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Development of Hematopoietic Stem Cell Activity in the Mouse Embryo

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Summary

The precise time of appearance of the first hematopoietic stem cell activity in the developing mouse embryo is unknown. Recently the aorta–gonad–mesonephros region of the developing mouse embryo has been shown to possess hematopoietic colony-forming activity (CFU-S) in irradiated recipient mice. To determine whether the mouse embryo possesses definitive hematopoietic stem cell activity in the analogous AGM region and to determine the order of appearance of stem cells in the yolk sac, AGM region, and liver, we transferred these embryonic tissues into adult irradiated recipients. We report here the long-term, complete, and functional hematopoietic repopulation of primary and serial recipients with AGM-derived cells. We observe potent hematopoietic stem cell activity in the AGM region before the appearance of yolk sac and liver stem cell activity and discuss a model for the maturation of stem cell activity in mouse embryogenesis.

Introduction

Hematopoietic cell development begins in the mouse embryo at approximately day 7 of gestation and is thought to originate in the yolk sac and proceed to the fetal liver before becoming resident in the adult bone marrow. The first visible differentiated hematopoietic cells in the conceptus are of the erythroid lineage. They appear in the 7.5 day post coitum (dpc) yolk sac (Russell and Bernstein, 1966; Russell, 1979) and later at 9 dpc, in the fetal liver (Johnson and Jones, 1973; Houssaint, 1981). Committed multilineage progenitors, colony-forming unit-culture (CFU-C), for erythroid and myeloid lineages have been reported in in vitro cultures of yolk sac as early as 7–8 dpc (Moore and Metcalf, 1970; Johnson and Barker, 1985; Wong et al. 1986). Fetal liver was also found to produce committed myeloid precursors at 9 dpc and onwards (Moore and Metcalf, 1970; Samoylina et al., 1992). In multilineage (erythroid–myeloid) in vivo progenitor assays, colony-forming unit-spleen (CFU-S) activity has been reported in 8 dpc yolk sac (Moore and Metcalf, 1970), although many investigators have been unable to detect such activity until 9 dpc (Perah and Feldman, 1977; Symann et al., 1978; Samoylina et al., 1990; Medvinsky, 1993). Recently, the aorta–gonad–mesonephros (AGM) region of the embryo has been demonstrated to be a novel site of hematopoiesis in the mouse embryo and to contain CFU-S (erythroid–myeloid) progenitors beginning at 9 dpc (Medvinsky et al., 1993). Progenitor activities in the AGM, yolk sac, and fetal liver were compared for the production of in vivo macroscopic colonies on the spleen at 8 days ($CFU\text{-}S_8$) and 11 days ($CFU\text{-}S_{11}$) posttransplantation. The first statistically significant $CFU\text{-}S_8$ progenitors appear at the 24 somite pair stage concurrently in the embryo body and the yolk sac. Both the number and frequency of CFU-S progenitors in the AGM region surpasses that of yolk sac and peaks in the 38–40 somite pair stage of the 10 dpc embryo. This activity reaches a maximum in the AGM region just before the activity can be found in the fetal liver, suggesting that the AGM region may be the source of liver multilineage progenitors. However, these assays are specific only for the myeloid and erythroid lineages, not the lymphoid lineages. Progenitors for T lymphoid lineage cells have been found in the embryonic yolk sac beginning at 8–9 dpc using in vitro thymic organ cultures (Liu and Auerbach, 1991) or stromal cell cocultures (Palacios and Imhof, 1993). In vitro cocultures of yolk sac (Cumano et al., 1993; Palacios and Imhof, 1993; Huang et al., 1994), embryo body (Ogawa et al., 1988; Huang and Auerbach, 1993), and the splanchnopleuric mesoderm (Godin et al., 1993) with stromal cell lines that support the differentiation of B lymphoid cells have demonstrated B cell activity beginning as early as 8–9 dpc in these tissues. Thus, all hematopoietic lineages can be produced by early embryonic tissues. However, a common embryonic progenitor–stem cell for all these lineages cannot be examined using these assays.

The most stringent and reliable measure of pluripotential hematopoietic stem cell (HSC) activity has been through the generation of adult radiation chimeras. Complete and long-term hematopoietic repopulation of adult mice has previously been demonstrated for whole (Micklem et al., 1966) and stem cell–enriched (Spangrude et al., 1988) adult bone marrow and fetal liver (Jordan et al., 1990). In addition, pluripotential and long-term HSC activity has been observed from late 11 dpc yolk sac, long term after transplantation into lethally irradiated mouse recipients (Moore and Metcalf, 1970; Huang and Auerbach, 1993). The reports of HSC activity in yolk sac at earlier times in mouse embryonic development are sparse either because the procedures used for the identification of such cells in vivo are technically difficult or because such activity is limited. While two groups failed to find HSC activity in 9 dpc yolk sac (Sonoda et al, 1983; Harrison et al., 1979), two other groups have shown that such yolk sac activity prior to 11 dpc can lead to the long-term high level repopulation of only the erythroid lineage (Toles et al., 1989) or low level lymphoid-restricted repopulation (Weissman et al.,

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1977, 1978). These cells were delivered transplacentally or to the yolk sac of the embryo. Thus, the onset and temporal expansion of pluripotential HSC activity (equivalent to the stem cell activity found in the adult bone marrow) in the early developing mouse embryo, particularly in the AGM region, is unknown and in the yolk sac is uncertain.

In birds, adult hematopoietic cells are derived from intrabody stem cells (Dieterlen-Lievre, 1975; Dieterlen-Lievre and Martin, 1981), which are thought to reside in the mesodermal region containing the dorsal aorta (Cormier and Dieterlen-Lievre, 1988). As shown in elegant grafting experiments between quail embryos and chick yolk sacs, intraembryonic cells but not those of the yolk sac contribute to the complete adult avian blood system. The amphibian system has an analogous intraembryonic site, which contributes adult hematopoietic stem cell activity (Turpen et al., 1981; Maeno et al., 1985). Since the analogous site in the developing mouse embryo has not been examined for adult repopulating HSC activity, we performed direct transplantation of mouse AGM cells into lethally irradiated mouse recipients to test for complete long-term repopulating potential. We report here the results of radiation chimeras produced with embryonic tissues of 8–11 dpc mouse embryos. We demonstrate the complete, multilineage, long-term repopulation of such mice with cells of the AGM region and observe potent HSC activity in this intraembryonic region prior to the detection of stem cell activity in the yolk sac and the fetal liver. These in vivo results, in combination with previous in vitro progenitor and CFU-S data, suggest a novel model for mammalian HSC maturation.

