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Definitive Hematopoiesis Is Autonomously Initiated by the AGM Region

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Summary

The adult hematopoietic system of mammals is a dynamic hierarchy of cells with the hematopoietic stem cell at its foundation. During embryonic development, the source and expansion potential of this cell remain unclear. Two sites of hematopoietic activity, the yolk sac and aorta–gonad–mesonephros (AGM) region, function in mouse ontogeny at the pre-liver stage of hematopoiesis. However, cellular interchange between these tissues obscures the embryonic site of hematopoietic stem cell generation. Here we present the results of a novel in vitro organ culture system demonstrating that, at day 10 in gestation, hematopoietic stem cells initiate autonomously and exclusively within the AGM region. Furthermore, we provide evidence for the in vitro expansion of hematopoietic stem cells within the AGM region. These results strongly suggest that the AGM region is the source of the definitive adult hematopoietic system, which subsequently colonizes the liver.

Introduction

During mouse embryogenesis, hematopoietic events beginning in the yolk sac (YS) at day 7.5 in gestation (Russell and Bernstein, 1966) shift to the fetal liver and later to the spleen and bone marrow (Moore and Metcalf, 1970; Johnson and Moore, 1975). Until recently, it had been generally accepted that this picture reflected the consecutive migration of pluripotential hematopoietic stem cells (HSC) from the YS to the definitive hematopoietic territories. Although primitive hematopoiesis and committed hematopoietic progenitors can be detected in the YS as early as 7–8.5 days postcoitum (dpc) (Moore and Metcalf, 1970; Johnson and Barker, 1985; Wong et al., 1986; Liu and Auerbach, 1991; Cumano et al., 1993; Palacios and Imhof, 1993; Huang and Auerbach, 1993), the lack of definitive colony-forming unit-spleen (CFU-S) progenitors and long-term repopulating HSCs (LTR-HSCs) in the YS until late 9 dpc (Sonoda et al., 1983; Perah and Feldman, 1977; Symann et al., 1978; Samoylina et al., 1990; Medvinsky et al., 1993) and 11 dpc (Moore and Metcalf, 1970; Müller et al., 1994), respectively, has brought this widely held dogma into controversy (reviewed by Medvinsky, 1993; Dzierzak and Medvinsky, 1995).

Recently, in the mouse embryo, a pre-liver intraembryonic site of potent definitive hematopoietic activity has been identified. This axial, mesodermally derived region of the mouse embryo containing the dorsal aorta, genital ridge/gonads, and pro/mesonephros (aorta–gonad–mesonephros [AGM]) has been shown to harbor adult-type multipotent hematopoietic progenitors (CFU-S) and pluripotential LTR-HSCs. Comparisons of activity harbored in this intraembryonic hematopoietic site with that in the YS have revealed several differences in dynamics as well as potency. While CFU-S progenitors appear simultaneously in the YS and the AGM region at late 9 dpc (Medvinsky et al., 1993), temporal quantitation studies comparing the number of CFU-S progenitors in the YS, AGM region, and fetal liver have revealed that CFU-S progenitor number and frequency in the AGM region greatly exceed that in the YS from 9 dpc to late 10 dpc and that CFU-S progenitor numbers and frequencies decrease dramatically in the AGM at 11 dpc, while there is a concomitant increase in liver CFU-S (Medvinsky et al., 1993). Furthermore, definitive LTR-HSC activity has been found in the AGM region at late 10 dpc, at a time slightly earlier than in the YS and fetal liver (Müller et al., 1994). While these results indicate that the intraembryonic AGM region is the most potent pre-liver site of definitive hematopoietic activity, the direct measurement of CFU-S and LTR-HSCs within different parts of the embryo as a means of identifying the primary source of definitive hematopoietic activity is not adequate owing to the active interchange of cells via the circulation and possible interstitial migration, as was suggested in amphibian embryos (Turpen and Knudson, 1982).

Previously, the ontogenic source of the definitive adult hematopoietic system has been determined in nonmammalian vertebrates by orthotopic embryo-grafting experiments (reviewed by Dieterlen-Lievre and Le Douarin, 1993). Two sites of hematopoiesis have been found to exist early in the embryonic development of birds and amphibians. In the avian species, it was found in chick–quail (Dieterlen-Lievre, 1975) and chick–chick embryo grafts (Lassila et al., 1978) that the intraembryonic region containing the dorsal aorta is responsible for definitive hematopoiesis, while the YS produces only transient embryonic hematopoiesis. Similarly, in amphibians in grafts between the intraembryonic dorsal lateral plate and the ventral blood islands (YS analog), it was found that the intraembryonic region containing the pronephros is the major source of definitive hematopoiesis (Turpen et al., 1981; Turpen and Knudson, 1982; Kau and Turpen, 1983; Maeno et al., 1985). While the orthotopic embryo-grafting method has yielded conclusive results in these nonmammalian vertebrates, the in utero development of the mouse embryo prohibits such experiments at the present time and requires alternative methods of investigation.

Therefore, to examine the source of the definitive adult hematopoietic system in the mouse embryo, we developed a novel in vitro organ culture approach. Here we describe the results of isolated organ cultures of 9, 10, and 11 dpc YS, AGM, and liver. We demonstrate that CFU-S progenitor activity is not only maintained but

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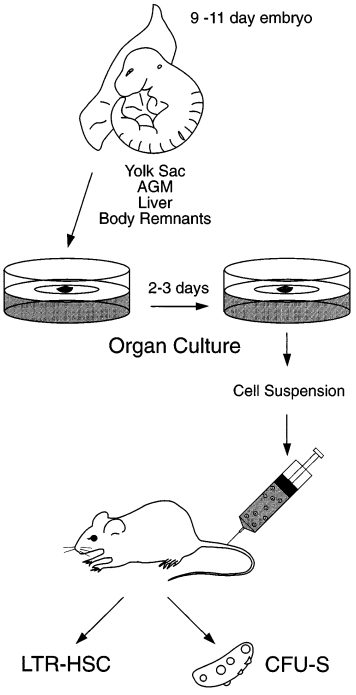

Figure 1. Experimental Design

We separately explanted 9, 10, and 11 dpc tissues (AGM, YS, liver, head/heart, and body remnants) from (CBA × C57Bl/10)F1 embryos onto filters supported by stainless steel mesh stands at the air–liquid interface. Tissues were cultured for 2–3 days and dissociated, and cells assayed for CFU-S_{11} and LTR-HSC activity as previously described (Medvinsky et al., 1993; Müller et al., 1994). For LTR-HSC activity, the sex of the embryos was detected by PCR during the culture period and only male embryonic tissues were transplanted (for PCR details see Experimental Procedures).

autonomously generated and increased in AGM cultures at 10 and 11 dpc, while YS supports only weak CFU-S activity. Moreover, we report that at 10 dpc the AGM region exclusively initiates abundant LTR-HSC activity and is totally independent of influences from the YS and the liver at this time. We demonstrate large increases in LTR-HSC activity in cultured AGM over directly assayed AGM region, which strongly suggest that the AGM region is the pre-liver ontogenic source of the definitive adult hematopoiesis in the mouse and strongly support the view that the fetal liver is seeded by HSCs generated in the AGM region.

