with 10 ng/ml IL3, 50 ng/ml SCF, 2 ng/ml IL6, 5 ng/ml Tpo, and 2 U/ml Epo. Hematopoietic colonies were scored after 6–8 days of culture in 5% CO_2 at 37°C. BFU-Es, CFU-GEMMs, CFU-GMs, and CFU-Ms were counted in the total CFU-C count, whereas CFU-Es were scored separately.

Competitive Repopulation Assay

Cells from the fetal hematopoietic organs were transplanted in limiting dilutions (3–0.001 embryo equivalents, ee) together with 1 × 10^5 unfractionated CD45.1 support/competitor BM cells into irradiated CD45.2 adult female recipients via retro-orbital injection. Irradiation was split into two 550 rad doses, 3 hr apart. The recipient mice were maintained in sterile cages with irradiated diet and antibiotic water (Baytril) for the first 4 weeks. Peripheral blood was analyzed at 4–6 weeks, 10–12 weeks, and 5–12 months after transplantation. Bone marrow, spleen, and thymus were analyzed at 10–12 weeks or 5–8 months after transplantation. For serial transplantations, 1:10 and 1:100 of unfractionated BM cells from the primary recipients was harvested at 10–12 weeks or 5–8 months and transplanted into irradiated secondary recipients together with competitor cells, as described above.

Flow Cytometry

Hematopoietic reconstitution of peripheral blood, BM, spleen, and thymus by fetal cells was analyzed by flow cytometric analysis (FACS), by using FITC (fluorosceinisothiocyanate)-conjugated CD45.2 and biotin-conjugated CD45.1 visualized by SAv (streptavidin)-APC (allophycocyanin). For lineage analyses, PE (phycoerythrin)-conjugated antibodies against B220 (B cells), Gr1 and Mac1 (granulocytes and macrophages), and CD4 and CD8 (T cells) were used. The immunophenotype of hematopoietic cells in fetal organs was analyzed with PE-conjugated c-kit antibody and biotin-conjugated CD34 antibody, visualized by SAv-APC. All monoclonal antibodies were purchased from BD Pharmingen.

Immunophenotyping of HSCs and Progenitors

The surface phenotype of placenta and fetal liver HSCs and clonogenic progenitors was determined by FACS, followed by transplantation and methylcellulose colony assay, respectively. Collagenase-treated organs were stained for CD34 and c-kit as indicated above, and cells in each organ were sorted into four subfractions (R1–R4, Figures 4A and 4B). Equal embryo equivalents (0.2–0.3 ees for the placenta and 0.05–0.2 ees for the fetal liver) of each subfraction were transplanted into irradiated recipients, and reconstitution in peripheral blood >12 weeks was assessed by flow cytometry. Similarly, each subfraction was analyzed for clonogenic progenitor activity.

Quantitation of HSC/RUs

The quantity of HSCs in each organ was assessed as reconstituting units/embryo equivalent (RUs/ee), calculated by ([reconstituted recipients]/[total recipients])/(transplanted dose). Peripheral blood reconstitution over 10–12 weeks was regarded as positive HSC/RUs.

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