

ES cells have only a limited lymphopoietic potential after adoptive transfer into mouse recipients

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SUMMARY

While hematopoietic stem cells from adult and fetal stages of murine development are capable of long term reconstitution of all mature blood lineages *in vivo*, embryonic hematopoietic stem cell repopulation *in vivo* has proved difficult. It is thought that there are many fewer hematopoietic stem cells in the embryo than in the fetal/adult stages of mouse development and that these cells possess a different developmental potential. One source of such cells are embryonic stem (ES) cells which can differentiate into most mature blood lineages *in vitro*. We have therefore used transplantation of differentiated ES cells to assess the hematopoietic potential of embryonic hematopoietic cells *in vivo*. We demonstrate here that precursors obtained from *in vitro* cultures of

normal ES cells can contribute only to restricted and limited hematopoiesis in a mouse without leading to tumour formation. Repopulation occurs for greater than 6.5 months at levels ranging from 0.1% to 6% in B and T cell lineages in peripheral blood. In contrast to *in vitro* colony data demonstrating the myeloid lineage developmental potential of ES cells, no donor-derived myeloid repopulation was observed in CFU-S assays and no macrophage and mast cells were found in long term repopulated recipients. Thus, the hematopoietic potential of ES cells *in vivo* is limited to low levels of repopulation and is restricted to the lymphoid lineage.

Key words: ES cells, lymphopoiesis, repopulation

INTRODUCTION

Murine hematopoiesis begins by day 7 of embryogenesis and proceeds throughout the entire life span of the animal to provide at least eight distinct lineages of mature blood cells. All of these cells, which differ in morphology and function are derived from pluripotent hematopoietic stem cells (Russell, 1979; Dexter and Spooner, 1987). During embryonic stages of mammalian development, hematopoiesis occurs in the yolk sac and appears to be limited in lineage complexity and magnitude when compared to the blood system during fetal and/or adult stages (Moore and Metcalf, 1970; Perah and Feldman, 1977). The cells in the embryonic differentiation pathway are thought to originate from hematopoietic stem cells. However, such stem cells are difficult to isolate due to their low abundance during this developmental stage (Wong et al., 1986; Moore and Metcalf, 1970) and thus the developmental potential of embryonic hematopoietic stem cells has not been well characterized. It is possible that embryonic hematopoietic stem cells may possess different developmental potential than fetal liver or adult bone marrow cells and provide a better source of transplantable cells for the blood system. Recently, it has been demonstrated that adult bone marrow hematopoietic stem cells are lacking in devel-

opmental potential for at least two T cell subsets (V 3 and V 4) and one B cell subset (B-1a) as compared to fetal liver hematopoietic stem cells (Ikuta et al., 1990; Kantor et al., 1992). Thus, it is important to determine if embryonic hematopoietic stem cells can contribute to fetal and adult hematopoiesis. At present, it is unclear whether a single class of embryonic hematopoietic stem cell can produce all the blood lineages found in embryos, as well as those found during fetal and adult stages and whether the developmental program is induced by microenvironment (Cudennec et al., 1981) and/or controlled by proposed clock mechanisms (Wood et al., 1985). Also, it is uncertain whether there is a direct lineage relationship between embryonic hematopoietic stem cells and fetal/adult stem cells and it has not been determined how embryonic hematopoietic stem cells may be expanded to the numbers found in fetal/adult stages. Therefore, it is of great interest to isolate and characterize embryonic hematopoietic stem cells.

ES cells (Evans and Kaufman, 1981; Martin, 1981) possess hematopoietic activity after differentiation *in vitro* (Doetschman et al., 1985; Schmitt et al., 1991; Wiles and Keller, 1991; Burkert et al., 1991; Lindenbaum and Grosfeld, 1990). Differentiation potential for most hematopoietic lineages has been demonstrated by *in vitro* culture of ES cells with hematopoietic growth factors (Wiles

and Keller, 1991; Burkert et al., 1991) and by intraperitoneal injection into mice of differentiated ES cells infected with oncogenic retroviruses (Chen et al., 1992). Previously, full and complete *in vivo* repopulation has been reported with cultured mouse blastocysts (Hollands, 1988). Thus, these studies suggest that ES cells could provide a plentiful source of normal embryonic hematopoietic stem cells for such developmental studies. ES cells provide an efficient *in vitro* system for clonal genetic manipulation and expansion and therefore, it is of importance to determine whether they can give rise, *in vivo*, to normal multipotential hematopoietic cells.

