Definitive hematopoietic stem cells first develop within the major arterial regions of the mouse embryo

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The aorta–gonad–mesonephros (AGM) region is a potent hematopoietic site within the mammalian embryo body, and the first place from which hematopoietic stem cells (HSCs) emerge. Within the complex embryonic vascular, excretory and reproductive tissues of the AGM region, the precise location of HSC development is unknown. To determine where HSCs develop, we subdissected the AGM into aorta and urogenital ridge segments and transplanted the cells into irradiated adult recipients. We demonstrate that HSCs first appear in the dorsal aorta area. Furthermore, we show that vitelline and umbilical arteries contain high frequencies of HSCs coincident with HSC appearance in the AGM. While later in development and after organ explant culture we find HSCs in the urogenital ridges, our results strongly suggest that the major arteries of the embryo are the most important sites from which definitive HSCs first emerge.
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Introduction

Definitive hematopoietic stem cells (HSCs) are the cells responsible for the continuous production of all mature blood cells during the entire adult life span of an individual (Lemischka, 1991; Spangrude *et al*., 1991). They are clinically important cells in transplantation protocols used in therapies for blood-related diseases. Experimentally, HSCs are defined as cells that can confer long-term reconstitution of the entire hematopoietic system of an irradiated adult recipient. To understand the biology of HSCs better, particularly the factors leading to their induction, expansion and maintenance, much research has focused on their developmental origins (Dzierzak *et al*., 1998). During mouse ontogeny, the first HSCs appear in the intra-embryonic aorta–gonad–mesonephros (AGM) region at day 10 of gestation (E10) (Muller *et al*., 1994; Medvinsky and Dzierzak, 1996). From E11 onwards, HSCs are also found in the yolk sac and fetal liver (Moore and Metcalf, 1970; Huang and Auerbach, 1993; Muller *et al*., 1994; Medvinsky and Dzierzak, 1996). However, the AGM region is the most potent source of adult repopulating HSCs (Medvinsky and Dzierzak, 1996), which are thought to colonize the fetal liver and subsequently the bone marrow, the main site of hematopoiesis in the adult.

The AGM region is an anatomically complex tissue forming several organ systems in addition to hematopoietic cells. The AGM is derived from the mesodermal germ layer and generally extends from the forelimbs to the hindlimbs of the E9.5–E12.5 mouse embryo. The first discernible tissue formed in the corresponding region of the E8 mouse embryo is the vasculature of the paired dorsal aortas (Rugh, 1990; Kaufman, 1992). At E8.5, the aortas are linked to the yolk sac vasculature through the vitelline artery, and at E9 the paired aortas fuse to form a single midline dorsal aorta (Garcia-Porrero *et al*., 1995). The umbilical artery forms the connection between the dorsal aorta and the placenta. The urogenital system begins to form at E9 (Rugh, 1990; Kaufman, 1992). Differentiation of the embryonic excretory system begins with the pronephric ducts at E9 and the mesonephric ducts at E10. By late E11, the pro/mesonephric ducts are regressing and by E12 the metanephros (definitive kidney) has become prominent in the caudal aspect of the embryo. The gonads begin to be discernible at E10 (Rugh, 1990; Kaufman, 1992) and primordial germ cells (PGCs) migrate into them. By E12 the gonads have enlarged significantly and are prominent ventral to the mesonephroi. As hematopoietic cells have been detected in association with each of the three developing organ systems (see below), it is of great interest to determine where within the actively differentiating AGM region the first HSCs develop. Insight into the spatial and temporal occurrence of this process is a prerequisite for further studies into the cellular and molecular interactions involved in the generation of definitive HSCs.

Descriptive studies performed initially in the chick (Dieterlen-Lievre and Martin, 1981) and subsequently in mammalian embryos (Garcia-Porrero *et al*., 1995; Tavian *et al*., 1996) suggest that the aorta may be the site where definitive hematopoietic cells emerge. These investigations demonstrated that discrete clusters of hematopoietic cells are closely associated with the ventral wall of the dorsal aorta. In the mouse and the human these clusters are found from E9.5 to E11.5 (Garcia-Porrero *et al*., 1995) and from E27 to E40 (Tavian *et al*., 1999), respectively, and cells within the clusters express hematopoietic markers (Tavian *et al*., 1996; Wood *et al*., 1997; Garcia-Porrero *et al*., 1998; Marshall *et al*., 1999; North *et al*., 1999). Genetic analyses support the hypothesis that the intra-aortic clusters represent definitive hematopoietic cells. For example, mutation of the *Tie-2* (Takakura *et al*., 1998) and *Cbfa2* (known also as *AML1* and *Runx1*) (North *et al*., 1999) genes disrupts both definitive hematopoiesis and the formation of intra-aortic clusters. Finally, *in vivo* labeling experiments in the chick suggest a precursor–progeny

relationship between aortic endothelial cells and intra-aortic clusters (Jaffredo *et al.*, 1998).

Several descriptive and functional studies indicate that the urogenital ridges (UGRs) may also serve as hematopoietic tissues. In non-mammalian vertebrates the presumptive mesonephric anlagen generate hematopoietic precursors and the pro/mesonephros serves as a hematopoietic site (Turpen *et al.*, 1981). The abundant presence of hematopoietic cells together with the expression of hematopoietic related genes such as *SCL, GATA-2, Flk-1* and *Fli-1* in the amphibian (Walmsley *et al.*, 1994; Bertwistle *et al.*, 1996) and fish (Gering *et al.*, 1998; Brown *et al.*, 2000) pronephros suggests that this site is important in the formation of the definitive adult hematopoietic system. In the mouse, CFU-S$_8$ (colony-forming unit-spleen day 8) (Medvinsky *et al.*, 1996) and CFU-S$_{11}$ (M.de Bruijn, M.Peeters, T.Luteijn, P.Visser, N.Speck and E.Dzierzak, submitted) are found in the UGRs, as well as in the aorta, starting at E10 of development. It is unclear whether CFU-S are seeded into or are generated *in situ* in the UGRs since the circulation is contiguous and blood cells actively move throughout the embryo beginning at E8.5 (Cumano *et al.*, 1996). Several hematopoietic markers are expressed in the mouse UGRs at the time of AGM HSC generation, including SCL (Sanchez *et al.*, 1999) and GATA-3 (Lakshmanan *et al.*, 1999), which are expressed in all mesonephric tubules, Ly-6E (Sca-1) in the anterior mesonephric tubules (Miles *et al.*, 1997), and CD34 throughout the mesonephros (Wood *et al.*, 1997). In addition, GATA-2 appears to play a critical role in urogenital development (Zhou *et al.*, 1998). However, the functional relevance of these genes in the development of HSCs within the UGRs of mammals remains to be established.