Results

Isolated Embryonic Tissues Can Initiate and Expand CFU-S_{11} in Culture

Using an experimental approach whereby isolated embryonic tissues can be cultured separately from other tissues so that cells cannot migrate or circulate among hematopoietic sites, we examined the AGM region, YS, and liver for the generation of definitive CFU-S progenitors at day 11 posttransplantation (CFU-S_{11}). As shown in Figure 1, the organ culture system was established with whole embryonic tissues cultured on semipermeable filters at the air–medium interface for 2–3 days followed by dispersion into a single-cell suspension and injection into lethally irradiated mice.

When 9 dpc tissues from 13 embryos were individually cultured and cell suspensions were injected, no CFU-S_{11} were found in the recipient mice. However, beginning at early and mid–10 dpc, significant CFU-S_{11} activity could be found in cultured AGM and YS, but not in liver, control head/heart, or body remnants (Table 1). At the 32–33 somite pair stage, the number of CFU-S_{11} per cultured AGM was found to be 4.8 as compared with 0.5 CFU-S_{11} per cultured YS, representing a greater than 9-fold difference in progenitor number between the tissues. At the 34–35 somite pair stage, the number of CFU-S_{11} in cultured AGM regions (9.3 CFU-S_{11} per tissue) again surpassed the number in cultured YS (1.0 CFU-S_{11} per tissue) by a factor of 9. When 11 dpc embryo (42 somite pair) tissues were examined, large numbers of CFU-S_{11} were found in the AGM region as compared with the YS and liver. As shown in Figure 2, the resultant spleen colonies from one representative experiment were 3-fold more abundant per cultured AGM region (31.4 CFU-S per tissue) than cultured YS (9.2 CFU-S per tissue) and 15-fold more per cultured AGM than per cultured liver (1.8 CFU-S per tissue). The weights of the spleens of the various recipient groups differ dramatically and correspond directly with the number of CFU-S_{11} produced. Finally at 12 dpc, large numbers of CFU-S_{11} are also found in cultured liver (0.2 embryo liver equivalents produced confluent spleen colony growth; data not shown).

To determine whether the organ culture system provides a normal supportive environment for CFU-S_{11} progenitors, we compared CFU-S_{11} numbers from cultured tissues with directly transplanted tissues (Medvinsky et al., 1993). The cumulative results from numerous experiments at early– and mid–10 dpc and 11 dpc are shown in Figure 3. At 10 dpc, uncultured AGM region harbors 0.9–1.2 CFU-S_{11} progenitors (Medvinsky et al., 1993). However, cultured AGM regions from 32–33, 34–35, and 36–38 somite pair stage embryos yielded 4.8, 6.2, and 9.6 CFU-S_{11} per tissue, respectively. Thus, within the cultured AGM region, CFU-S_{11} numbers are increased 4- to 9-fold over the numbers of CFU-S_{11} per uncultured, directly transplanted AGM. While it was capable of maintaining CFU-S_{11} progenitors, no comparable increases in CFU-S_{11} numbers were found in cultured 10 dpc YS. At 11 dpc, CFU-S_{11} numbers in the AGM region were again increased (6-fold) during the culture period, while cultured YS and liver show only small increases over uncultured tissues. These data demonstrate that, beginning at 10 dpc, the CFU-S_{11} progenitor pool is initiated and expanded autonomously in cultured AGM tissue and to a greater potency than in isolated YS and liver, and they are consistent with the idea that the AGM region is a major generator of CFU-S_{11} progenitors.

Cultured AGM Tissue Can Autonomously Generate and Expand LTR-HSCs

The successful maintenance and growth of CFU-S_{11} progenitors in organ cultures led to experiments testing for definitive adult LTR-HSC activity. The in vivo long-term reconstitution of lethally irradiated mouse recipients is the only assay at present to reveal LTR-HSCs unequivocally. As shown in Figure 1, tissues from male embryos were cultured for 2–3 days and cells were injected into

Table 1. Development of CFU-S_{11} in Organ Culture of 10 dpc Tissues

Somite Pairs	Tissue	CFU-S_{11} per Embryo Equivalent	CFU-S_{11} per 10^6 cells	Total Number of Cells Injected ($\times 10^6$)
32–33	Yolk sac	0.5	7.2	0.28
	AGM	4.8	86.4	0.22
	Head/heart	0	0	1.90
	Liver	0	0	0.03
	Body Remnants	0	0	3.30
34–35	Yolk sac	1	12.5	0.24
	AGM	9.3	53.8	0.52
	Head/heart	0	0	5.10
	Liver	0	0	0.03
	Body Remnants	0.3	0.5	1.90

Embryonic tissues were cultured as described in Figure 1 and tested for CFU-S_{11} generation. The numbers of CFU-S_{11} per embryo equivalent and CFU-S_{11} per 10^6 cells are shown. In two representative experiments, the total numbers of 32–33 and 34–35 somite pair embryos used for organ cultures were four and three, respectively. Each group of tissues was pooled and transplanted into two irradiated adult recipient mice. The total number of injected live cells from pooled cultured tissue is indicated. Temporal dynamics (at 10 and 11 dpc) of the in vitro CFU-S_{11} progenitor production is shown in Figure 3 for cultured YS, AGM region, and liver. In recipient mice transplanted with cultured body remnants, we found some CFU-S_{11} production, which likely represents contamination with the AGM region (as the AGM region is juxtaposed to the somites and lateral walls of the embryo).

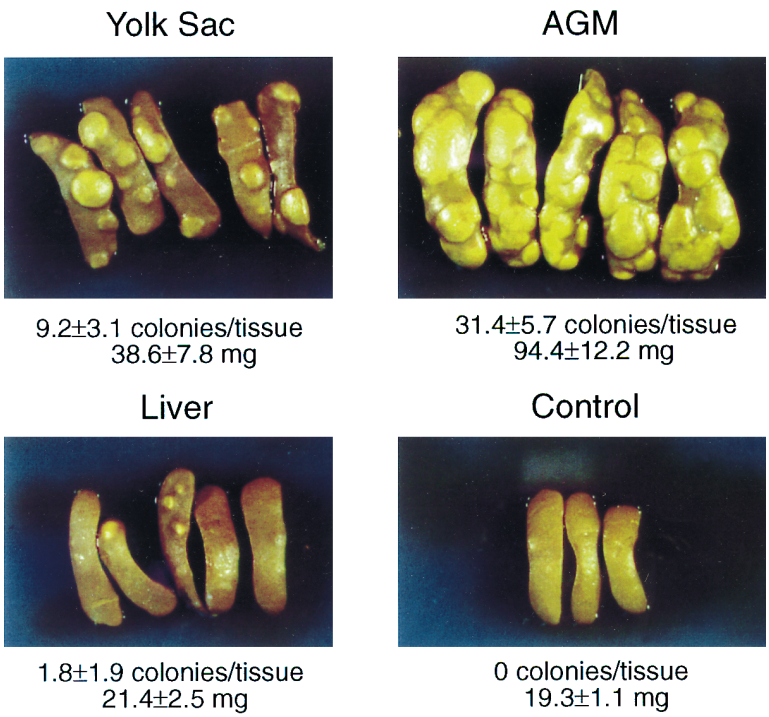

Figure 2. Analysis of CFU-S_{11} Activity in Cultured Embryonic Tissues

Spleens containing CFU-S_{11} generated by cultured 11 dpc embryonic hematopoietic tissues are shown. The AGM region, YS, and liver from five mid–11 dpc (CBA × C57Bl/10)F1 embryos were cultured separately for 3 days as described in Figure 1. Each group of tissues was pooled and transplanted into five irradiated (CBA × C57Bl/10)F1 adult recipients, with 1 embryo equivalent of tissue per recipient. Note that the spleens of the recipients that were transplanted with the cultured AGM region demonstrate 3 times as many colonies as those that received YS transplants and 15 times as many colonies as those that received liver transplants. The weights of the spleens of different groups of recipients differ dramatically in correspondence with CFU-S_{11} productivity of the cultured tissues. No colonies were observed in the control noninjected recipients.