In order to study ES cell-derived hematopoiesis *in vivo*, we have employed conventional transplantation assays using intravenous injection into recipient mice (Spangrude et al., 1988; Jordan et al., 1990; Capel et al., 1989). We demonstrate here that normal, differentiated ES cells can contribute to B and T lymphocyte lineages *in vivo* but not myeloid lineages, either long term or in CFU-S assays. They contain restricted lymphopoietic precursor activity capable of only low level but long term repopulation of recipient mice. These results indicate the limited *in vivo* hematopoietic potential of ES cells and suggest interesting differences between ES cells and fetal/adult hematopoietic precursors.

MATERIAL AND METHODS

Growth and differentiation of ES cells

ES-cells, AB-1 (McMahon and Bradley, 1990), CCE (Evans and Kaufman, 1981), D3 (Doetschman et al., 1985) and AB-1.6 (an AB-1 cell clone that was stably transfected with a neomycin and HSV1-TK gene), all of 129/Sv male origin, were grown and differentiated as previously described (Lindenbaum and Grosveld, 1990). Only cells of low passage number were used. These cells retain their totipotency as demonstrated by the ability to form germline chimeras and are able to produce all hematopoietic lineages *in vivo* in such mice (M. Lindenbaum, P.P. Pandolfi and F. Grosveld, personal communication). After removal of STO feeder cells, ES cells were differentiated as a suspension culture in 0.5-1% Pokeweed mitogen mouse spleen conditioned medium (Terry Fox Laboratory) as a source of hematopoietic growth factors. In a typical experiment 8-10 bacterial plates (10 cm) were seeded with $1-2 \times 10^5$ ES cells/ml, grown under differentiation conditions and disaggregated by either trypsin treatment (1/1 dilution in PBS, 30 minutes on ice), collagenase treatment (Wong et al., 1986) or by repeatedly flushing through a 21 gauge needle. Fractionation, as described by Jordan et al. (1990) was done on a 55% Percoll (Pharmacia) gradient. Approximately 96% of the cells were found in fraction a, and between 1% and 2% in each of fractions b and c. After density fractionation on a Percoll gradient, between 0.8×10^5 and 3.6×10^6 cells were injected intravenously into recipient mice. All animal care was in accordance with the Animals Scientific Procedures Act, Home Office London, UK.

Injection into recipient animals

Newborn W^v/W^v and SCID mice were injected intravenously as described (Capel et al., 1989). Transplants were dependent upon the availability of newborns, therefore the length of time of ES cell differentiation before injection varied. Percoll fractionation allowed us to inject the neonatal recipient animals more efficiently, since cell aggregates and the cells forming aggregates were depleted in density fractions b and c. This allowed the animals to survive the injection process. In comparison to this, less than 50%

of the animals injected with fraction a or unfractionated cells survived. These animals died within minutes of injection from respiratory distress and trauma. All surviving animals were used for repopulation analysis. For CFU-S experiments, adult 129 mice (5-8 weeks of age) were irradiated in two doses by a Cobalt source (2×500 rads, 4 hour interval) and were injected intravenously in the tail vein. All animals were obtained from the SPF breeding facility at the National Institute for Medical Research, London.