It has also been suggested that PGCs in the developing gonads may contribute to the hematopoietic lineage (Rich, 1995). Mouse PGCs not only express the c-kit receptor tyrosine kinase (Geissler *et al.*, 1988), a common hematopoietic marker expressed on HSCs (Sanchez *et al.*, 1996), but can also, under certain culture conditions, yield embryonic stem (ES)-like cells that can be differentiated *in vitro* into hematopoietic cells (Rich, 1995). In summary, the available data suggest that the aorta, mesonephros and gonads are all possible sites for the development of hematopoietic cells. However, the precise site from which definitive HSCs first emerge is unknown.

Here we present the results of experiments in which we determine the localization of HSCs within the mouse AGM region. We subdissected AGM regions from genetically marked E11–E12 embryos into parts containing either the aorta or the UGRs, and tested cell suspensions prepared from these tissues for functional HSCs by *in vivo* transplantion into irradiated adult mice. We find that at E11 HSCs reside only in the aorta region of the subdissected AGM. Results of transplantation experiments with other major embryonic vessels, the vitelline and umbilical arteries, indicate that these tissues also contain HSCs. Thus, the major arterial regions of the mouse embryo are the first and most potent tissues involved in the development of HSCs. At E12 the UGRs contain HSCs as well. Data obtained from the transplantation of cultures of E11 UGR explants suggest that the HSCs increase

autonomously in the UGRs. The studies presented here are the first to demonstrate functionally that definitive HSCs develop from the major arterial regions (dorsal aorta, vitelline and umbilical vessels) of the vertebrate embryo and strongly support a close relationship between the developing hematopoietic and vascular systems.

Results

HSCs develop within the dorsal aorta and surrounding mesenchyme of the subdissected E11 AGM

To locate HSCs within the AGM region we subdissected it longitudinally, separating the dorsal aorta with its surrounding mesenchyme from the UGRs (Figure 1). E11 was chosen as the earliest time for analysis since the developing tissues are well demarcated, allowing precise subdissections. Also, at E11 HSCs are present in sufficiently high numbers to be frequently detected in freshly isolated tissues (Muller *et al.*, 1994). To compare the frequency of HSCs in the aorta with that in the UGRs, cell suspensions were made from the subdissected tissues of genetically marked embryos and injected intravenously at limiting dilutions into irradiated adult recipient mice. Mice were analyzed for donor-type hematopoietic reconstitution at 4–9 months post-transplantation by a donor-specific semi-quantitative PCR assay on DNA isolated from peripheral blood.

As shown in Table I, direct transplantation of 1 or 0.3 embryo equivalents (e.e.) of E11 aorta and mesenchyme yielded high level donor-type repopulation. Successfully transplanted recipients of either 1 or 0.3 e.e. of dorsal aorta (7/17 and 4/15, respectively) showed high level engraftment (average 90%) in the peripheral blood (Figure 2A). In contrast, at the same embryonic donor tissue equivalents, HSCs could not be detected, not even at low levels (<10%) of engraftment in recipients transplanted with UGR cells (Table I). To determine whether a small number of HSCs are present in the UGRs, high numbers of UGR tissue equivalents were also transplanted. No donor cell derived engraftment was found when up to 5 e.e. of UGR cells were transplanted, suggesting that at E11 the UGR contains very little or no HSC activity.

To test whether the transplantation of E11 aorta region cells results in complete, multilineage hematopoietic repopulation, recipient mice were killed at >4 months post-transplantation and several hematopoietic tissues and cell lineages were tested for the presence of the donor genetic marker. As shown in Figure 2A, PCR analysis of DNAs from all samples shows high level (up to 100%) contribution by donor-marked cells. Furthermore, the bone marrow of such primary recipient mice yields high level hematopoietic engraftment when transplanted into secondary recipients (data not shown), fulfilling the stringent criteria of definitive HSCs. Thus, adult-type HSCs first develop within the dorsal aorta area of the AGM region.

HSCs are also present in the UGRs at E12

We previously found a change in the anterior–posterior distribution of HSCs from E10 to E11 in the mouse AGM region (Medvinsky and Dzierzak, 1996; A.Medvinsky, personal communication). At E10 HSCs were found only in the anterior half of the AGM region, whereas at E11 they

**Table I.** HSC activity in directly transplanted and cultured E11 and E12 aorta and UGR

Tissue	Embryo equivalent	No. of mice repopulated/total transplanted[a]			
		E11	E12	E11 cultured[b]	E12 cultured[b]
Aorta[c]	1	7/17	17/29	17/28	6/6
	0.3	4/15	1/6	8/20	0/6
UGR	4.3[d]	0/3			
	1	0/10	5/26	18/26	2/6
	0.3	0/9	0/4	8/19	2/5
AGM	1	7/8	7/9	9/11	4/5
	0.3	1/6	1/2	5/11	4/5