lethally irradiated female recipients. At 2 and 8 months posttransplantation, recipient peripheral blood DNA was tested for the presence of the donor male marker. In the representative experiment shown in Figure 4A, the YMT2/B male marker was detected to high levels (greater than 10%) in 2 out of 2 recipients receiving cultured 10 dpc AGM, but not in any of the 2 and 3 recipients receiving cultured YS or cultured liver, respectively. The cumulative results of 13 experiments (Table 2) demonstrate that at day 10 in gestation only cultured AGM can repopulate lethally irradiated recipients to high levels (greater than 10%). Moreover, the efficiency of high level donor cell repopulation of recipients from cultured 35–38 somite pair AGM was greatly increased (89%; 24 positive out of 27 transplanted, with about 2 embryo equivalents) when compared with the direct transplantation (3%; 3 positive out of 96 transplanted, with 1.2 embryo equivalents) of AGM region cells of this stage (Müller et al., 1994). Thus, in correspondence with autonomous generation and increases in number of CFU-S_{11} progenitors in cultured AGM, LTR-HSCs are also autonomously generated and greatly increased (approximately 15-fold, taking into account the embryo equivalents transplanted) within the isolated 10 dpc AGM region during organ culture.

While levels of donor cell engraftment greater than 10% generally represent full multilineage repopulation, we tested whether numerous transplant recipients of cultured 10 dpc AGM contained the donor-derived marker in all hematopoietic tissues and the lymphoid

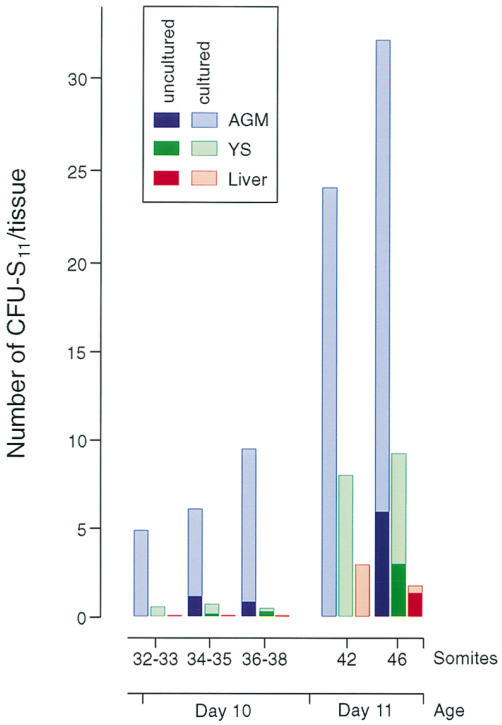

Figure 3. Histogram Analysis of CFU-S_{11} Generation Potential in Organ Cultures and Directly Transplanted Uncultured 10 and 11 dpc Embryonic Tissues

For design of the experiment see Figure 1. The cumulative CFU-S_{11} results of cultured pooled AGM regions, YS, and livers from 10 dpc (32–33, 34–35, and 36–38 somite pair) and 11 dpc (42 and 46 somite pair) embryos are shown in light blue, green, and red, respectively. Results were obtained from one to six organ culture experiments performed with four 32–33, ten 34–35, 18 36–38, two 42, and four 46 somite pair embryos. The range of values for CFU-S_{11} per tissue from embryos was as follows: 34–35 somite pair AGM (3.3–9.0); 34–35 somite pair YS (0.4–1.0); 36–38 somite pair AGM (7–14); 36–38 somite pair YS (0–1.5). The CFU-S_{11} results of directly transplanted uncultured AGM, YS, and liver from 10 dpc (34–35 and 36–38 somite pair) and 11 dpc (46 somite pair) embryos (Medvinsky et al., 1993) are shown in dark blue, green, and red, respectively. CFU-S_{11} experiments with directly transplanted uncultured 32–33 and 42 somite pair tissues were not performed. The cultured AGM region shows overproduction of CFU-S_{11} compared with numbers detected in uncultured, directly transplanted AGM region. The data are consistent with the idea that the AGM region is a major generator of CFU-S_{11} activity that accumulates under conditions preventing their emigration and subsequent colonization of liver and other tissues. The X axis indicates the age and somite number of the embryos used for explantation to organ culture and for direct analysis of uncultured tissues. The Y axis indicates the number of CFU-S_{11} per tissue.

and myeloid lineages at greater than 8 months posttransplantation. Peripheral blood, bone marrow, thymus, spleen, lymph node, splenic macrophage and B cell, peritoneal macrophage, and bone marrow mast cell DNA was examined for the presence of the Y chromosome marker by semiquantitative polymerase chain reaction (PCR) analysis. As shown in Figure 4B for four representative recipients, we found that all tissues and the T and B lymphoid and myeloid lineages were repopulated to 100% with donor-derived cells. Also, cultured bone marrow macrophages and mast cells were found to be 100% donor derived (data not shown). Thus, the organ culture conditions do not affect the high level multilineage potency of LTR-HSCs arising from cultured 10 dpc AGM and yield results identical to those previously reported with directly transplanted 10 dpc AGM region cells (Müller et al., 1994).

LTR-HSC potential at the following stage of development (11 dpc) was examined by organ culture of YS, liver, body remnants, and AGM region. A limiting dilution experiment revealed high level LTR-HSC activity in cultured YS and AGM, but not in liver or body remnants (Figure 5). While 0.25 embryo equivalents of YS and AGM resulted in high level (greater than 10%) repopulation, only the AGM region was able to give some repopulation with 3-fold fewer cells (0.08 embryo equivalents), indicating a difference in the number of LTR-HSCs, the potency of the LTR-HSCs between these tissues, or both.

Finally, to determine the earliest stage at which LTR-HSC activity can be generated, we cultured 9 dpc embryonic tissues. In two experiments with tissues from a total of 18 embryos, no repopulating activity was found in cultured AGM, liver, or YS. Taken together, these results demonstrate that HSC activity is autonomously initiated and expanded in the AGM region, but not in the YS or liver, beginning at 10 dpc. From at least 10 dpc onward, the generation and expansion of the LTR-HSC in the AGM region is not dependent upon migration of cells from the YS. In addition, this initial increase of definitive adult LTR-HSC activity in the AGM region is not dependent on the liver microenvironment.