PCR and Southern blot analysis

Due to the great sensitivity of the polymerase chain reaction (PCR) method, we used positive displacement pipettes and reagents and materials specifically designated for use in PCR. Pre- and post-PCR steps were carried out in separate rooms. Our PCR conditions produced a linear relationship between the percentage of male genomic DNA used and the signal strength of the PCR product (after hybridization with appropriate probes) when quantitated by the Molecular Dynamics PhosphorImager. For analysis, 0.2 µg of genomic DNA was added to a stock mixture containing Taq polymerase (1 unit/reaction, Cambio) and PCR buffer (Cambio), PCR primers (for the combination Sry-/myogenin-primers, 50 ng and 200 ng respectively and for the combination HSV1-TK-/myogenin-primers, 100 ng and 100 ng) and nucleotides (0.2 mM each dNTP). The PCR primers had the following sequence (5' - 3'): Sry-primers TCA TGA GAC TGC CAA CCA CAG and CAT GAC CAC CAC CAC CAA (Koopman et al., 1991), myogenin-primers TTA CGT CCA TCG TGG ACA GC and TGG GCT GGG TGT TAG TCT TA and the TK-primers CCT TCC GGA GGA CAG ACA CA and GCG GGC GAT TGG TCG TAA TC. PCR cycles for the combination Sry-/myogenin-primers were: 10 minutes 94°C, 35-37 cycles of 94°C for 10 seconds, 65°C for 30 seconds followed by 5 minutes 72°C (Koopman et al., 1991); PCR cycles for the combination HSV1-TK- / myogenin-primers were: 5 minutes 94°C, 35-37 cycles of 94°C for 1 minutes, 58°C for 1.5 minutes, 72°C for 4 minutes followed by 10 minutes 72°C. PCR cycles were done in a Techne PHC-2 thermocycler. PCR products were 441 bp (Sry), 380 bp (HSV1-TK) and 245 bp (myogenin). The products were analysed on 2% agarose gels, blotted and hybridized with an Sry-specific probe (p422, gift of J. Gubbay), a myogenin gel purified fragment probe from PCR amplified genomic DNA and a TK-specific probe (2.8 kb *Bam*HI/*Bgl*III fragment of the HSV-1 TK gene) as described (Maniatis et al., 1982). Percentage engraftment with donor ES cells was determined by quantitation of PCR Southern blot signal on the PhosphorImager. Genomic DNAs of peripheral blood, CFU-S, whole spleens and other mouse hematopoietic tissues were prepared as described in Maniatis et al. (1982). A Y-chromosome-specific probe (pY-2, Lamar and Palmer, 1984) was used to detect male-specific signals in CFU-S-derived DNA by Southern blot analysis.

In vitro cultures

B cell mitogenic growth conditions were: 10 µg/ml LPS (Sigma) in complete medium; T cell mitogenic growth conditions were: 10-40 U/ml murine IL-2 (Biosource), 5 µg/ml Concanavalin A (Sigma) in complete medium. Complete medium was RPMI 1640, 5% FCS, 2 mM L-glutamine, 10 mM Hepes, 10 U/ml penicillin, 10 µg/ml streptomycin, 0.1 mM 2-mercaptoethanol. Cells were grown for 1-2 weeks at 37°C and 5% CO₂. After stimulation and harvesting of cells, control experiments by fluorescence activated cell sorter (FACS) showed that at least 60-70% of the splenocytes cultured under T cell growth conditions were positive for Thy-1 and at least 70% of the splenocytes grown under B-cell growth conditions were positive for B220. Genomic DNA was prepared from the harvested cells and analysed by PCR as described above.

Peritoneal macrophages were cultured in complete medium with 10% L929-conditioned medium for 4 days. Mast cell cultures were

established from non-adherent bone marrow cells. Cells were cultured for greater than 4 weeks in RPMI 1640, 10% FCS, non-essential amino acids, 40 μ M 2-mercaptoethanol and 10-20 U of IL-3. Confirmation of mast cells was made by staining with May-Grünwald/Giemsa dye.

Flow cytometric analysis

Expression analysis for hematopoietic cell-surface antigens was performed by flow-cytometry on a Becton Dickinson FACSTAR Plus analyser using monoclonal antibodies anti-Thy 1.2 (Sigma), anti-L3T4 (CD4; Becton Dickinson), anti-Lyt 2 (CD8, Becton Dickinson), anti-H-2K^b (Pharmingen) and anti-B220 (Pharmingen). Monoclonal antibodies: CD8 and B220 antibodies were directly conjugated to fluorescein, the antibody CD4 was labelled with phycoerythrin. Biotinylated H-2K^b antibody was reacted with streptavidin-phycoerythrin for detection on the FACSTAR. The monoclonal antibody anti-Thy 1.2 was directly conjugated to fluorescein. Briefly, 2×10^5 cells were washed in MEM, 5% FCS and 0.01% azide. Antibody was added to the cells for 30 minutes on ice. Cells were washed two times and fixed in 1.1% formaldehyde/PBS solution. At least 10,000 cells were recorded.

Ouchterlony analysis

Serum was obtained from coagulated peripheral blood samples after centrifugation (7500 revs/minute, 4°C) for 5 minutes. Approximately 6 μ l of each serum sample was loaded in preformed wells of Ouchterlony plates (2% agar, 0.1% NaN₃ in 1 \times PBS) as indicated in Fig. 7. Igh allotype-specific antiserum (gift of D. Dresser) was loaded into the center well of each Ouchterlony plate. Plates were incubated overnight at room temperature.