[a]Only mice with ≥10% donor repopulation in the peripheral blood at 4–9 months after transfer were included as positive. Data are from a total of 21 independent experiments.
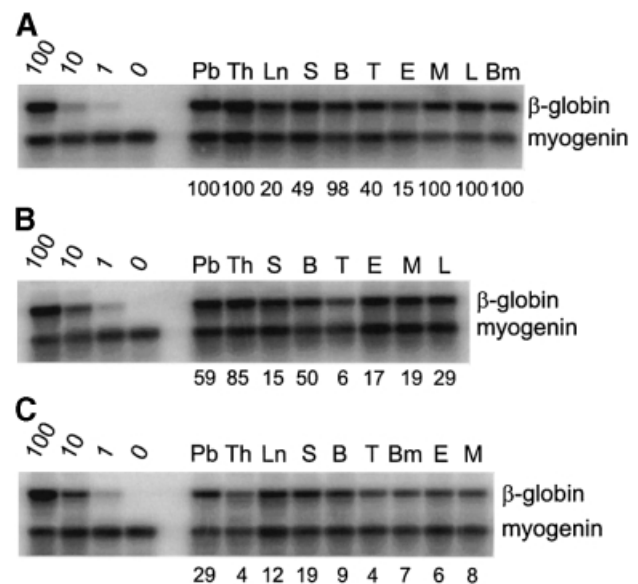[b]Organ explants were cultured for 3 days prior to transplantation.
[c]Aorta region: includes surrounding mesenchyme.
[d]An average of 4.3 e.e. were transplanted in two experiments (range 4–5).
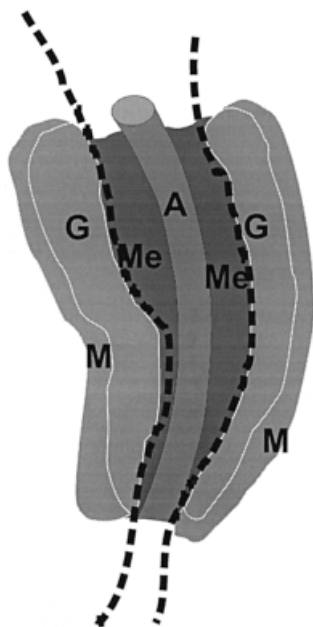
Fig. 1. Schematic representation of subdissected regions of the AGM. E11 and E12 AGM tissue is dissected along the dotted lines to separate the dorsal aorta and surrounding mesenchyme from the urogenital ridges, the latter of which contain the developing gonads and pro/mesonephroi. A, dorsal aorta; Me, mesenchyme; G, gonad; M, mesonephros.

Fig. 2. Multilineage repopulation analysis of transplant recipients engrafted with aorta- or UGR-derived HSCs. Southern blot analysis was performed on products obtained from donor-marker specific PCRs. PCR reactions were performed on DNA isolated from hematopoietic tissues and sorted hematopoietic cells from representative transplant recipients receiving cells from (**A**) E11 aorta, (**B**) E12 aorta and (**C**) E12 UGR. At >4 months post-transplantation, transplant recipients were killed and hematopoietic tissues isolated. Lymphoid, myeloid and erythroid lineage cells were sorted and DNAs examined for the presence of the donor cell genetic marker (human β-globin). Percentage contribution was controlled by mixes of the donor-transgene marker with unmarked DNA (100, 10, 1 and 0%). Donor-marker contribution was determined by PhosphorImager quantitation of specific ^{32}P hybridizing probe. The donor signal was normalized to the hybridization signal for the myogenin PCR product. Percentage donor marker contributions are indicated below each lane. Pb, peripheral blood; Th, thymus; Ln, lymph nodes; S, spleen; B, splenic B lymphocytes; T, splenic T lymphocytes; E, bone marrow erythroid precursors; M, bone marrow myeloid precursors; L, bone marrow lymphoid precursors; Bm, bone marrow.

were spread throughout the AGM region. Here we examined whether HSC activity expands in a lateral direction during development. We dissected E12 AGMs into aorta and UGRs, and transplanted the cells as described above. In contrast to the E11 UGR, transplantation of 1 e.e. of E12 UGR cells did result in high level (40–100%) donor-type HSC repopulation in five out of 26 mice (Table I). Multilineage hematopoietic repopulation of all hematopoietic tissues was found (Figure 2C), and secondary transplantion of bone marrow from the primary UGR transplanted mice yielded high level peripheral blood contribution (40% engraftment). Thus, the UGR harbors and/or generates true HSCs at E12. However, the highest

number of HSCs is still found in the aorta region (17 out of 29 mice were high level engrafted with 1 e.e. of E12 aorta; Table I). Repopulation by aorta region-derived HSCs is multilineage (Figure 2B) and the HSCs can be serially

Table II. Spatial distribution of HSCs within the E12 UGR

Tissue	No. of mice repopulated/total transplanted[a]
Gonads	1/10
Mesonephroi	4/11
Metanephroi	1/2[b]
UGR[c]	3/7
Aorta[d]	9/11
AGM[c]	5/6

[a]Transplanted mice received 1–2 e.e. of subdissected tissue. Only mice with ⩾10% donor repopulation in the peripheral blood at 4–5 months after transfer were included as positive. Data are from a total of two independent experiments.
[b]Five embryo equivalents transplanted per mouse.
[c]Neither UGR nor AGM include metanephroi.
[d]Aorta region: includes surrounding mesenchyme.

transplanted into secondary recipients yielding up to 80% peripheral blood contribution (not shown). Hence, the high frequency of HSCs in the E12 aorta region suggests that it remains the major site of HSC development.

The complex differentiation of the excretory and reproductive systems within the UGR begins around E10/11. At E12 it is possible to dissect the gonads cleanly from the pro/mesonephros. In addition, the metanephroi (definitive kidneys) are now easily identified and isolated. To determine in which part of the UGR HSCs are localized, we isolated gonads, mesonephroi and metanephroi from E12 genetically marked embryos and transplanted cell suspensions of these tissues into irradiated adult recipients. At >4 months post-transplantation, hematopoietic engraftment was found mainly in mice transplanted with 1–2 e.e. of mesonephroi (four out of 11 recipients) (Table II). Only one out of 10 recipients transplanted with 1–2 e.e. of gonads was positive. Interestingly, one out of two recipient mice transplanted with a high dose (5 e.e.) of metanephric cells was positive. These data demonstrate that the majority of HSCs in the E12 UGR are harbored in the mesonephroi.