Results

The AGM Region of Developing Mouse Embryo Contains Long-Term Repopulating Hematopoietic Cell Activity

One criterion for the assessment of hematopoietic stem cell activity is the long-term engraftment of the blood system of recipient mice. It has been shown that clonal stabilization of stem cell–derived hematopoietic repopulation occurs only 4–6 months posttransplantation (Jordan and Lemischka, 1990). The ability of embryonic tissues, particularly AGM region cells, to contribute to the prolonged hematopoietic repopulation of lethally irradiated adults was tested using cells from genetically marked embryos. A β-globin transgene and the Y chromosome sequences *Y-2* and *Sry* were used in these studies as markers for donor embryonic cells. As shown in Figure 1, embryos could be generated such that the donor genetic markers would be present only in contributing embryonic cells and not in maternal blood cells (a potential source of contaminating engrafting cells). Heterozygous or homozygous males transgenic for the human β-globin locus were mated with nontransgenic females. Embryo body or AGM region cells, as well as yolk sac, liver, circulating, and body remnant cells were prepared from a pooled mixture of male/female, transgenic/nontransgenic embryos at 8, 9, 10, or 11 dpc and intravenously injected as a single cell suspension into lethally irradiated female recipients. Tissues from

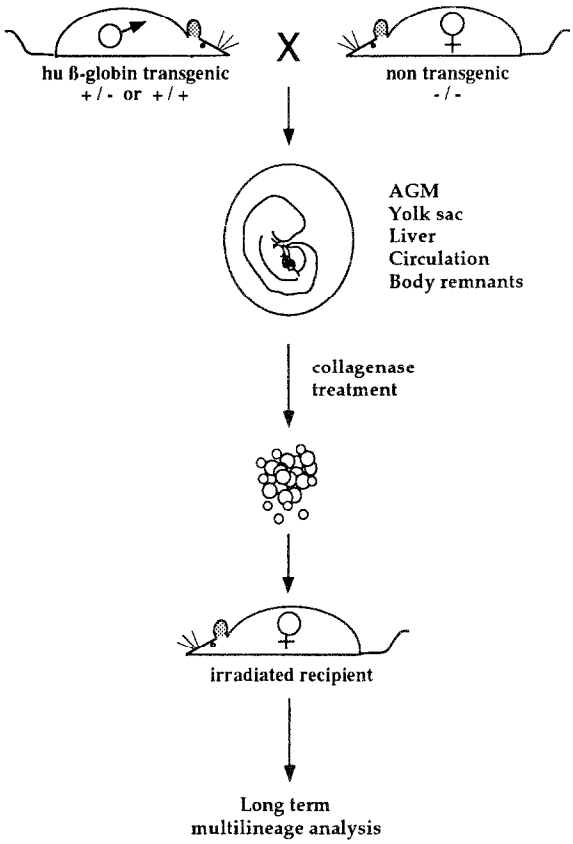

Figure 1. Strategy for the Generation of Genetically Marked Embryos and Injection of Embryonic Tissues into Recipients

To avoid contamination of embryonic tissues by maternal blood-derived hematopoietic progenitor cells, males heterozygous or homozygous for the human β-globin locus (Strouboulis et al., 1992) were used to produce genetically marked embryos. The embryonic tissues were dissected as described by Medvinsky et al. (1993), pooled, and collagenase-treated, and intravenously injected into lethally irradiated female recipient mice.

the individual embryos included in each experimental pool were typed (by polymerase chain reaction [PCR] or Southern blot) for the presence of the transgene or Y chromosome marker so as to determine the percentage of marked cells injected. A limiting dose of unmarked syngeneic female bone marrow cells (Harrison, 1980) was also injected to aid the recipients in short-term survival following irradiation. Short-term and long-term repopulation analyses were performed on recipient peripheral blood DNA by PCR for the presence of the *Sry* male-specific gene.

As shown in Figure 2A, blood DNA samples from animals receiving the various embryonic tissues were negative (<0.1%) for donor *Sry* signal when derived from 8 or 9 dpc embryos. Only when 10 dpc tissues (Figure 2B) or 11 dpc tissues (Figure 2C) were transplanted did recipients yield *Sry*-positive signal. Three animals receiving 10 dpc AGM cells were *Sry* positive, while those receiving body remnant, circulating, or liver cells were negative. The 10 dpc AGM recipients were repopulated to varying degrees (<0.1% to >10%) at 1 month posttransplantation. At 3 months postinjection, engraftment persisted only in the

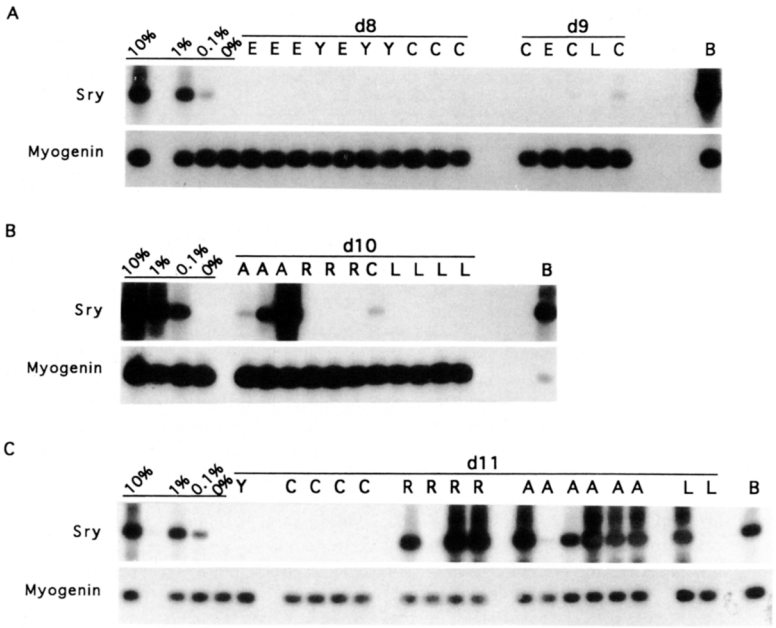

Figure 2. Temporal Analysis of Recipient Peripheral Blood after Injection of 8 dpc through 11 dpc Embryonic Cells

Female recipients were injected with (A) 8 or 9 dpc, (B)10 dpc, and (C) 11 dpc embryonic tissues generated from matings of heterozygous male β-globin transgenic mice. Peripheral blood was examined by PCR analysis at 3 months (for 8 dpc recipients), 3.5 months (for 9 dpc recipients), 1 month (for 10 dpc recipients), and 3 months (for 11 dpc recipients) posttransplantation. The embryonic tissues transplanted were the following: (E), whole embryo; (Y), yolk sac; (C), embryonic circulating cells; (L), fetal liver; (A), AGM; (R), body remnants; and (B), control adult bone marrow. Autoradiograms of Southern blots after gel electrophoresis are shown. Filters were hybridized with *Sry* and myogenin (for DNA normalization) probes and the contribution standards were derived by diluting male into female genomic DNA. The two <1% positive 10 dpc AGM recipients lost their male *Sry* signal by 3 months posttransplantation. The third animal (>10% repopulated) is animal 9.2 shown in Figure 3A and is the predecessor of secondary and tertiary animals shown in Figure 6. All bone marrow control recipients were completely repopulated.