Generation and Expansion of LTR-HSCs Occurs in the Anterior AGM Region

At 10 dpc, the mesodermal region that forms the AGM is composed of the dorsal aorta, the pronephros, mesonephros, and the genital ridge. At later stages of development, the metanephros (the primordium for the adult kidneys) and the gonads form here (Kaufman, 1992). The data obtained from amphibian and fish models suggest posterior to anterior migration of hematopoietic cells (Turpen and Knudson, 1982; Detrich et al., 1995). Therefore, it was of interest to localize along the anterior–posterior axis which region within the AGM is responsible for the LTR-HSC generation. We separated the anterior and posterior sections of the AGM region after dissection from 10 dpc male embryos (Figure 6A). The anterior AGM region contains the pronephros, part of the mesonephros, the dorsal aorta, and the genital ridge. The posterior section contains part of the mesonephros, the dorsal aorta, and the genital ridge. These sections were cultured for 2 days and then tested for LTR-HSC activity in lethally irradiated female mouse recipients. The level of donor-derived reconstitution was determined by Y chromosome–specific PCR analysis of peripheral blood DNA (Figure 6B). In two separate experiments at more than 4 months posttransplantation, the cultured anterior section repopulated all recipients (5 out of 5) to a much higher percentage (10%–100%) than the cultured posterior section. No recipients transplanted with posterior AGM region cells were found to be repopulated to greater than 5%, and, as found in previous experiments, cultured intact YS and liver

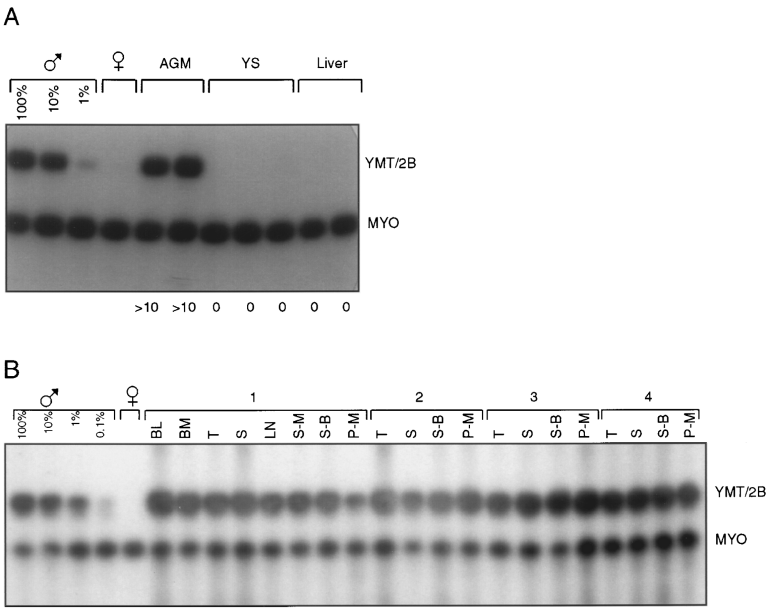

Figure 4. Peripheral Blood and Multilineage PCR Analysis of Transplant Recipients Receiving 10 dpc Cultured AGM Region

After organ culture (as described in Figure 1), AGM region, YS, and liver tissues were analyzed for LTR-HSC activity by transplantation into female irradiated recipients. Only male embryonic tissues were used for transplantation.

(A) At 6 months posttransplantation, peripheral blood DNA samples were prepared as previously described, and donor male cell contribution was assessed semiquantitatively using myogenin gene–specific oligonucleotides as the DNA normalization control (Müller and Dzierzak, 1993; Medvinsky et al., 1993). Southern blot analysis was performed for YMT2/B- and myogenin (MYO)-specific fragments. Autoradiograms are shown. Controls for donor cell contribution include dilutions of male DNA into female DNA and are as indicated: 100%, 10%, and 1%. The percentage contribution in each of the peripheral blood samples is indicated at the bottom of each lane. Note that only strong YMT2/B signal is observed in mice transplanted with cultured AGM region.

(B) Four recipient mice (numbered 1–4) were sacrificed at more than 8 months posttransplantation of day 10 cultured AGM cells. The following tissues were dissected and cell lineages isolated: peripheral blood (BL), bone marrow (BM), thymus (T), spleen (S), lymph nodes (LN), splenic macrophages (S-M), splenic B cells (S-B), and peritoneal macrophages (P-M). Quantitation of specific hybridizing YMT2/B and myogenin PCR products after Southern blot transfer reveals that all tissues and cell lineages from the four transplant recipients are 100% donor cell derived. Tissues (thymus, spleen, and bone marrow) from five negative recipients injected with 10 dpc cultured YS were also analyzed, and no contribution of donor cells was found (data not shown).

showed no repopulating activity. Additional experiments were performed to determine whether CFU-S_{11} activity in the AGM region would colocalize with the LTR-HSCs. When a mixture of 10 dpc AGM regions from 14 embryos (33–38 somite pairs) was cultured as described above, no difference in CFU-S_{11} number was found between the anterior (2.8 CFU-S_{11} per tissue) and posterior (2.4 CFU-S_{11} per tissue) regions. In a second experiment with five late 10 dpc embryos (38–40 somite pairs), we found 12.6 CFU-S_{11} per anterior AGM and 8.6 CFU-S_{11} per posterior AGM region. Thus, there is no strict correlation in the spatial development of CFU-S progenitors and LTR-HSCs. These results demonstrate that the anterior section of the 10 dpc AGM region, which consists of the pro/mesonephros and part of the dorsal aorta and genital ridge, is most important for the autonomous generation and expansion of definitive LTR-HSC activity.

Table 2. LTR-HSC Activity in Organ Cultures of 10 dpc Tissues

Tissue	Positive/Total at 2 Months[a]	Positive/Total at 8 Months
AGM	34 of 36	24 of 27
Yolk sac	0 of 16	0 of 16
Liver	0 of 10	0 of 10
Body remnants	0 of 7	0 of 4

The number of reconstituted donor-positive recipients and the total number of recipients transplanted with cultured embryonic tissues are shown. Tissues from 10 dpc embryos (35–38 somite pairs) were explanted to organ cultures as described in Figure 1. Cells obtained from organ cultures of only the male tissues were transplanted into lethally irradiated female recipients. The LD_{50} dose of irradiation was determined by animals injected with body remnant control cells. The number of reconstituted donor-positive recipients was determined by YMT2/B-specific PCR analysis of peripheral blood DNA at the posttransplantation times shown as described in Experimental Procedures. Semiquantitative PCR showed the above number of recipients were reconstituted with greater than 10% donor cells. We also observed some reconstituted animals with about 1% donor contribution as follows: 3 of 27 with cultured AGM region, 1 of 16 with cultured YS, and 1 of 4 with cultured body remnants. A small number of recipients were reconstituted with about 0.1% donor cells. The number of embryo tissue equivalents transplanted per recipient was 1–2.5 in 12 out of 13 experiments. In one experiment, 3.7 embryo tissue equivalents was transplanted.

[a] Not all transplant recipient animals were analyzed at 2 months.