HSC numbers increase autonomously in cultured E11 UGRs

There are several possible explanations for the 1 day delay in the appearance of HSCs in the UGR. (i) The UGR is seeded by definitive HSCs between E11 and E12. (ii) The UGR is seeded by very low numbers of HSCs that migrated there before E11. These HSCs then expand in the UGR, reaching detectable levels by E12. (iii) HSCs develop autonomously in the UGR. (iv) The UGR is seeded by pre-definitive HSCs by E11 (Medvinsky and Dzierzak, 1999). To begin to discriminate between these possibilities we isolated E11 UGRs and performed an organ explant culture before transplantation. We previously showed that the organ explant culture allows for the expansion of detectable HSC activity (Medvinsky and Dzierzak, 1996). Separation of the E11 UGR from the remaining embryonic tissues, followed by explant culture, will prevent migration of HSCs into the UGR between E11 and E12. As shown in Table I, a remarkable increase in HSC activity was found in the cultured E11 UGR compared with the uncultured E11 UGR (18/26 versus 0/10; 1 e.e./mouse). Cultures of E11 aorta region, performed
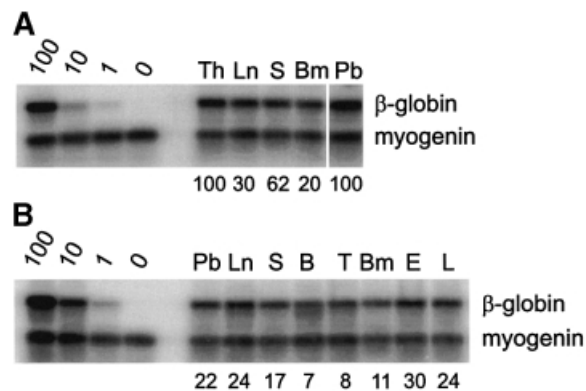
Fig. 3. Multilineage repopulation analysis of transplant recipients engrafted with cultured aorta- or UGR-derived HSCs. DNAs were isolated from hematopoietic tissue and sorted hematopoietic cells from representative transplant recipients receiving cells from (**A**) E11 cultured aorta and (**B**) E11 cultured UGRs. At >4 months post-transplantation, transplant recipients were killed and hematopoietic tissues isolated. Lymphoid, myeloid and erythroid lineage cells were sorted and DNAs examined for the presence of the donor cell genetic marker (human β-globin) as described in Figure 2.

for comparison, also showed an increase in HSC activity, though less dramatic than the increase in the UGR cultures. The engraftment by cultured E11 UGR and aorta is due to definitive HSCs, since they confer multilineage hematopoietic repopulation (see Figure 3). HSC activity is maintained at the same levels in E12 cultured UGR compared with uncultured E12 UGR (2/6 versus 5/26). These explant culture data indicate that HSC numbers can be dramatically increased in E11 UGR, independent of HSC influx from the aorta region between E11 and E12. The HSCs in the UGR must therefore have either migrated to the UGR in very limited numbers by E11, or developed autonomously in the UGR during the culture period.

To determine whether signaling between the aorta and UGR influences in a positive or negative manner the HSC activity in either tissue at E11, we prepared co-cultures of genetically marked aorta with unmarked UGR explants, and of unmarked aorta with marked UGR explants. One subdissected UGR was placed on each side of the aorta region to restore the normal AGM architecture. Following 3 days of co-culture the cells were tested for HSC activity (Table III). The repopulating activity found in 1 e.e. of UGR cultured alone (8/14 recipients) versus 1 e.e. of UGR cultured with aorta (7/14) was similar. The repopulating activity in the 1 e.e. of aorta cultured alone (7/14) compared with that of 1 e.e. of aorta cultured in the presence of the UGR (5/14) was also similar. Thus, HSC generation in either the aorta or UGR appears to occur at the same efficiency regardless of whether the aorta and UGR are cultured alone or in direct juxtaposition, suggesting that there is neither an activating nor repressing signal delivered by one tissue that impacts on HSC generation in the other.

The spatial and temporal expression pattern of Cbfa2 in the AGM coincides with the presence of HSCs

The expression patterns of known markers of definitive HSCs have been studied to identify the source of HSCs in

Table III. HSC activity in co-cultured E11 aorta and UGR

Marked tissue	Embryo equivalent	No. of mice repopulated/total transplanted[a]	
		Cultured alone	Co-cultured[b]
Aorta	1	7/14	5/14
	0.3	4/12	3/11
UGR	1	8/14	7/14
	0.3	5/11	4/14