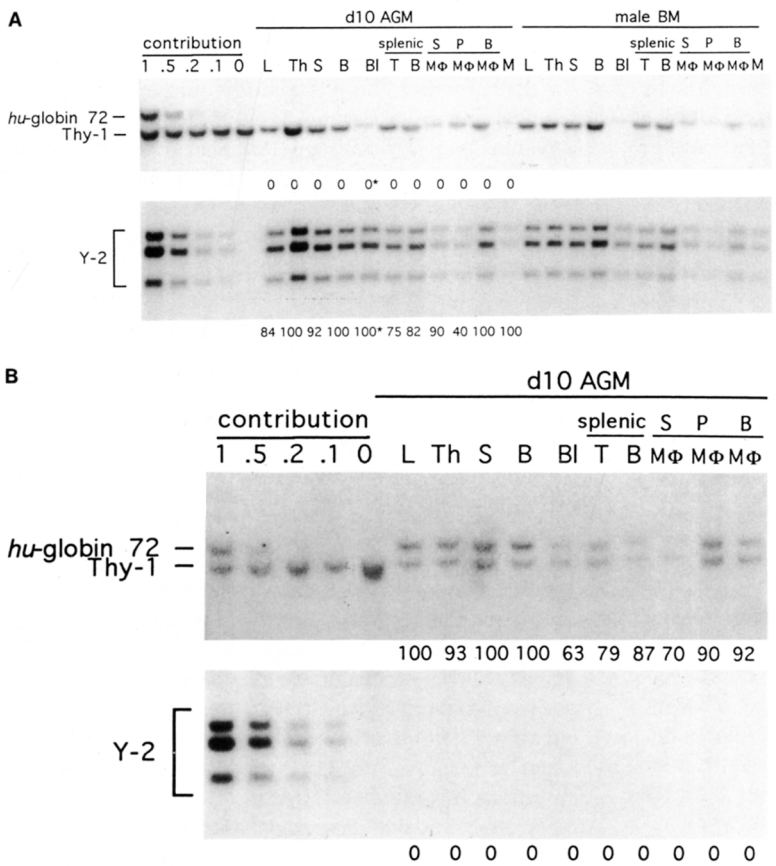

Figure 3. Multilineage Marker Analysis of the Long-Term 10 dpc AGM Repopulated 9.2 Mouse and B.2 Mouse

Recipient 9.2 (A) was sacrificed and analyzed at 7.5 months postinjection of one embryo equivalent (1.8×10^4) AGM cells. Of the embryos used in this experiment, 50% were male as determined by *Sry* PCR. However, none of the embryos were positive for the globin transgene. Recipient B.2 (B) was sacrificed and analyzed at 8 months postinjection of one embryo equivalent (3.7×10^4 cells) of AGM cells. Of the embryos used, 50% were male as determined by *Sry* PCR and all embryos were globin transgenic since a homozygous male was used for the mating. Recipient mouse DNAs were isolated from the following tissues: (L), lymph nodes; (Th), thymus; (S), spleen; (B), bone marrow; (Bl), peripheral blood; (T), splenic T cells; (B), splenic B cells; (S), splenic macrophages; (P) peritoneal macrophages; (B), bone marrow macrophages; and (M), bone marrow mast cells, cut with EcoRI, blotted as previously described (Strouboulis et al., 1992), and hybridized with the human globin and *Thy-1* probes and rehybridized with a *Y-2* probe. Contribution controls indicate 1, 0.5, 0.2, 0.1, and 0 copies of the globin transgene (12 kb hybridizing band) or copy equivalents of the *Y-2* repetitive gene (2.1, 1.9, and 1.5 kb hybridizing bands). The endogenous *Thy-1* gene (8.2 kb hybridizing band) was used as a DNA normalization control. Quantitation of donor-derived repopulation was determined by densitometry and phosphorimaging and percentage of engraftment values are indicated under each lane. The value for peripheral blood repopulation (*) could not be determined accurately, since degradation of high molecular weight DNA took place during preparation of the sample. Tissue DNA samples from a transplanted adult bone marrow recipient are also shown. This female recipient was transplanted at the same time as the 9.2 mouse, received 4×10^6 male adult bone marrow cells, and was fully engrafted.

Table 1. Frequencies of Long-Term Repopulating Activity in the Embryo

Embryo age	Donor embryonic tissues	Number of positive recipients / Number of recipients	Embryo equivalents per recipient	Frequency of repopulation
Day 10	112 AGM	3/96	1.16	3%
	121 yolk sac	0/74	1.63	0%
	66 liver	0/43	1.53	0%
	33 circulation	0/27	1.22	0%
Day 11	18 AGM	11/19	0.95	73%
	14 yolk sac	10/17	0.82	59%
	10 liver	6/15	0.67	40%
	13 circulation	0/9	1.45	0%

Embryonic tissues from 10 and 11 dpc were dissected and AGM, yolk sac, liver, and circulating cells were isolated. The number of donor embryonic tissues transplanted, the number of positive recipients out of the number of surviving transplant recipients, the average number of embryo equivalents per recipient, and the frequency of repopulation are indicated. Transplantation recipients were examined by *Sry* or human globin-specific PCR for the presence of donor male or transgenic cells in peripheral blood DNA at greater than 6 months postinjection. Each animal was independently analyzed at least two times and only animals with signal representing greater than 10% repopulation by donor cells are considered positive. Fourteen individual transplantation experiments were performed using 10 dpc embryonic tissues and four individual transplantation experiments were performed using 11 dpc tissues. Embryos were pooled before injection of single cell suspensions into female recipients. The number of cells in one embryo equivalent of AGM was $1.4–5 \times 10^4$, yolk sac was $3–9.2 \times 10^4$, liver was $0.9–6.7 \times 10^4$, and the circulation was $1–18 \times 10^4$. In four experiments with 10 dpc tissues, one embryo equivalent was injected per recipient and in eight experiments, the embryo equivalents injected ranged from 9–0.03 per recipient. In two experiments with 11 dpc tissues, one embryo equivalent was injected per recipient and in two experiments, the embryo equivalents injected ranged from 3–0.03 per recipient. A limiting number of normal, sygeneic female bone marrow (2×10^4) or spleen (2.5×10^5) cells were coinjected to promote short-term survival of recipients. Only 50% of injected control animals survived, while all irradiation controls (no cells injected) died within 14 days. Each experiment also contained adult (male) bone marrow control recipients, which always tested positive for long-term complete engraftment.

The three positive recipients resulting from the injection of 10 dpc AGM cells were derived from embryonic cells of the 39–41, 34–37, and 34–35 somite pair stage and were further examined as shown in Figures 3 and 4. The 11 dpc embryonic tissues were obtained from embryos with >42 somite pairs.

high signal animal yielding >10% donor repopulation (data not shown). Since this PCR assay was linear only to the 10% level, Southern blot analysis of the highly engrafted 10 dpc AGM recipient demonstrated 100% repopulation (Figure 3A). Also, the low level signal (<0.1%) found in the 10 dpc embryonic circulation transplanted animal was absent in this recipient when it was retested at 3 months posttransplantation. Thus, 10 dpc AGM cells appear to be the most potent embryonic hematopoietic reconstituting cells when compared with other transplanted 10 dpc embryonic cells. Interestingly, at 11 dpc when other investigators have found high level repopulating activity in the yolk sac, we have found high level (>10%) repopulation of recipients receiving 11 dpc AGM, body remnant, or liver cells (Figure 2C). Thus, long-term repopulating activity is present in most embryonic tissues at 11 dpc.