Discussion

Despite a long history of studies on developmental hematopoiesis, the embryonic origin of the definitive hematopoietic system in adult mammals is unknown. For over 20 years it has been widely accepted that the YS, in which the first differentiated hematopoietic cells can be detected, is the source of the founder HSCs for the fetal liver and subsequently the adult bone marrow (Moore and Metcalf, 1970). However, studies using avian (Dieterlen-Lievre, 1975; Lassila et al., 1978) and amphibian embryos (Turpen et al., 1981; Turpen and Knudson, 1982; Kau and Turpen, 1983; Maeno et al., 1985) have demonstrated that an independent source of hematopoietic activity functions within the embryo proper. In these species, it has been clearly shown that YS-derived hematopoiesis is transitory (possibly providing for the immediate needs of the embryo) and does not supply the adult with full hematopoietic potential. Hence, the

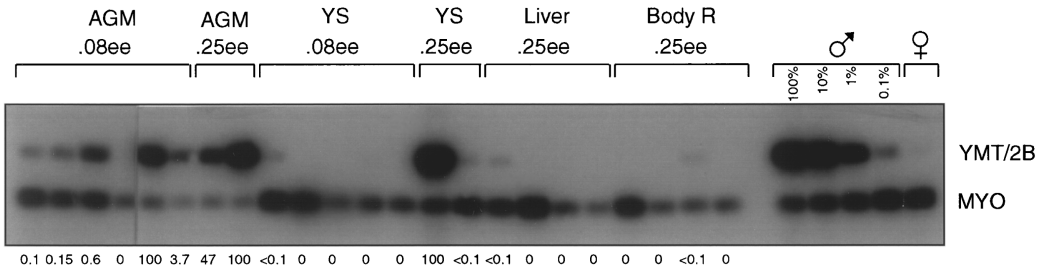

Figure 5. PCR Analysis of Recipient Peripheral Blood for Donor Cell Engraftment by Cultured 11 dpc Embryonic Tissues

At more than 6 months posttransplantation, recipients receiving 11 dpc cultured AGM region, YS, liver, and body remnant cells were analyzed for donor cell contribution by peripheral blood DNA PCR specific for YMT2/B and myogenin. Either 0.25 embryo equivalents (ee) or 0.08 ee of cultured tissues was injected into female recipients. Controls for donor cell contribution are mixes of male and female DNA and are indicated as 100%, 10%, 1%, and 0.1%. Percentage contribution of donor-derived cells was determined by phosphorimaging and is indicated for individual recipients at the bottom of each lane. High level repopulation was found after transplantation of 0.25 ee of cultured AGM or cultured YS and 0.08 ee of cultured AGM, but not when cultured liver or body remnants (Body R) were transplanted.

hematopoietic hierarchy of the YS is limited in its complexity and incomplete compared with that in the definitive hematopoietic territories. (Dzierzak and Medvinsky, 1995). In the mouse, this is consistent with the failure of numerous research groups to uncover definitive cells such as CFU-S progenitors and LTR-HSCs within the early YS (Sonoda et al., 1983; Perah and Feldman, 1977; Symann et al., 1978; Samoylina et al., 1990; Medvinsky et al., 1993; Müller et al., 1994; Harrison et al., 1979). Recently, an alternative site of potent hematopoietic activity has been identified in the mouse embryo (Medvinsky et al., 1993; Godin et al., 1993) that is the anatomical analog of the intrabody definitive hematopoietic source in avian and amphibian embryos. The AGM region, which at earlier stages of development represents intraembryonic splanchnopleura, has become a strong alternative candidate for the origin of definitive hematopoiesis in the mammalian embryo. Temporal analysis of lympho/hematopoietic progenitors at 8 dpc (Godin et al., 1995) and definitive CFU-S_{11} progenitors (Medvinsky et al., 1993) and LTR-HSCs (Müller et al., 1994) at 10 dpc strongly suggest their temporal appearance more or less in parallel in both the AGM region and the YS of the mouse embryo. Although the more potent CFU-S_{11} and LTR-HSC activity of the 10 dpc AGM region suggests that this site may be the generator of definitive HSCs, these studies neither reveal the primary embryonic source of these cells nor disclose the relationship between the embryonic hematopoietic tissues, which may actively exchange cells through the circulation or interstitial migration.

In the present study, we have exploited a whole-organ culture approach, which prevents possible cellular interchange between the individual hematopoietic tissues of the embryo and preserves the potentially important cellular microenvironment of the explant. It has been shown in other developmental systems that in vitro organ culture of embryonic rudiments can be used for ontogenic investigations (Kinoshita and Asashima, 1995; Jenkinson et al., 1982; Kratochwil, 1983; Tickle and Eichele, 1994; Serra and Moses, 1995). The in vitro organ culture described here has enabled us to discriminate reproducibly between the YS and AGM region for the initiation and expansion of definitive HSCs and progenitors.

We have demonstrated that CFU-S_{11} progenitors can be maintained during the 2–3 day culture of whole, intact AGM, YS, and liver from 10, 11, or 12 dpc embryos. However, only the cultured AGM region can significantly increase the number of CFU-S_{11} progenitors within it during the organ culture period. In contrast with the normal embryo, it appears that CFU-S generated within the isolated cultured AGM region are unable to emigrate and disseminate throughout the embryo (Medvinsky et al., 1996). Thus, they accumulate in situ and surpass the numbers that can be observed in uncultured AGM region (Medvinsky et al., 1993). Also, the significant delay of onset and the low numbers of CFU-S_{11} detected in the isolated cultured liver and YS strongly suggest colonization of these tissues by AGM-generated CFU-S_{11} progenitors.

Of greater significance in the establishment of the definitive hematopoietic system, and in strong support of the CFU-S findings, are the results of the long-term in vivo transplantations of cells from organ culture. We have shown that at 10 dpc only the AGM region is able to initiate and expand definitive adult LTR-HSC activity. In general, uncultured 10 dpc AGM cells can not reliably repopulate adult recipients; we previously found only 3 out of 96 recipients donor-cell positive with directly transplanted AGM region cells (Müller et al., 1994). Here we have shown that 10 dpc AGM region in organ culture can expand LTR-HSCs to numbers that can consistently provide complete hematopoiesis to the adult (in 24 out of 27 recipients), while cultured YS failed to yield LTR-HSC activity until 11 dpc and did so at a lower frequency than cultured AGM. Taken together, these data provide strong evidence that the AGM region is the first (and possibly the only) tissue initiating development of LTR-HSCs at the pre-liver stage of hematopoiesis. It seems likely that LTR-HSCs appearing in the YS and in other tissues of the embryo at 11 dpc are the result of dissemination of LTR-HSCs from the AGM region. This would be much like the dissemination observed in avian embryos, in which heterospecific chimeras between quail embryo and chick blastoderm revealed quail cells in the YS blood islands shortly after grafting (Martin et al., 1978).

Orthotopic embryo-grafting experiments in avian and amphibian species clearly demonstrate two separate