[a]Only mice with ≥10% donor repopulation in peripheral blood at 4–6 months after transfer were included as positive. Data are from a total of five independent experiments.
[b]Marked aorta regions were cultured for 3 days with unmarked UGRs, and marked UGRs with unmarked aortas. Marked and unmarked co-cultured tissues were injected together.
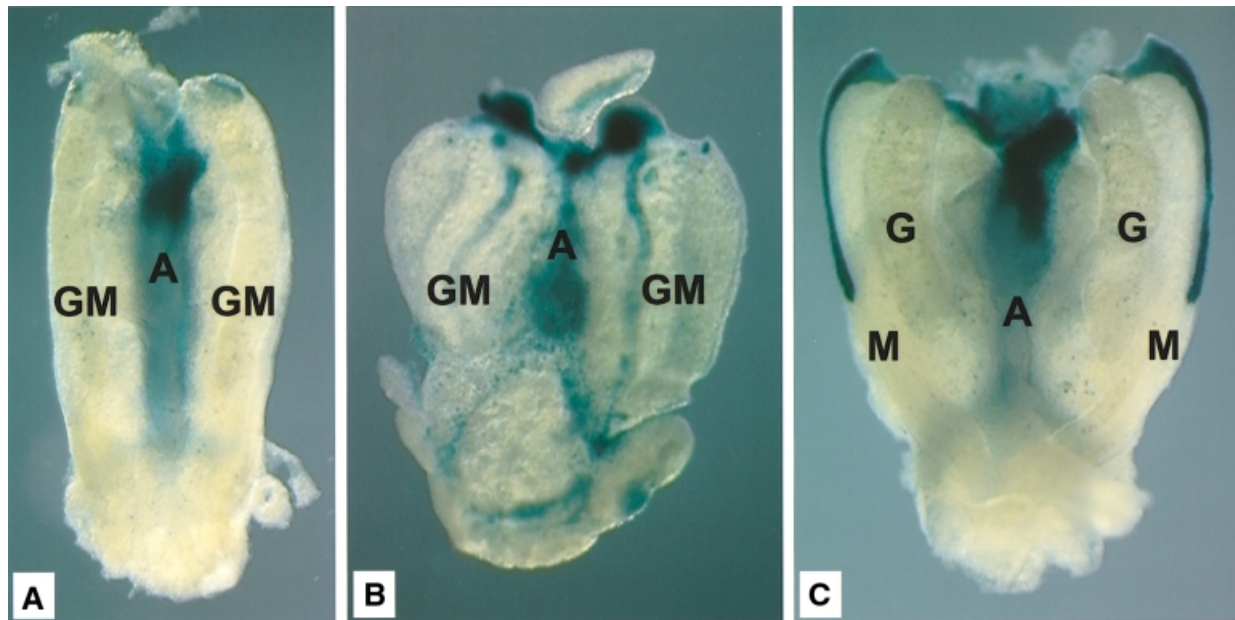
Fig. 4. *Cbfa2-LacZ* expression in subregions of the AGM. X-gal staining was performed on (**A**) E11 AGM directly after dissection, (**B**) E11 AGM after 3 days in organ explant culture and (**C**) E12 AGM directly after dissection. Abundant blue staining is observed in the area containing the dorsal aorta and mesenchyme in all three tissues. However, the UGRs stained brightly only at E12 or after explant culture of E11 AGMs. The stripe of expression in the anterior mesonephros at E12 is from the Mullerian duct. Whether HSC activity is located in the strongly X-gal-positive Mullerian ducts or elsewhere in the mesonephroi remains to be determined.

complex tissues. One such recently described marker is CBFα2, a transcription factor important in the development of definitive hematopoietic cells. Homozygous disruption of the *Cbfa2* gene leads to a complete deficiency in all definitive hematopoietic lineages in the developing mouse embryo, resulting in fetal liver anemia and lethality at E12 (Okuda *et al.*, 1996; Wang *et al.*, 1996). No HSCs are found in the AGM of *Cbfa2*-deficient embryos, suggesting that the AGM is the site of the primary defect (Z.L.Cai, M.de Bruijn, X.Ma, B.Dortland, T.Luteijn, J.R.Downing and E.Dzierzak, submitted). *Cbfa2* is expressed in the ventral endothelium of the dorsal aorta, the ventral para-aortic mesenchyme and in the clusters of hematopoietic cells associated with the ventral wall of the aorta (North *et al.*, 1999). Furthermore, *Cbfa2* is required for the formation of intra-aortic hematopoietic clusters (North *et al.*, 1999). Taken together, these results suggest that *Cbfa2* is expressed by the first HSCs as they arise in the mouse embryo. Since we

observe HSCs in the UGRs of E11 cultured and E12 uncultured AGM regions, we tested whether *Cbfa2* was expressed in the UGRs at these times.

Embryos that express *lacZ* from the *Cbfa2* gene (North *et al.*, 1999) were dissected and stained with the β-galactosidase substrate X-gal. As shown in Figure 4A, a representative E11 AGM region shows a high degree of staining in the aorta and surrounding mesenchyme. However, little staining (at the anterior-most tips of the pronephros) is observed in the UGR, although serial sections reveal the presence of isolated *Cbfa2*-expressing cells scattered throughout the UGR (not shown). At E11 HSCs are found in the directly transplanted aorta, but not in the UGRs (Table I). In contrast, strong X-gal staining is observed in the UGRs of E11 cultured and E12 uncultured AGMs (Figure 4B and C, respectively), times at which functional HSCs are present there (Table I). In the UGRs of the E12 AGM, *Cbfa2* expression is most prominent in the Mullerian ducts in the mesonephroi, again coincident

Table IV. HSC activity in directly transplanted vitelline/umbilical arteries

Tissue	Embryo equivalent	No. of mice repopulated/total transplanted[a]		
		E10.5	E11.5	E12.5
Vitelline/umbilical	2.5–5	4/35[b]		
	3		3/7	22/24
	1		3/8	10/22
	0.3		1/3	2/16
	0.1			2/8
AGM	2.5–3	1/12		16/16
	1		4/6	14/22
	0.3		3/6	6/14
	0.1		1/3	0/10
Liver[c]	3	0/3	2/2	
	1		2/6	
	0.3		1/2	

[a]With one exception (see below), only mice with ≥10% donor repopulation at 4–7 months after transfer were included as positive. Data are from a total of 12 independent experiments.
[b]One mouse transplanted with 5 e.e. of umbilical arteries was engrafted at high levels (100%) at 3 months post-transplantation, but died before the >4 month bleed could be performed.
[c]Liver was used in these experiments as a positive control and thus is not mentioned in the text of the Results.
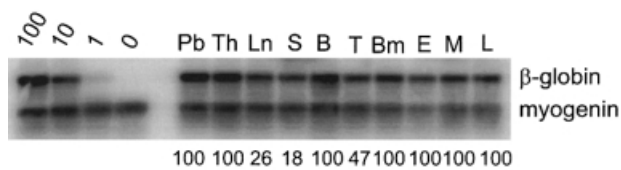
Fig. 5. Multilineage repopulation analysis of a transplant recipient engrafted with E10 vitelline and umbilical arteries. At >4–7 months post-transplantation, transplant recipients that received vitelline and umbilical arteries were killed and hematopoietic tissues isolated. The results from a representative multilineage analysis are shown. Lymphoid, myeloid and erythroid lineage cells were sorted and DNAs examined for the presence of the donor cell genetic marker (human β-globin) as described in Figure 2.

with the presence of HSCs in the mesonephroi (Table II). Thus, *Cbfa2* expression coincides with the spatial and temporal presence of HSCs in the aorta and UGRs.