The Frequency of Long-Term Repopulating Hematopoietic Cell Activity in Developing Embryonic Tissues

To establish more firmly the onset of AGM long-term hematopoietic repopulating activity compared with other embryonic tissues (particularly the yolk sac) and to examine further the frequencies of engraftment by the different embryonic tissues, we performed additional transplantation experiments. As shown in Table 1 at greater than 4 months posttransplantation of 10 dpc tissues, 3 highly positive (>10% donor repopulated) recipients were obtained from the transplantation of 112 AGM regions into 96 recipients (~1.16 embryo equivalents per recipient). In contrast, when 121 yolk sacs or 66 livers were transplanted into 74 or 43 recipients (average of 1.63 and 1.53 embryo equivalents each), respectively, no mice were positive for the donor marker. Short term after transplantation we observed numerous low level donor positive recipients. Seven recipients receiving AGM cells and three recipients receiving yolk sac cells were found to be low level repopulated (between 1% and 5%) at 2 months but lost all donor-derived cells when examined later. Only the three 10 dpc AGM recipients originally testing >10% donor positive remained positive long term after transplantation. These experiments suggest that the AGM region acquires detectable long-term high level repopulating activity at 10 dpc (between the 34 and 41 somite pair stage) before yolk sac and liver and is limiting since only 3% of the recipients are high level engrafted.

When 11 dpc AGM cells were transplanted (~0.95 embryo equivalents per recipient), repopulation of recipients was found at a much higher frequency (73%) than with 10 dpc AGM cells. In addition, 11 dpc yolk sac and liver cells were found to engraft long-term (≥3 months) recipients to high levels (>10%) and at high frequencies: 59% and 40%, respectively (average of 0.82 and 0.67 embryo equivalents per recipient). No long-term repopulation was observed in recipients of 10 or 11 dpc embryonic circulating cells. Thus, we have confirmed the high level repopulating frequency of day 11 pc yolk sac cells seen by other investigators (Moore and Metcalf, 1970; Huang and Auerbach, 1993) and demonstrated that yolk sac is not the only day 11 embryonic tissue with high frequency stem cell activity; AGM region and liver are also highly active at this time in embryonic development.

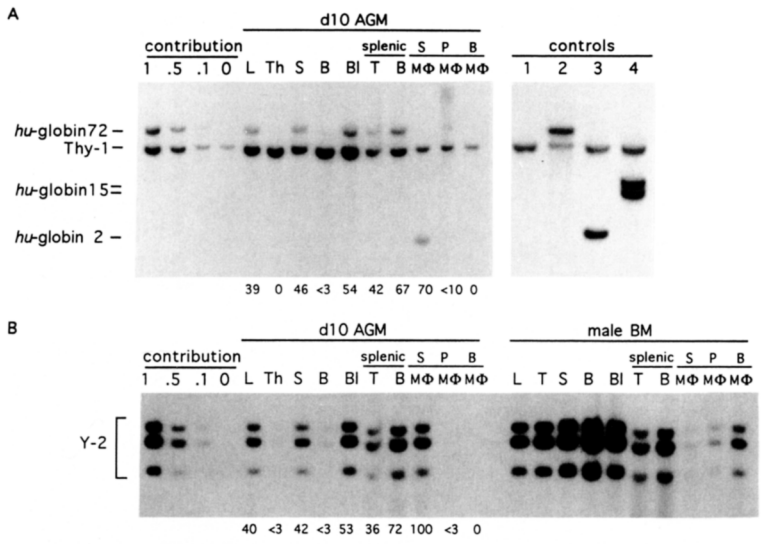

Figure 4. Multilineage Marker Analysis of the Long-Term Engrafted I.2 Mouse Injected with a Mixture of 10 dpc Embryonic Cells

Recipient I.2 was sacrificed and analyzed at 7 months postinjection of one embryo equivalent of a mix of AGM (1.2×10^4) cells from the 34–35 sp stage, liver (0.96×10^4) cells from the 32–35 sp stage, and yolk sac (5×10^4) cells from the 34–35 sp stage. Of the AGM cells used (total of 8 pooled embryos), 50% were derived from male embryos and 50% were globin transgene line 72 positive embryos. Of the liver cells used (total of 5 pooled embryos), 40% were derived from male embryos and 20% from globin transgene line 2 positive embryos. Of the yolk sac cells (total of 12 pooled embryos), 75% were derived from male embryos and 58% from globin transgene line 15 positive embryos. DNAs were isolated from hematopoietic tissues and enriched lineages, analyzed as described in Figure 3, and hybridized with human globin and *Thy-1* probes (A) and then reprobed with a *Y-2* fragment (B). Control lanes in (A) demonstrate transgenic line–specific EcoRI integration fragments: lane 1, nontransgenic DNA; lane 2, DNA from a line 72 transgenic mouse; lane 3, DNA from a line 2 transgenic mouse; and lane 4, DNA from a line 15 transgenic mouse. Contribution controls indicate 1, 0.5, 0.2, 0.1, and 0 copies of the globin transgene or copy equivalents of the *Y-2* repetitive gene. Quantitation of donor globin and *Y-2* signal were performed by phosphorimaging and densitometry with the *Thy-1* hybridization as a normalization control. The *Thy-1* signal in the contribution controls varies due to the total amount of DNA per lane: lane 1, 10 μg male DNA; lane 2, 5 μg male DNA; lane 3, 1 μg male DNA; and lane 4, 0.5 μg female DNA. Percentage donor engraftment is indicated under each lane. Tissue DNA samples from an adult bone marrow recipient control are also shown in (B). This female recipient was transplanted at the same time as the I.2 mouse, received 4×10^6 male adult bone marrow cells, and was fully engrafted.

Repopulation of AGM Recipient Mice Is Multilineage

A definitive characteristic of hematopoietic stem cell activity is the repopulation of all the mature blood lineages of the recipient. After the initial peripheral blood DNA PCR screening, we examined the extent of multilineage repopulation in the three positive primary recipients that received 10 dpc AGM cells. Hematopoietic tissues (blood, thymus, spleen, and bone marrow) and specific cell lineages (B and T cells, macrophages, and mast cells) were assayed by Southern blot analysis for the presence of the Y chromosomal and the β-globin transgene markers. When males heterozygous for the transgene are used for the generation of donor embryos (see Figure 1), the two independently segregating markers (Y chromosome and globin transgene) should be individual in 25% and overlapping in 25% of the injected donor cells (total of 75% of donor cells are genetically marked).