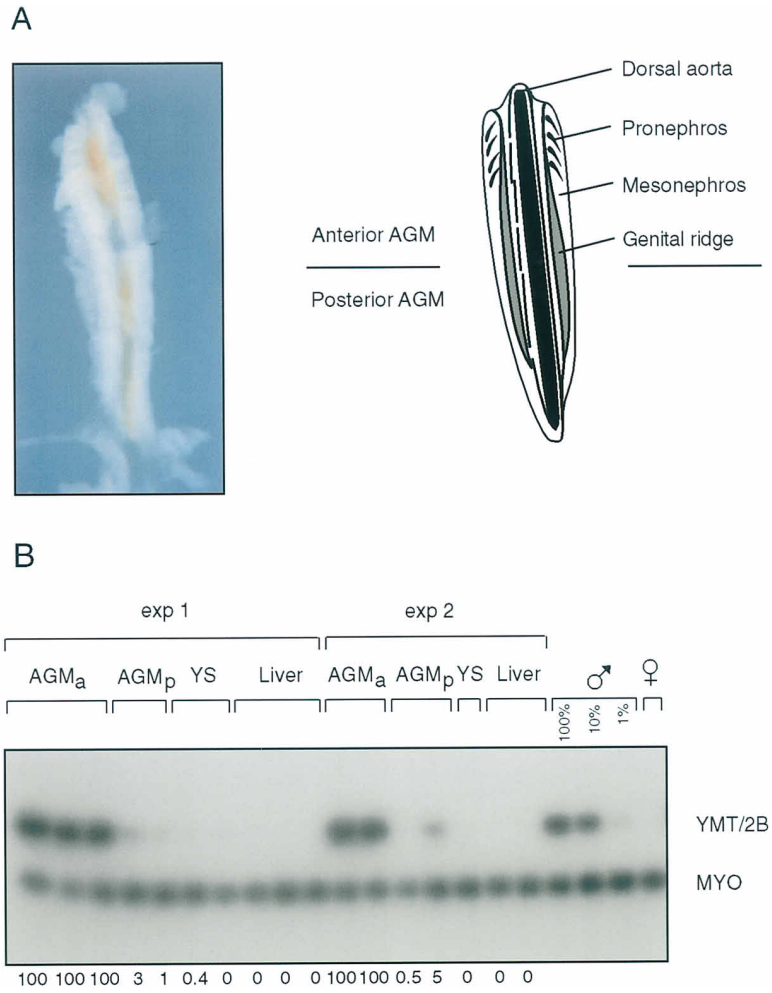

Figure 6. PCR Analysis of Peripheral Blood DNA from Transplant Recipients Receiving Cultured 10 dpc Anterior or Posterior AGM Region

We cultured 10 dpc embryonic tissues as decribed above (Figure 1) with the exception of the AGM region.

(A) The whole AGM region was dissected from the embryo; the schematic drawing indicates the tissue constituting this region: pronephros, mesonephros, genital ridge, and dorsal aorta. The AGM region was further dissected across the midline into anterior and posterior portions, cultured for 2–3 days, and transplanted into irradiated recipients.

(B) Southern blot analysis of PCR-amplified peripheral blood DNA from recipients receiving cultured AGM_a (anterior), AGM_p (posterior), YS, or liver at more than 4 months posttransplantation. Semiquantitative analysis was performed on samples from two separate experiments. Controls include mixes of male and female DNA as indicated (100%, 10%, and 1%), and percentage contribution of male donor cells in individual female recipients are shown for each lane. This analysis revealed that 5 out of 5 recipients receiving cultured AGM_a cells were 100% donor cell engrafted, while 4 recipients receiving cultured AGM_p cells were only 0.5%–5% donor cell engrafted. Cultured YS and liver cells were unable to engraft even to low levels.

mesodermal sites of hematopoietic development in the embryo, and thus evolutionary conservation among species would favor the birth of pre-stem cells in situ in the mouse AGM region. At the moment we cannot exclude the possibility that the organ culture conditions we used are incompatible with HSC initiation in the YS. However, this seems unlikely since it would imply that the early mouse embryo simultaneously employs two very distinct mechanisms for the generation of definitive hematopoiesis in the two different sites. Another more realistic possibility is that there may exist within the conceptus, at an undetermined site, a pre-LTR-HSC with multilineage potential that for some reason (lack of homing receptors, low proliferative potential in an adult microenvironment, etc.) cannot repopulate adult recipients to high levels. Numerous events, such as a progressive increase in expansion potential, maturation to an adult phenotype, or both, may occur in the AGM region to generate high level LTR-HSC activity. This would be consistent with previous studies demonstrating limited and/or low level repopulation with YS cells (Sonoda et al., 1983; Harrison et al., 1979; Weissman et al., 1978; Toles et al., 1989) and differentiated embryonic stem cells (Müller et al., 1994) when transplanted into an embryonic and neonatal environment, respectively, and the presence of multilineage progenitors in the para-aortic splanchnopleura (Godin et al., 1993). Recent in vitro work on a multipotential AA4.1-positive cell from the mouse splanchnopleura suggests that this may be a candidate pre–stem cell (Godin et al., 1995). It is also a formal possibility that primordial germ cells (PGCs) may play a role in the generation of LTR-HSCs. The colocalization of PGCs with CFU-S in the embryo body supports this notion (Medvinsky et al., 1993, 1996), as does the finding that PGCs retain plasticity to form embryonic stem cells (Matsui et al., 1992) and possibly hematopoietic progenitors in vitro (Rich, 1995). It is interesting that the most potent initiation of LTR-HSCs in the organ culture system is localized to the anterior AGM region. This may suggest a posterior to anterior gradient of activity and/or a migration of cells in the mouse, possibly to the pronephric region (the most anterior structure of the embryonic kidney), which has been shown to play an important role in the development of the definitive hematopoietic system in amphibians (Turpen et al., 1981; Turpen and Knudson, 1982; Kau and Turpen, 1983; Maeno et al., 1985).

It has been generally thought that the maturation of LTR-HSC activity in the embryo occurs within the fetal liver, since increasing stem cell activity has been found there from 11 dpc onward (Müller et al., 1994; Fleischman et al., 1982; Morrison et al., 1995; Jordan et al.,

1990). However, the high level of LTR-HSC activity that develops in cultured 10 dpc AGM suggests that each individual AGM region is able autonomously to produce a pool of HSCs sufficient to provide the adult organism with hematopoietic cells throughout life in the absence of the fetal liver microenvironment. Subsequent to this burst of activity in the AGM region, LTR-HSC expansion in the liver (1–2 days later) overtakes the AGM region, thus suggesting that colonization of the fetal liver by AGM LTR-HSCs is followed by further expansion and maturational steps. The significance of such excessive secondary expansion of LTR-HSCs in the fetal liver is not clear, taking into account the oligoclonality of adult hematopoiesis (Jordan and Lemischka, 1990; Jordan et al., 1990). The mechanisms of primary (AGM) and secondary (fetal liver) expansion of HSCs, as well as the comparative properties of HSCs from these two embryonic sources, need to be elucidated further.

Since we have observed at least a 15-fold enhancement in LTR-HSC activity from cultured 10 dpc AGM region when compared with uncultured AGM, this work provides a demonstration of expansion of LTR-HSC activity in vitro. At this time we cannot distinguish between two possible means by which LTR-HSC numbers increase in the AGM region during the culture period: by maturation of a pool of pre–stem cells to definitive LTR-HSCs or by clonal expansion of a single or a few starting HSCs. Whatever the mechanism, in contrast with Dexter-type long-term bone marrow cultures, in which LTR-HSCs are maintained but not expanded (Harrison et al., 1987), the unique temporal and spatial properties of the embryonic AGM region should lead to a better understanding of the induction or expansion factors (or both) necessary for the initiation of the definitive adult hematopoietic system in mammals. The molecular mechanisms by which the embryo attains HSC expansion may provide potentially useful applications for gene therapy.

Finally, the in vitro organ cultures offer an advantageous test system for identifying specific developmental hematopoietic defects in numerous recently reported homologous recombination knockout mice (Pandolfi et al., 1995; Tsai et al., 1994; Okuda et al., 1996; Scott et al., 1994). Such homozygous mutant mice die between 11 and 16 dpc and show a lack of definitive hematopoiesis. Analysis of YS, AGM, and liver from mutant 10–11 dpc embryos in the organ culture system described here should indicate whether deficiencies occur in the pre–stem cell, stem cell, progenitor cell, or microenvironmental compartments. We will continue to use the in vitro organ culture system for pre-LTR-HSC identification within chimeric organs, for tracing the migration and differentiation of LTR-HSCs, and for the study of the molecules involved in LTR-HSC initiation, expansion, and maturation.