HSCs are present in the vitelline and umbilical arteries at high frequency beginning at E10.5
The enrichment of HSCs in the aorta region of the AGM prompted us to extend our analyses to the other major arteries present at this time in the mouse conceptus: the vitelline and umbilical vessels. Histological and functional studies (Garcia-Porrero *et al.*, 1995; Takakura *et al.*, 1998), as well as the *Cbfa2* expression pattern (North *et al.*, 1999), suggested the presence of definitive hematopoietic cells in these vessels, but the presence of HSCs had not been assessed. We isolated the vitelline and umbilical vessels from E10 through E12 embryos and assayed them by adoptive transfer for the presence of HSCs. At early E10 (<35 somite pairs) no HSC activity is detectable in the vitelline and umbilical arteries even when large numbers of cells (up to 4.5 e.e.) are transplanted. However, beginning at E10.5, HSCs are found in limiting numbers (Table IV). Direct transplantation of 2.5–5 e.e. of the vitelline and umbilical vessels resulted in four highly engrafted recipients out of 35 transplanted. The frequency of successfully engrafted recipients increases at E11 (three

out of eight recipients with 1 e.e.) and remains high at E12 (10 out of 22 with 1 e.e.). Mice engrafted with vitelline and umbilical arteries show long-term multilineage hematopoietic repopulation of all hematopoietic tissues (Figure 5), as well as high level hematopoietic repopulation of secondary recipients with bone marrow from primary engrafted animals (not shown). These results demonstrate that the vitelline and umbilical vessels harbor true HSCs. To determine whether HSCs are generated autonomously in this tissue, explant cultures were attempted, but were not successful. Nevertheless, since HSCs were not found in E10–12 body remnants or in the circulating blood by direct transplantation (our unpublished results; Muller *et al.*, 1994), the data indicate that the vitelline and umbilical vessels are in fact a potent source of definitive HSCs. At all time points the frequencies of HSCs in the vitelline plus umbilical vessels and AGM regions transplanted from the same embryos were very similar. Thus, the vitelline and umbilical arteries, along with the dorsal aorta region of the AGM, are a major source of definitive HSCs.

Discussion

Vascular arterial localization of definitive HSCs
The ontogenic association of hematopoietic cells with the vasculature has long been observed in the blood islands of the mammalian yolk sac (Sabin, 1917) and together with genetic studies has led to the notion that endothelial and hematopoietic cells differentiate from a common progenitor. The targeted disruption of the vascular endothelial growth factor (*VEGF*) (Carmeliet *et al.*, 1996; Ferrara *et al.*, 1996) and the *Flk-1* receptor tyrosine kinase (Shalaby *et al.*, 1995, 1997) genes in mouse embryos results in complete deficiencies in both the vascular and hematopoietic systems, strongly implicating a close developmental relationship between these lineages. However, the acceptance of an intra-embryonic origin for adult mammalian hematopoiesis is only recent

(Dzierzak and Medvinsky, 1995). Hence, the origin of adult hematopoietic cells (and in particular definitive HSCs) within the specific vascular regions of the mammalian embryo body is highly speculative. Many descriptive, immuno- and histochemical analyses in mouse and human embryos using markers associated with definitive HSCs and progenitors have demonstrated clusters of hematopoietic cells in close association with the floor of the dorsal aorta (Garcia-Porrero et al., 1995; Tavian et al., 1996). The expression of hematopoietic markers such as CD34, SCL, CD31, AA4.1 and CBFα2 is shared with endothelial cells lining the dorsal aorta (Baldwin et al., 1994; Wood et al., 1997; Marshall et al., 1999; North et al., 1999; Petrenko et al., 1999; Sanchez et al., 1999) and closely parallels results observed in the avian species (Drake et al., 1997; McNagny et al., 1997; Jaffredo et al., 1998). Targeted disruption of *Tie-2* selectively disrupts vascular remodeling and definitive hematopoiesis (Sato et al., 1995; Takakura et al., 1998), and disruption of the *Cbfa2* gene blocks both definitive hematopoiesis (Okuda et al., 1996; Wang et al., 1996) and the formation of intra-aortic clusters (North et al., 1999). Taken together, these studies strongly suggest that the mammalian dorsal aortic endothelium and/or surrounding mesenchymal cells are the source of definitive hematopoietic cells. However, none of these studies directly investigated whether functional definitive HSCs, the putative source of the adult hematopoietic system, are localized at high frequencies within the mammalian dorsal aorta.

We demonstrate here that the dorsal aorta with its surrounding mesenchyme is indeed a potent site of functional HSC generation. Subdissections of the E11 AGM region show that only the aortic region contains definitive HSCs at this time in development. At a later developmental time, the aortic region remains the most potent source of HSCs within the AGM region. We also show for the first time that functional definitive HSCs are present within the vitelline and umbilical arteries. The presence of HSCs in these vessels at E10.5 coincides with their first appearance in the AGM. Furthermore, our data show that the number of HSCs within the vitelline and umbilical vessels appears to be similar to the number of HSCs in the AGM region. Thus, the first and most potent sites of definitive HSC activity are associated with the major vasculature of the mouse embryo: the dorsal aorta and the vitelline and umbilical arteries.