Long-term engraftment of mouse 9.2 is shown in Figure 3A. This recipient, which received one embryo equivalent of AGM cells (1.8×10^4) from a pooled mixture of three male and three female embryos (all were nontransgenic and of the 39–41 somite pair stage) is highly repopulated in all the hematopoietic tissues and lymphoid and myeloid lineages at 7.5 months postinjection. The marked donor-derived repopulating cells are male nontransgenic cells as determined by hybridization with Y chromosome (*Y-2*) and globin transgene-specific probes. Percentage of engraftment in each tissue and lineage varied and ranged from 40%–100% donor-derived cells as internally quantitated against *Thy-1* endogenous gene hybridization. Thus, one embryo equivalent of AGM cells are capable of full and complete repopulation of a lethally irradiated adult recipient.

The B.2 recipient mouse shown in Figure 3B demonstrates reconstitution with one embryo equivalent of AGM cells (3.7×10^4 cells) from a mixture of eight 34–37 somite pair embryos (four male, four female; eight transgenic). This animal is very highly engrafted in all hematopoietic tissues and lineages (macrophages and B and T lymphoid cells) at 8 months posttransplantation. Levels of engraftment ranged from 63%–100% in the hematopoietic tissues and 70%–92% in the enriched cultured lymphoid and myeloid lineages. These cells are derived from female transgenic-marked HSCs as determined by hybridization to the globin transgene probe (normalized by endogenous *Thy-1* signal) and lack of signal with the *Y-2* probe. The combined results of mouse 9.2 and B.2 demonstrate that neither genetic marker, Y chromosome, nor globin transgene has an effect on the long-term multilineage engraftment of these recipients and suggest that one or very few AGM cells is capable of full and complete reconstitution.

A third positive recipient mouse, I.2 is shown in Figure 4. This recipient received a mixture of embryonic cells (32–35 somite pairs) to examine the competitive hematopoietic repopulation potential of AGM cells as compared with liver and yolk sac cells. The injected mixture of cells was derived from three separate globin transgenic lines with differing transgene chromosomal integration sites: AGM cells from line 72, yolk sac cells from line 15, and liver cells from line 2. Thus, the origin of the repopulating cells in the recipient could be determined by EcoRI Southern blot analysis (Figure 4A, controls) for the unique fragments: a 12 kb fragment for line 72, 5.3 and 4.8 kb frag-

ments for line 15, and a 3.5 kb fragment for line 2. High level engraftment (36%–100%) is observed in most hematopoietic tissues and lineages as determined by globin transgene (Figure 4A) and *Y-2* probe (Figure 4B) hybridizations. Three main patterns emerge from these blots. The first and predominating pattern shows equivalent signals for both the globin transgene and *Y-2* markers in lymph node, spleen, blood, and splenic B and T cells. The hybridizing globin fragment migrates at 12 kb (line 72), indicating repopulation by AGM-derived cells. Thus, most hematopoietic cells are derived from male AGM cells. The second pattern is observed in a single lineage, the splenic macrophages of this recipient. These cells are male derived (*Y-2* positive) but do not carry the line 72 globin transgene marker. Instead, a globin hybridizing fragment migrating at 3.5 kb indicates splenic macrophage production from line 2, fetal liver cells. This marker is probably beneath the limits of detection in whole spleen DNA and suggests the presence of a low frequency long-term single lineage repopulating cell in 10 dpc liver. The liver at this early stage of development has been found to be capable of producing macrophages on adult bone marrow stroma in Dexter-type cultures (A. M. and N. L. Samoylina, unpublished data). Finally, the third pattern is found in the bone marrow, thymus, and peritoneal and bone marrow macrophages. While no or faint globin (line 72) and *Y-2* hybridizing signals are seen, it appears that most of these cells are derived from female nontransgenic cells and could be donor or endogenous in origin. Thus, this recipient is repopulated by at least three stem/progenitor cells. These results strongly suggest that in 10 dpc hematopoietic tissues, the most competent cells for long-term high level multilineage hematopoietic engraftment under our stringent transplantation conditions are from the AGM region.

In addition, we have performed multilineage analysis on long-term donor-repopulated recipients receiving 11 dpc AGM region, yolk sac, or liver cells. All three embryonic tissues were capable of high level repopulation of all hematopoietic tissues and lineages analyzed (data not shown).

Functional Repopulation of the Erythroid Lineage in Long-Term Repopulated AGM Recipients

While we have shown that AGM cells are capable of producing myeloid and lymphoid lineage cells in vivo, we sought to determine whether erythroid lineage cells are also produced. It was also of interest to determine whether AGM stem cell–derived erythroid cells would be capable of appropriate developmental expression of globin genes. The use of the complete human globin transgene locus allowed us to detect the presence of functional donor-derived human globin in erythrocytes by S1 RNA analysis and to determine whether embryonic, fetal, and/or adult globins were being produced in the peripheral blood of AGM recipient mice (Figure 5). When tested at 8 months posttransplantation, the peripheral blood of one out of two animals receiving 10 dpc AGM cells was found to be highly positive for adult human β-globin expression (Figure 5, lanes 4 and 5). No human ε or γ-globin expression was observed. The positive 10 dpc AGM recipient was the B.2 mouse, described in Figure 3B. When quantitative expression of the human β-globin transgene of the peripheral blood of the B.2 mouse was compared with that of a fully engrafted bone marrow control recipient (100% expression), human β-globin RNA was produced to the 66% level (66% of the B.2 blood cells produce RNA to the level expected of this single copy transgene; see Strouboulis et al., 1992). This level is consistent with the percentage of engraftment (63%) determined by DNA analysis of B.2 peripheral blood (Figure 3B). In addition, a recipient receiving 11 dpc AGM cells was found to express human β-globin at 2 months postinjection (Figure 5, lane 6). In this recipient, as well as numerous other recipients, the level of hu-

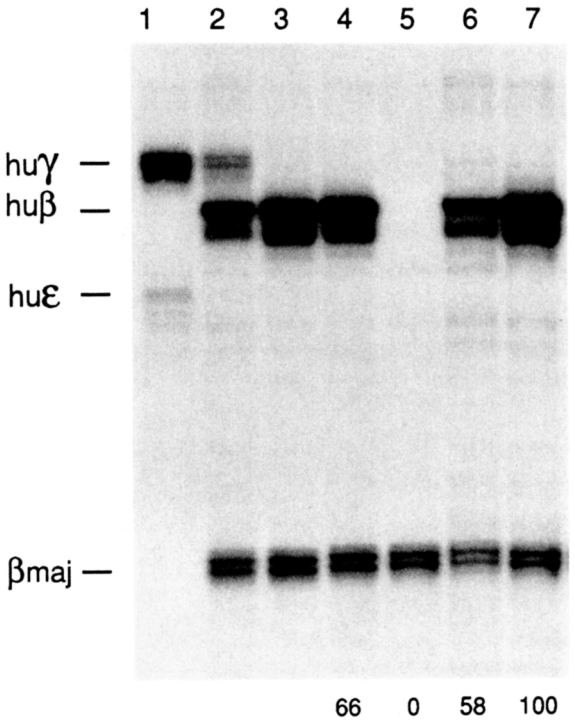

Figure 5. Expression of the Human ε, γ, and β Transgenes in the Peripheral Blood of AGM Transplantion Recipients by S1 Nuclease Protection Analysis