Experimental Procedures

Animals and Tissues

Early hematopoietic and nonhematopoietic tissues from (CBA × C57BL/10)F1 embryos were used for organ cultures (see below and Figure 1). The AGM region, YS, liver, and head/heart, as well as other pooled tissues (body remnants), were dissected from 9, 10, and 11 dpc embryos in L-15 media supplemented with 5% fetal calf serum. Embryo age was determined starting with 0 dpc on the morning of vaginal plug discovery. In various experiments, somite pairs were counted to determine embryo age more accurately. Following organ culture, the embryonic tissues were treated with 0.04% collagenase, pipetted into a single-cell suspension, counted, and transplanted intravenously into irradiated (1000 rads of a ^{60}Co source) adult female (CBA × C57BL/10)F1 mice. This procedure was performed as described earlier (Müller et al., 1994) except that no supporting cells were injected. Mice were housed in positive pressure cabinets and received neomycin (0.16 g per 100 ml) with drinking water for the first month following irradiation and transplantation. Animals were obtained from the specific pathogen–free breeding facilities at the National Institute for Medical Research, London. All animal procedures were in accordance with the Animal Scientific Procedures Act, 1986 (United Kingdom).

Organ Cultures of Embryonic Tissues

We explanted 9, 10, and 11 dpc tissues (AGM, YS, liver, head/heart, and body remnants) from (CBA × C57BL/10)F1 embryos separately onto Durapore 0.65 μm filters (Millipore) supported by stainless steel mesh stands at the air–liquid interface (see Figure 1). The filters were washed and sterilized in several changes of boiling water before use in culture experiments. Tissues were cultured at 37°C in 5% CO_2 in myeloid long-term culture media (Stem Cell Technologies, Inc.) supplemented with hydrocortisone succinate (Sigma) to yield a final concentration of 10^{-6} M. After 2–3 days in culture, the tissues were dissociated with 0.04% collagenase, and cells were counted and transplanted intravenously into irradiated adult recipients as previously described (Medvinsky et al., 1993; Müller et al., 1994) for CFU-S_{11} and LTR-HSC assays. During the organ culture period, the sex of the embryos was detected by PCR analysis using oligonucleotides designed by A. Rattigan and P. Burgoyne (personal communication) that amplify a 342 bp fragment of the YMT2/B gene on the Y chromosome.

Analysis of the Mice Transplanted with Embryonic Tissues

For CFU-S_{11} assays, the recipient mice were sacrificed on day 11 posttransplantation, and their spleens were removed and fixed in the Bouen solution. Spleen colonies were counted under the dissecting microscope. For the LTR-HSC assay, the peripheral blood of recipient mice was analyzed two times, once at 1.5–2.5 months and then at 5.5–8 months posttransplantation. Blood (100–200 μl) was collected from the tail vein of the mice and proteinase K digested, followed by phenol–chloroform extraction and isopropanol precipitation as previously described (Müller et al., 1994). Donor male cell contribution was assessed semiquantitatively by PCR using YMT2/B primers and myogenin gene–specific oligonucleotides as the DNA normalization control (Müller and Dzierzak, 1993). The reactions for the combination of YMT2/B and myogenin oligonucleotides were initial heating at 95°C for 5 min, followed by 30 cycles at 94°C for 10 s, 60°C for 30 s, and 72°C for 35 s and a final single cycle at 37°C for 10 min. The cycles were performed in a Techne PHC-2 thermocycler. The sizes of the amplified products were 342 bp (YMT2/B) and 245 bp (myogenin). The products were separated on 2.0% agarose gels and transferred to nylon membranes followed by hybridization with ^{32}P-labeled YMT2/B- and myogenin-specific probes. The percentage of engraftment was determined by quantitation of radioactive fragments on a phosphorimager (Molecular Dynamics) and plotting against a graph derived from control samples of serial dilutions of male DNA as described previously (Müller et al., 1994).

Multilineage Analysis of Donor Cell Contribution

To test the contribution of donor HSCs from 10 dpc cultured AGM into the different lympho/hematopoietic lineages in long-term transplanted mice, we sacrificed and analyzed four positive animals. DNAs were made from the following tissues and cells and were analyzed by PCR as described previously (Müller et al., 1994): peripheral blood, bone marrow, thymus, spleen, lymph nodes, B cells, macrophages, and mast cells. In brief, B cells from spleen cell suspensions (nonadherent fraction) were enriched in culture by stimulation with 10 μg/ml lipopolysaccharide (Sigma) for 3 days; macrophages were initially enriched as an adherent cell fraction from the peritoneum and spleen, followed by expansion in culture with 10%

L929–conditioned medium (source of macrophage colony-stimulating factor) for 4–10 days; mast cells were cultured for 5 weeks in 10% WEHI-3–conditioned medium. B cells were determined to be 97.5% enriched as determined by antibody staining specific for B220 and FACS analysis. Macrophages were stained directly in culture with Mac-1 antibody, and 100% of cells were found to be positive for this marker. Mast cells were stained with IgE antibody (Clone SPE-7; Sigma), followed by a secondary PE-conjugated goat anti-mouse antibody (Southern Biotechnology Associates, Inc.), and 53%–81% of cells were found to be positive by FACS analysis. Ficoll gradient fractionation was performed to remove dead cells from the B cell cultures.

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References

Cumano, A., Furlonger, C., and Paige, C.J. (1993). Differentiation and characterization of B-cell precursors detected in the yolk sac and embryo body of embryos beginning at the 10- to 12-somite stage. Proc. Natl. Acad. Sci. USA *90*, 6429–6433.

Detrich, H.W., Kiernan, M.W., Chan, F.Y., Barone, L.M., Yee, R., Rundstadler, J.A., Pratt, S., Ransom, D., and Zon, L.I. (1995). Intraembryonic hematopoietic cell migration during vertebrate development. Proc. Natl. Acad. Sci. USA *92*, 10713–10717.

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

Dieterlen-Lievre, F., and Le Douarin, N.M. (1993). Developmental rules in the hematopoietic and immune systems of birds: how general are they? Semin Dev. Biol. *4*, 325–332.

Dzierzak, E., and Medvinsky, A. (1995). Mouse embryonic hematopoiesis. Trends Genet. *11*, 359–365.

Fleischman, R.A., Custer, R.P., and Mintz, B. (1982). Totipotent hematopoietic stem cells: normal self-renewal and differentiation after transplantation between mouse fetuses. Cell *30*, 351–359.

Godin, I.E., Garcia-Porrero, J.A., Coutinho, A., Dieterlen-Lievre, F., and Marcos, M.A.R. (1993). Para-aortic splanchnopleura from early mouse embryos contains B1a progenitors. Nature *364*, 67–70.

Godin, I., Dieterlen-Lievre, F., and Cumano, A. (1995). Emergence of multipotent hematopoietic cells in the yolk sac and para-aortic splanchnopleura in mouse embryos, beginning at 8.5 days postcoitus. Proc. Natl. Acad. Sci. USA *92*, 773–777.

Harrison, D.E., Astle, C.M., and DeLaitre, J.A. (1979). Processing by the thymus is not required for cells that cure and repopulate W/W^v recipients. Blood *54*, 1152–1157.

Harrison, D.E., Lerner, C.P., and Spooncer, E. (1987). Erythropoietic repopulating ability of stem cells from long-term marrow cultures. Blood *69*, 1021–1025.

Huang, H., and Auerbach, R. (1993). Identification and characterization of hematopoietic stem cells from the yolk sac of the early mouse embryo. Proc. Natl. Acad. Sci. USA *90*, 10110–10114.