Remarkably, these are the only vessels in both avian and mouse embryos in which clusters of hematopoietic cells were found to be attached to the lumenal wall (Dieterlen-Lievre and Martin, 1981; Garcia-Porrero et al., 1995). In addition, the CBFα2 transcription factor is expressed in the clusters of hematopoietic cells and in the endothelial cells lining both the ventral wall of the dorsal aorta and the vitelline and umbilical arteries (North et al., 1999). The hematopoietic cells appear to be budding from the aortic endothelium, thus suggesting an endothelial precursor to definitive hematopoietic cells. Direct labeling studies in chick embryos show that cells lining the dorsal aorta, most likely endothelial cells, give rise to intra-aortic hematopoietic clusters (Jaffredo et al., 1998). If hemogenic endothelial cells do in fact exist (Dieterlen-Lievre, 1998; Nishikawa et al., 1998a,b), they may be present only at specific developmental times and anatomical positions.

Since the first adult repopulating HSCs appear in the dorsal aorta when it is an intact and functioning vessel, it is likely that the immediate precursor to the definitive HSC is an endothelial cell that maintains plasticity and potential for the hematopoietic lineage. However, at this developmental time the aorta is continuing to increase in length as well as diameter (Kaufman, 1992). Hemangioblasts may still be present and contribute to this vascular growth as well as give rise to HSCs in the inductive microenvironment of the existing ventral endothelium. Another alternative for the direct precursor to adult repopulating HSCs may be a type of multipotential hematopoietic cell that acquires characteristics needed for adult hematopoietic repopulation. Putative precursors may include the neonatal repopulating cells found in the E9 AGM and yolk sac (YS) (Yoder et al., 1997), or the *in vitro* clonable multipotential progenitors found in E7.5 paraaortic splanchnopleura and E8.5 YS (Cumano et al., 1996).

Sublocalization of other hematopoietic progenitors within the AGM

Recently, several other hematopoietic progenitors have been sublocalized within the mouse AGM region. In general, these immature and more mature hematopoietic progenitors are localized within the aortic region. The spatial and temporal mapping of CFU-S_8 show that these progenitors are present in both the aorta and the UGRs at E10 (Medvinsky et al., 1996). Although the frequency of CFU-S_8 is always highest in aortic tissue, by late E10 the absolute number of CFU-S_8 in the UGRs is higher than in the aorta (2- to 4-fold), suggesting that these progenitors may be made in the aortic area but preferentially colonize and/or expand in the UGR. The distribution of the more immature CFU-S_{11} progenitors over the E10 and E11 AGM has been found to be slightly higher in absolute numbers and in frequency in the aorta region. Later, at E12 in development, equal frequencies are found in the aorta and UGRs. Thus, CFU-S predominate early in the aorta region, with activity spreading to the UGRs.

B-cell precursors also predominate (in absolute number and frequency) in the aorta region, but are present as well in the mesonephros, gonad and mesentery of E10 embryos (Godin et al., 1999). Additionally, *in vitro* clonable multilineage precursors are found in the aorta region (Godin et al., 1999), but no data are available from the other subdissected regions. Thus, the aorta region contains the greatest numbers of HSCs as well as immature and more mature progenitors, supporting the notion that this vascular region (together with the vitelline and umbilical arteries) is the most potent generator of the definitive adult hematopoietic system. It is interesting to speculate that definitive HSCs, along with the variety of other functional hematopoietic progenitors detected in the aorta region, may reside together in the hematopoietic clusters associated with the arterial walls, as suggested by differential expression of glycoprotein IIb–IIIa on the cells within the aortic clusters (Ody et al., 1999).

The mesonephros as a site of hematopoietic cell growth

The presence of functional definitive HSCs in the mesonephros of the mouse embryo is a curious but not unexpected finding. The pronephros in amphibians and

fish is a well-described site of hematopoiesis (Turpen *et al.*, 1981; Walmsley *et al.*, 1994; Bertwistle *et al.*, 1996; Gering *et al.*, 1998). IgM-positive B cells are found in the mesonephros of the chick (Zettergren and Cutlan, 1992) and also in *Rana* pronephros and mesonephros (Zettergren, 1982). Macrophages are detected in UGRs of E11.5 mouse embryos (De Felici *et al.*, 1986), and as described above, hematopoietic progenitors are also found in the UGRs of E10 and E11 mouse embryos. Since the mesonephros is formed from intermediate mesoderm (Larsen, 1997), the presence of HSCs in the mesonephros suggests that plasticity for the hematopoietic lineage may be maintained in the mesoderm. Indeed, many hemato-poietic-associated genes (*Cbfa2*, *GATA-2*, *GATA-3*, *SCL*, *Sca-1*, *CD34*, *Flk-1*) (Miles *et al.*, 1997; Wood *et al.*, 1997; Robert *et al.*, 1998; Zhou *et al.*, 1998; Lakshmanan *et al.*, 1999; North *et al.*, 1999; Sanchez *et al.*, 1999) are expressed in the developing excretory system (mesone-phros, metanephros) and particularly in the Wolffian and Mullerian ducts. Since vasculogenesis rather than angio-genesis is thought to occur in the UGRs (Robert *et al.*, 1998), it is interesting to speculate that these vascular precursors may still possess hematopoietic potential and form some HSCs *in situ*.

Alternatively, localized migration of hematopoietic cells (or cells with hematopoietic potential) into the E11 mesonephros may occur, since it has been reported that cells in the mouse embryo readily migrate from the mesonephros to the gonads at E10/E11 (Martineau *et al.*, 1997; Perez-Aparicio *et al.*, 1998). If the source of definitive HSCs in the mouse is the aorta, the presence of these cells in the mesonephros (and at low frequencies in the gonads and metanephros) would support a lateral pattern of interstitial migration. Regardless of the mech-anism by which HSCs appear in the UGR, the UGR is clearly a potent site for HSC activity, as is shown by the dramatic increase in HSC activity in E11 UGR explant cultures. The nature of this increase, be it expansion of limited numbers of HSCs already present or *de novo* generation of HSCs in isolated UGR, remains to be established.