S1 nuclease protection was performed with probes specific for human γ-, β-, and ε-globin transgene RNAs and endogenous mouse β major globin RNA (erythroid lineage control). Protected fragments are indicated at left. RNA samples protected in each lane are as follows: lane 1, 10 dpc line 72 transgenic yolk sac RNA (38 somite pairs); lane 2, 12 dpc line 72 transgenic fetal liver RNA; lane 3, line 72 transgenic adult spleen RNA; lanes 4 and 5, peripheral blood RNA from two animals injected with line 72 transgenic (34–37 somite pair) 10 dpc AGM cells at 8 months posttransplantation; lane 6, peripheral blood RNA from an animal injected with (44 somite pair) 11 dpc AGM cells at approximately 2 months posttransplantation; lane 7, peripheral blood RNA from an animal injected with human globin transgenic adult bone marrow at approximately 2 months posttransplantation. Quantitation of expression levels of human β-globin transgene (indicated beneath each sample lane) were performed by densitometry using mouse β major expression as an internal control. Since the expression of this transgene is known to be copy number–dependent (Strouboulis et al., 1992), 100% expression indicates the level of a single copy of the human β-globin transgene compared with the diploid level expression of the endogenous mouse β major gene. The peripheral blood RNA sample in lane 4 is from the B.2 recipient shown in Figure 3B.

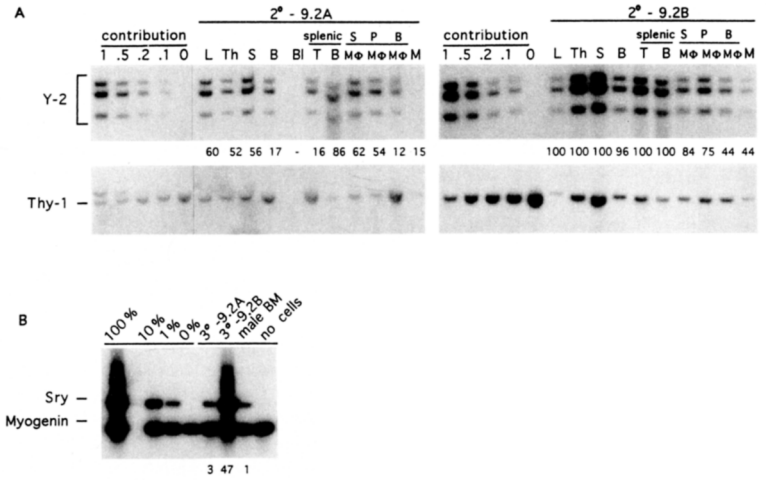

Figure 6. Analysis of Secondary and Tertiary Transplantation Recipients Receiving AGM-Derived Hematopoietic Stem Cells

At 7.5 months posttransplantation of 10 dpc AGM cells, animal 9.2 was sacrificed and 3 × 10^6 bone marrow cells were injected into two lethally irradiated secondary recipients, 9.2A and 9.2B. At 4 months posttransplantation, the secondary recipients were sacrificed and (A) numerous hematopoietic tissues and lineages were examined by Southern blot analysis for the presence of the Y chromosome marker. Abbreviations for the tissues and methods are the same as in Figure 3. Contribution controls indicate copy equivalents of the *Y-2* repetitive gene. Endogenous *Thy-1* gene hybridization was used as a normalization standard. Quantitation was performed by phosphorimaging and densitometry and percentage donor engraftment indicated under each lane. No peripheral blood DNA (minus) was obtained for recipient 9.2A and the percentage engraftment of bone marrow macrophages in both secondary recipients was low due to fibroblast overgrowth in the culture.

(B) PCR analysis of peripheral blood DNA from tertiary recipients 7 weeks after injection of 3 × 10^6 bone marrow cells from secondary transplant mice 9.2A and 9.2B. Contribution controls indicate 100%, 10%, 1%, and 0% *Sry* signal, with myogenin as the normalization standard. The lane marked male BM and no cells indicates peripheral blood DNA obtained from recipients at 7 weeks posttransplantation of 4 × 10^6 male bone marrow cells or 2.5 × 10^5 female spleen cells, respectively. Quantitation was performed by densitometry and percentage engraftment indicated under sample lanes.

man β-globin expression in peripheral blood was found to correspond to the level of engraftment when DNA analysis was performed (data not shown). Thus, repopulation in the erythroid lineage with genetically marked cells, in addition to the repopulation shown in the myeloid and lymphoid lineages, indicates the presence of multilineage repopulating cells in the AGM region of the embryo.

Serial Transplantation of AGM-Derived Cells Results in Long-Term Multilineage Repopulation

The most stringent test for the presence of long-term self-renewing stem cell activity in the AGM region is the serial transplantation of bone marrow from the primary recipients into secondary recipients for long-term multilineage repopulation. Such transplantation was performed at 8 months postengraftment of mouse 9.2 (Figure 3A). Initially, the CFU-S 11 activity of the donor cells was measured and resulted in all colonies positive for the *Y-2* sequence (data not shown). Long-term repopulation of secondary recipients was also tested by transplantation of bone marrow cells (3 × 10^6) from primary recipient 9.2 into lethally irradiated adult recipients. As shown in Figure 6A, all hematopoietic tissues and lineages of two secondary recipients, 9.2A and 9.2B, were repopulated with donor marked cells at 4 months posttransplantation. Hematopoietic tissue and lineage engraftment of animal 9.2A ranged from 12%–86%, while that of animal 9.2B ranged from 44%–100%. When bone marrow cells (3 × 10^6) from secondary animals 9.2A and 9.2B were transplanted into tertiary recipients (Figure 6B), again repopulation was observed. The 9.2B bone marrow contributed to 47% repopulation of peripheral blood-nucleated cells and 9.2A bone marrow contributed to 3% repopulation at 7 weeks posttranplantation as determined by *Sry*-specific PCR. These data indicate that AGM-derived hematopoietic cell activity can be serially transplanted and that the level of engraftment of the long-term secondary recipients corresponds well to the percentage of donor-derived cells in the bone marrow of the primary recipient (see Figure 3A).