Jenkinson, E.J., Franchi, L.L., Kingston, R., and Owen, J.J.T. (1982). Effect of deoxyguanosine on lymphopoiesis in the developing thymic rudiment *in vitro*: application in the production of chimeric thymus rudiments. Eur. J. Immunol. *12*, 583–587.

Johnson, G.R., and Barker, D.C. (1985). Erythroid progenitor cells and stimulating factors during murine embryonic and fetal development. Exp. Hematol. *13*, 200–208.

Johnson, G.R., and Moore, M.A.S. (1975). Role of stem cell migration in initiation of mouse foetal liver haematopoiesis. Nature *258*, 726–728.

Jordan, C.T., and Lemischka, I.R. (1990). Clonal and systemic analysis of long-term hematopoiesis in the mouse. Genes Dev. *4*, 220–232.

Jordan, C.T., McKearn, J.P., and Lemischka, I.R. (1990). Cellular and developmental properties of fetal hematopoietic stem cells. Cell *61*, 953–963.

Kau, C.L., and Turpen, J.B. (1983). Dual contribution of embryonic ventral blood island and dorsal lateral plate mesoderm during ontogeny of hematopoietic cells in *Xenopus laevis*. J. Immunol. *131*, 2262–2266.

Kaufman, M.H. (1992). The Atlas of Mouse Development (London: Academic Press), pp. 465–468.

Kinoshita, K., and Asashima, M. (1995). Effect of activin and lithium on isolated *Xenopus* animal blastomeres and response alteration at the midblastula transition. Development *121*, 1581–1589.

Kratochwil, K. (1983). Embryonic induction. In Cell Interactions and Development: Molecular Mechanisms, K.M. Yamada, ed. (New York: John Wiley & Sons), pp. 99–122.

Lassila, O., Eskola, J., Toivanen, P., Martin, C., and Dieterlen-Lievre, F. (1978). The origin of lymphoid stem cells studied in chick yolk-sac embryo chimeras. Nature *272*, 353–354.

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.

Maeno, M., Tochinai, S., and Katagiri, C. (1985). Differential participation of ventral and dorsolateral mesoderms in the hematopoiesis of *Xenopus*, as revealed in diploid-triploid or interspecific chimeras. Dev. Biol. *110*, 503–508.

Martin, C., Beaupain, D., and Dieterlen-Lievre, F. (1978). Developmental relationships between vitelline and intra-embryonic hematopoiesis studied in avian 'yolk sac chimeras'. Cell Differ. *7*, 115–130.

Matsui, Y., Zsebo, K., and Hogan, B.L.M. (1992). Derivation of pluripotential embryonic stem cells from murine primordial germ cells in culture. Cell *70*, 841–847.

Medvinsky, A.L. (1993). Ontogeny of the mouse hematopoietic system. Semin. Dev. Biol. *4*, 333–340.

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

Medvinsky, A.L., Gan, O.I., Semenova, M.L., and Samoylina, N.L. (1996). Development of day-8 colony-forming units-spleen hematopoietic progenitors during early murine embryogenesis: spatial and temporal mapping. Blood *87*, 557–566.

Moore, M.A.S., and Metcalf, D. (1970). Ontogeny of the haematopoietic 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., Hemmati, H.D., Wandycz, A.M., and Weissman, I.L. (1995). The purification and characterization of fetal liver hematopoietic stem cells. Proc. Natl. Acad. Sci. USA *92*, 10302–10306.

Müller, A.M., and Dzierzak, E.A. (1993). ES cells have only a limited lymphopoietic potential after adoptive transfer into mouse recipients. Development *118*, 1343–1351.

Müller, A.M., Medvinsky, A.L., Strouboulis, J., Grosveld, F., and Dzierzak, E. (1994). Development of hematopoietic stem cell activity in the mouse embryo. Immunity *1*, 291–301.

Okuda, T., van Deursen, J., Hiebert, S.W., Grosveld, G., and Downing, J.R. (1996). AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal live hematopoiesis. Cell *84*, 321–330.

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.

Pandolfi, P.-P., Roth, M.E., Karis, A., Leonard, M.W., Dzierzak, E., Grosveld, F.G., Engel, J.D., and Lindenbaum, M.H. (1995). Targeted disruption of the GATA-3 gene causes severe abnormalities in the nervous system and in foetal liver hematopoiesis. Nature Genet. *11*, 40–44.

Perah, G., and Feldman, M. (1977). *In vitro* activation of the *in vivo* colony-forming units of the mouse yolk sac. J. Cell Physiol. *91*, 193–199.

Rich, I.E. (1995). Primordial germ cells are capable of producing cells of the hemopoietic system *in vitro*. Blood *86*, 463–472.

Russell, E.S., and Bernstein, S.E. (1966). Blood and blood formation. In Biology of the Laboratory Mouse, Second Edition, E.L. Green, ed. (New York: McGraw–Hill), pp. 351–372.

Samoylina, N.L., Gan, O.I., and Medvinsky, A.L. (1990). Development of the hematopoietic system: splenic colony forming units in mouse embryogenesis. Sov. J. Dev. Biol. *21*, 127–133.

Scott, E., Simon, M., Anastasi, J., and Singh, H. (1994). Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. Science *265*, 1573–1577.

Serra, R., and Moses, H.L. (1995). pRb is necessary for inhibition of N-myc expresion by TGF-β1 in embryonic lung cultures. Development *121*, 3057–3066.

Sonoda, T., Hyashi, C., and Kitamura, Y. (1983). Presence of mast cell precursors in the yolk sac of mice. Dev. Biol. *97*, 89–94.

Symann, M., Anckaert, M.A., Cordier, A., Rodhain, J., and Sokal, G. (1978). Murine yolk sac hematopoiesis studied with the diffusion chamber technique. Exp. Hematol. *6*, 749–759.

Tickle, C., and Eichele, G. (1994). Vertebrate limb development. Ann. Rev. Cell Biol. *10*, 121–152.

Toles, J.F., Chui, D.H.K., Belbeck, L.W., Starr, E., and Barker, J.E. (1989). Hemopoietic stem cells in murine embryonic yolk sac and peripheral blood. Proc. Natl. Acad. Sci. USA *86*, 7456–7459.

Tsai, F.-Y., Keller, G., Kuo, F.C., Weiss, M., Chen, J., Rosenblatt, M., Alt, F.W., and Orkin, S.H. (1994). An early haematopoietic defect in mice lacking the transcription factor GATA-2. Nature *371*, 221–226.

Turpen, J.B., and Knudson, C.M. (1982). Ontogeny of hematopoietic cells in *Rana pipiens*: precursor cell migration during embryogenesis. Dev. Biol. *89*, 138–151.

Turpen, J.B., Knudson, C.M., and Hoefen, P.B. (1981). The early ontogeny of hematopoietic cells studied by grafting cytogenetically labeled tissue anlagen: localization of a prospective stem cell compartment. Dev. Biol. *85*, 99–112.

Weissman, I.L., Papaioannou, V., and Gardner, R. (1978). Fetal hematopoietic origins of the adult hematolymphoid system. In Differentiation of Normal and Neoplastic Hematopoietic Cells (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press), pp. 33–47.

Wong, P.M.C., Chung, S.W., Chui, D.H.K., and Eaves, C.J. (1986). Properties of the earliest clonogenic hematopoietic precursors to appear in the developing murine yolk sac. Proc. Natl. Acad. Sci. USA *83*, 3851–3854.