While our studies do not directly address the question of PGCs as the precursors to HSCs, the frequency of PGCs in the gonads at E12 suggests that they are not a potent source of HSCs. Studies examining the migration and expansion of PGCs in mid-gestation mouse embryos estimate the number of PGCs in the gonads at E12 to be between 2000 and 2500 (Chiquoine, 1954). This number of PGCs, if they were to possess HSC potential, would be readily detected in the gonads by our adoptive transfer method. Our studies show very little hematopoietic repopulation with trans-planted gonadal cells. Although we transplanted low embryo equivalents of subdissected gonads and mesone-phroi, the number of HSCs found in the subdissected mesonephroi was always similar to the number of HSCs in the whole UGR. Thus, the low frequency of HSCs in the gonads renders it unlikely that the definitive adult hematopoietic system takes its origins from PGCs in this tissue at E12. However, fate mapping and the testing of PGCs in the microenvironment of the aorta, mesenchyme or mesonephros will have to be carried out to address this issue definitively.

In conclusion, we have demonstrated that the vascular regions, the dorsal aorta and the vitelline and umbilical arteries of the mid-gestation mouse embryo are the first and most potent generators of definitive HSCs. The anatomical link between functional HSCs and endothelial and mesenchymal cells in these arterial regions strongly supports the notion that a common progenitor for these lineages exists at this time in development. Our novel functional findings provide important cellular starting points for examining the molecular signals necessary for the generation of the first definitive HSCs.

Materials and methods

Embryo generation
To generate embryos, timed matings were set up between genetically marked male mice and wild-type (C57BL/10 × CBA)F$_1$ or C57BL/10 females. The day of vaginal plugging was counted as day 0. The genetically marked mouse lines used in this study were Ln72 [human β-globin transgenic (Strouboulis *et al.*, 1992)], BL1b [Ly-6E (Sca-1)/LacZ transgenic (Miles *et al.*, 1997)] and *Cbfa2*$^{lz/+}$ [*Cbfa2-lacZ* knock in (North *et al.*, 1999)]. For embryo generation, the transgene was always inherited through the male parent so as to avoid contribution by genetically marked maternal cells in transplantation assays. Animals were housed according to institutional guidelines, with free access to food and water. Animal procedures were carried out in compliance with the Standards for Humane Care and Use of Laboratory Animals.

Cell preparations
Pregnant mice were killed by cervical dislocation. Procedures for embryo and AGM dissection were essentially as described before (Muller *et al.*, 1994) and are given in detail by Dzierzak and de Bruijn (2000). Subdissections were performed using 27 G needles (Becton-Dickinson). Umbilical arteries and veins were severed with scissors at the points of attachment to the placenta and at the embryo proper. Vitelline vessels were also cut with scissors, along with a small external loop of gut. Circulating blood was washed from the vessels. Tissues were either incubated directly with collagenase (type I, Sigma; final concentration 0.12% v/v) for 1 h at 37°C in phosphate-buffered saline (PBS) supplemented with 10% fetal calf serum and penicillin/streptomycin, or cultured in explant organ culture for 3 days (Medvinsky and Dzierzak, 1996) prior to collagenase digestion. Cells were washed, resuspended in PBS, and transplanted intravenously into irradiated mice. To control for the contribution of circulating hematopoietic cells, body remnants or circulating embryonic blood were transplanted at the same cell doses as the test cell populations examined in these studies. No donor hematopoietic contribution could be detected in these transplanted control cell populations (our unpublished results). Cells were counted in a Bürker hemocytometer using Trypan blue to exclude dead cells. Cells were kept on ice at all times subsequent to collagenase digestion until the time of injection.

Analysis of long-term multilineage repopulating activity
Embryonic cell suspensions were assayed for the presence of definitive HSCs by intravenous transfer into irradiated adult recipients as described (Muller *et al.*, 1994; Dzierzak and de Bruijn, 2000). Briefly, (C57BL/10 × CBA)F$_1$ recipients (male or female) were exposed to a split dose of 900 rad of γ-irradiation from a ^{137}Cs source. Adult (C57BL/10 × CBA)F$_1$ spleen cells (2 × 10^5) were co-injected with the embryonic cells to promote short-term survival. Recipient mice were bled at 1 and >4 months after transfer and analyzed for percentage donor contribution by donor marker-specific PCR on DNA isolated from peripheral blood (Muller *et al.*, 1994; Miles *et al.*, 1997; Dzierzak and de Bruijn, 2000). Reconstitution was evaluated by ethidium bromide staining of agarose gels and in some cases by Southern blot hybridization as described previously (Muller *et al.*, 1994; Medvinsky and Dzierzak, 1996; Dzierzak and de Bruijn, 2000). To test for long-term multilineage hematopoietic repopulation, genomic DNA was isolated from peripheral blood, thymus, lymph node, splenic B and T cells, and bone marrow myeloid, lymphoid and erythroid cells. Percentage donor-cell contribu-tion was analyzed by PCR followed by Southern blotting. Antibodies used for sorting the different cell lineages from spleen and bone marrow were RA3-6B2 (anti-B220 for B cells), a combination of 53-6.7 and

H129.19 (anti-CD8a and anti-CD4, respectively, for T cells) and a combination of MEC13.3 and ER-MP20 (anti-CD31 and anti-Ly-6C, respectively, for bone marrow myeloid, lymphoid and erythroid cells; de Bruijn *et al.*, 1998). All antibodies were directly conjugated (PharMingen; ER-MP20–fluorescein isothiocyanate was a kind gift of Dr P.J.M.Leenen, Rotterdam, The Netherlands). Cells were sorted using a FACS Vantage SE (Becton-Dickinson). The purity of the sorted cells exceeded 95%.

Bone marrow cells of primary long-term (>4 months) reconstituted mice were transplanted into 900-rad-irradiated adult (C57BL/$10 \times CBA)F_1$ recipients (3×10^6 nucleated cells per mouse) to test for the ability of the donor HSCs to repopulate secondary recipients. Secondary recipients were analyzed at >1 month post-transplantation.

Analysis of Cbfa2 expression

$Cbfa2^{lz/+}$ mice were described previously (North *et al.*, 1999). β-galactosidase activity in freshly dissected or cultured AGM regions was monitored after staining with the X-gal substrate as described previously (North *et al.*, 1999).

Acknowledgements

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