Discussion

While previous investigations have lead to the general acceptance that the yolk sac functions as the primary and only site of hematopoiesis in the early (11 dpc) developing mouse embyo (Moore and Metcalf, 1970; Tavassoli, 1994; Huang et al., 1994), we have found the presence of potent and high frequency HSC activity in the AGM region, as well as the liver and the yolk sac. We have shown that the AGM-derived hematopoietic cells bear all the hallmark characteristics of adult bone marrow HSCs: long-term persistent engraftment, high level repopulation of all hematopoietic lineages, and the ability to repopulate secondary and tertiary recipients serially. In the 11 dpc AGM region, this HSC activity appears at a higher frequency than in liver or yolk sac. Earlier in development, at 10 dpc, we observed HSC activity only in the AGM region. Since only 3% of AGM recipients were engrafted, long-term high level repopulation was limiting, probably reflecting repopulation by as few as one HSC. Although we cannot exclude the possibility that HSCs exist at a low frequency in 10 dpc yolk sac and liver, these data favor the AGM region as the initial source of long-term repopulating cells and suggest that AGM HSCs may migrate to the fetal liver as well as the yolk sac by 11 dpc (Medvinsky, 1993). Alternatively, AGM HSCs may not be produced in situ, but this region may give competence to, maintain, or expand stem cells migrating from other sites such as the yolk sac. Interestingly, the AGM region is a site of stem cell factor and the c-kit receptor expression at 10 dpc (Matsui et al., 1990;

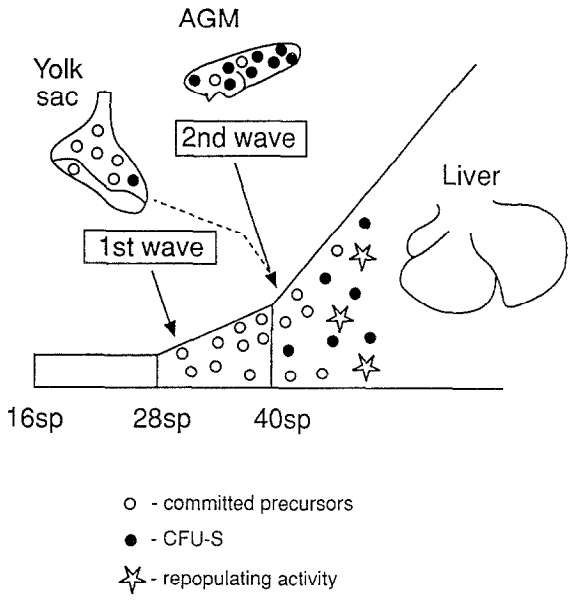

Figure 7. Proposed Waves of Hematopoietic Activity Entering the Fetal Liver during Embryonic Development

Representation of the putative sources of origin and temporal emergence of various measureable hematopoietic activities in the liver rudiment. Waves of hematopoietic activity in the yolk sac and the AGM region always precede the onset of committed precursor, CFU-S, and repopulating activities in the liver and predict a role for these embryonic tissues in establishment of fetal liver hematopoiesis.

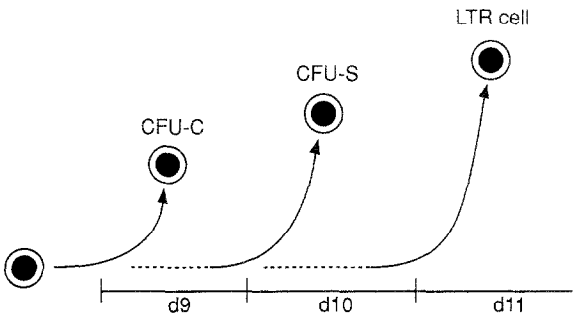

Figure 8. Model of Hematopoietic Stem Cell Maturation

The kinetic appearance of various hematopoietic activities in the early developing mouse embryo is shown. Long-term repopulating (LTR) activity is observable in the mouse embryo only at late 10 dpc, while CFU-C and CFU-S activity generally appear at 8 and 9 dpc, respectively. This could suggest a sequence in which a more primitive embryonic cell(s) is first capable of only committed activity to differentiated hematopoietic cells, then acquires the ability to form progenitor cells for CFU-S activity, and only later matures to full competence to become a multilineage long-term repopulating HSC. Alternatively, this sequence of appearance could be the result of low cell number or limits of detection of individual assays. In addition, it is unknown whether these functional hematopoietic activities are directly related through the progeny of a single cell or are independently established as indicated by the dotted lines.

Keshet et al., 1991). This ligand and receptor are known to play a role in primordial germ cell expansion (Godin et al., 1991; Dolci et al., 1991), hematopoiesis (Russell, 1979), and CFU-S production (Witte, 1990).

Surprisingly, we found that HSC activity in the embryo develops at a relatively late time in gestation (10 dpc) as compared with the appearance of the first differentiated hematopoietic cells in the embryonic yolk sac at 7.5 dpc, the first CFU-C progenitors at 8 dpc, and the first CFU-S progenitors at 9 dpc. Although slightly later in developmental time, these different hematopoietic cell types are found in the developing liver rudiment in the identical sequence (reviewed by Medvinsky, 1993). Previously, migration of hematopioetic cells from the yolk sac to the fetal liver has been strongly suggested (Johnson and Moore, 1975; Cuddenec et al., 1981; Houssaint, 1981). This, in combination with the various hematopoietic cell types arising in the yolk sac, AGM region, or both, is suggestive of waves of hematopoietic activity emerging in the fetal liver. As shown in Figure 7, committed precursors (CFU-C) arising in the yolk sac could be responsible for the first wave of hematopoietic activity observed in the liver at the 28 somite pair stage. Later in the second wave, CFU-S activity primarily from the AGM region could contribute to the appearance of CFU-S in the liver at the 40 somite pair stage. Finally, HSCs from the AGM region could contribute to stem cell activity in the liver at 11 dpc. Precedence for waves of hematopoietic activity has been previously established in the colonization of the avian thymus (Coltey et al., 1987, 1989).

How is it then that differentiated hematopoietic cells, committed progenitors, and CFU-S progenitors appear in the mouse embryo before long-term repopulating cells? Unlike the adult hematopoietic system, in which the classical hierarchical scheme illustrates stem cell to CFU-S progenitor to committed cell to differentiated cell production, these activities appear to develop in the reverse order in the embryo (Figure 8). Our observation that stem cell activity does not develop until 10 dpc raises a number of interesting possibilities: embryonic HSCs cannot functionally repopulate adult recipients; the frequency of the long-term repopulating cell in the early embryo compared with the committed progenitors is initially low and undetectable because of seeding inefficiency in the transplant recipients; embryonic HSCs are intrinsically programmed to produce large numbers of differentiated cells, then committed progenitors, and finally adult repopulating HSCs in this sequence; specific microenvironments direct a small number or pool of prestem or stem cells to various activities. In early developing yolk sac, they are directed to the formation of differentiated hematopoietic cells and only later, when other growth factors/receptors or microenvironments (intraembryonic) become available can intermediate progenitors (CFU-S) develop. Finally, definitive stem cell potential is acquired only after competency factors/specific microenvironments (for example, expansion or self-renewal) become available in the embryo.

These scenarios cannot be distinguished at the present time, nor can the number of initial prestem or stem cells be determined. We are presently establishing organ culture and stromal coculture systems along with clonal retroviral marking of 9, 10, and 11 dpc AGM region, yolk sac, and liver to address these questions. Also, the lineage relationship of the stem/prestem cells involved in produc-

tion of the various embryonic hematopoietic activities cannot be determined. Various cell surface markers, Sca-1, AA4.1, and Thy-1 have been described for HSCs of the adult mouse bone marrow and fetal liver. Recent studies by Huang et al. (1994) have shown that unlike fetal and adult HSCs, yolk sac cells from late day 10 embryos express *AA4.1* but not *Sca-1*. We have performed preliminary reverse transcriptase–PCR analysis of RNA from 10 dpc embryonic tissues using primers specific for the *Sca-1* gene (Ly-6A/E) and have found high expression in the AGM region and only very low expression in the yolk sac. We are presently attempting to enrich for HSCs from the AGM region using the Sca-1 and AA4.1 antibodies. It would be very interesting if AGM cells expressed both markers, perhaps indicating a more developmentally advanced stem cell phenotype. This marker could be extremely useful in determining the sequence of events (for example, expansion or migration) leading to the development of functional HSCs in the mouse embryo.

Experimental Procedures

Cell Preparations

(CBA × C57BL/10)F1 females were bred with transgenic heterozygous or homozygous males for the human globin gene (Strouboulis et al., 1992). The appearance of the vaginal plug was designated as day 0. Embryos were aseptically removed from uteri and dissected with tungsten needles in L-15 medium (Flow Lab) with 5% fetal calf serum under the dissection microscope. Individual embryonic tissues were further dispersed with the use of tungsten needles and then incubated for 1–1.5 hr in 0.1% collagenase with 10% fetal calf serum at 37°C. After gentle pipetting and washing, cells were injected intravenously in the tail vein of 2- to 3-month-old irradiated (CBA × C57BL/10)F1 female mice. The recipients were exposed to a split dose of 1000 rads of a ^{60}Co source. The embryo tissue cells were coinjected with either 2×10^4 female bone marrow cells or with 2.5×10^5 adult female splenocytes for immediate short-term radiation protection. These doses were limiting for long-term survival, as we found that about 50% of control-injected animals died within 2 months. All noninjected irradiation control mice died within 14 days. Mice were housed in positive pressure cabinets and received neomycin (0.16 g/100 ml) in their drinking water for the first 4 weeks following the irradiation. All animals were obtained from the specific pathogen-free breeding facility at the National Institute for Medical Research, London, and all animal procedures were in accordance with the Animals Scientific Procedures Act, 1986, United Kingdom.

DNA Preparation, PCR, and Southern Blot Analysis

Whole genomic DNA was isolated from the peripheral blood and tissues of recipient animals by proteinase K digestion, phenol–chloroform extraction, and isopropanol precipitation as described in Maniatis et al. (1982). Positive displacement pipettes or filter tips were used and reagents and materials were specially handled for utilization in PCR. The PCR reaction was done as follows: 200 ng of genomic DNA was added to a mixture containing 1 U Taq polymerase (Biotaq), PCR buffer (Biotaq), PCR primers (100 ng each), and 0.2 mM nucleotides (Perkin-Elmer Cetus). The sequences of the PCR primers are as follows: *Sry* primers, 5′-TCA TGA GAC TGC CAA CCA CAG-3′ and 5′-CAT GAC CAC CAC CAC CAC CAA-3′ (Koopman et al., 1991); myogenin primers, 5′-TTA CGT CCA TCG TGG ACA GC-3′ and 5′-TGG GCT GGG TGT TAG TCT TA-3′; and the human β-globin primers 5′-CTT CAG GTT CCC AGT GAG GAT G-3′ and 5′-GCT CCC TAA GGG GTA AAG AGT G-3′. The reactions for the combination *Sry*–myogenin PCR were initially heated at 94°C for 10 min, followed by 26–33 cycles of 94°C for 10 s, 65°C for 30 s, followed 10 min at 72°C; the cycle parameter for the human β-globin and myogenin primers was 94°C for 10 s, 26–33 cycles of 94°C for 10 s, 55°C for 30 s, 72°C for 1 min, followed by 10 min at 72°C. The cycles were done in a Techne PHC-2 thermocycler. The sizes of the amplified PCR products are 441 bp (*Sry*), 428 bp (human β-globin), and 245 bp (myogenin). The products were separated on 1.5% agarose gels, transferred to nylon membranes, and hybridized with an *Sry*-specific probe (p422, gift of J. Gubbay) and gel-purified myogenin, human β-globin fragments, or both after PCR amplification. A semilinear relationship was obtained between the amount of input DNA and signal strength of the PCR product after quantitation by phosphorimage analysis (A. M. M. and E. D., 1993).

For genomic Southern blots, 5 μg of genomic DNA were digested over night with EcoRI and separated on a 1% agarose gel. After blotting, the filters were hybridized with a 3.3 kb EcoRI probe specific for the human β-globin transgene (Blom van Assendelft et al., 1989) and a 1.3 kb ApaI *Thy-1* gene fragment (Giguere et al., 1985). After exposure to X-ray film, the filter was stripped and reprobed with a Y chromosome–specific probe (*pY-2*, Lamar and Palmer, 1984). All probes were radioactively labeled by nick translation (Amersham). The percentage engraftment was determined by quantitation of radioactive fragments on a phosporimager (Molecular Dynamics), densitometer (Chromoscan 3) or both.

In Vitro Cultures

To expand and support the growth of B cells, a single cell suspension from spleen was cultured in 10 μg/ml lipopolysaccharide (Sigma) in complete medium; to obtain splenic T cells, spleen cells were grown in complete medium supplemented with 10–40 U/ml murine interleukin 2 (IL-2) (Biosource) and 5 μg/ml Concanavalin A (Sigma). Complete medium consists of RPMI 1640, 5% FCS, 2 mM L-Glutamine, 10 mM HEPES, 10 U/ml penicillin, 10 μg/ml streptomycin, and 0.1 mM 2-mercaptoethanol. Cells were cultured for 3–4 days at 37°C and 5% CO_2. After cell harvest, the identity of the cells was verified by fluorescence-activated cell sorter (FACS) analysis. At least 80%–90% of the cells grown under T cell growth conditions were *Thy-1* positive. Of the splenocytes grown under B cell growth conditions, 80%–90% were positive for B220. Genomic DNA was prepared from the harvested cells and analyzed by PCR as described above.

Macrophages were obtained from the adherent cell fraction of peritoneal wash, bone marrow, or spleen cells and expanded in complete medium with 10% L929-conditioned medium for 4–7 days. Cells were lysed directly on the tissue culture plate for DNA preparation. Bone marrow–derived mast cells were established from the nonadherent fraction of bone marrow cells. Cultures were maintained for a period of 4 weeks in RPMI 1640, 10% FCS, nonessential amino acids, 40 μM β-mercaptoethanol, 2 mM L-Glutamine, 10 U/ml penicillin, and 10–20 U of IL-3. Mast cells were identified by staining with May–Grünwald/Giemsa dye.

RNA Analysis

Approximately 100 μl of peripheral blood was obtained from transplant recipients by tail vein nicking. Blood RNA was extracted with RNAzol B (Cinna/Biotecx) and expression analysis of globin genes was carried out as described in Strouboulis et al. (1992); all S1 probes are as previously described (Kollias et al., 1986; Fraser et al., 1990; Lindenbaum and Grosveld, 1990). Quantitation was performed by densitometry using the mouse β major signal as an RNA normalization control and human β-globin signal in Figure 6, lane 7 as the 100% expression control.

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