RESULTS

Differentiated ES cells do not form classical CFU-S

To examine the *in vivo* hematopoietic potential of differentiated ES cells, repopulation ability was tested in the CFU-S assay. Unmanipulated ES cells were differentiated in suspension culture for 6-13 days. Embryoid bodies containing blood islands were visible after approximately 9 days in culture. Cells were harvested, disaggregated, Percoll fractionated and intravenously injected as a cell suspension into lethally irradiated 129 mice. Percoll fractionation was employed since others have previously used this method for enrichment of fetal hematopoietic stem cells (Jordan et al., 1990). CFU-S were scored from day 9 to 11 posttransplantation as shown in Table 1. In only one experiment, where animals received day 13 differentiated ES cells, did we convincingly observe an increase in CFU-S day 10 as compared to the irradiation control mice which received no cells. The spleens receiving ES cells showed multilineage CFU-S and contained more colonies as compared to the irradiation control. However, the CFU-S were small and fewer in number when compared to the bone marrow control. CFU-S were observed with all three injected Percoll fractions as well as with injected unfractionated embryoid body cells.

We performed Southern blot analysis on DNA from the spleens of transplanted mice to determine whether the CFU-S were donor ES cell derived. Because individual CFU-S are clonally derived, the sensitivity of blots on spleen DNA should be great enough to detect the presence of ES-derived hematopoietic cells if they have the potential to form CFU-S. Using a probe for Y chromosome-specific sequences to

detect the presence of male ES cells in female 129 recipient mice, we found no positive signal from the donor cells (Fig. 1A) when compared to cells from bone marrow transplant (lanes 1 and 2) and Y chromosome contribution controls. Thus, the CFU-S generated in these mice are not clonally derived from ES cells. We further tested DNA from the recipients by sensitive PCR assay to detect the presence of small numbers of donor cells in the spleens or CFU-S (Fig. 1B). Hematopoietic repopulation analysis of such mice again relied on a Y-chromosome genetic marker, Sry, (Koopman et al., 1991) from the donor ES cells. We found no contribution from the donor differentiated cells.

Long-term repopulation with ES cells

Initial experiments with differentiated ES cells transplanted into lethally irradiated adult mice showed no evidence of classical CFU-S formation or long-term repopulation (Müller and Dzierzak, unpublished data). These adult animals may not be the optimal recipients for embryonic cells since lethal irradiation imposes strong selective pressure on the transplanted cells for rapid expansion and functional hematopoietic activity to promote survival. Therefore, we injected differentiated ES cells into neonatal SCID immunodeficient (Bosma et al., 1983) and W^v/W^v anemic, mast cell deficient (Russell, 1979) mice within 24

Table 1. CFU-S activity of embryoid bodies

Age of embryoid bodies (days)	6	8	9	9	10	11	11	13	13
Age of CFU-S (days)	9	11	10	11	9	9	9	10	11
Unfractionated	0	0	1	0	0	0	0	2	1
	2	0	0	0	1	0	0	3	0
		0	0	0		0		2	0
		0	3	0		0		5	0
								1	
Fraction a	0	0	1	0	3	0	0	4	4
	4	0	0	0	4	0	0	4	1
		0	2	0		0		1	0
		1		0		0		5	
Fraction b	3	0	0	1	0	0	0	3	0
	1	1	0	0	0	0	0	0	0
		1	0	0		0		2	1
		0	0	0		0		2	2
Fraction c	0	0	0	0	0	0	0	0	0
	1	0	0	0	0	0	0	2	0
		0	1	0		0		2	0
		0	0	0		0		3	0
Bone marrow	9	8-10	20	10	9	10	15	20	20
		20	20	10		15		20	20
			20			15		20	20
						10		20	
Irradiation control		0	0	0	0	0	0	0	0
		0	0			1		0	1
		1	0			0		2	0
		0	0			0		1	1
								2	

Each number represents the number of CFU-S per spleen. All spleens analysed for day 10 CFU-S from the experiment with day 13 embryoid bodies, were tested by Southern blotting (Fig. 1A) and some by PCR (Fig. 1B). Mice were injected with the following numbers of disaggregated embryoid body cells. Unfractionated: $0.24\text{--}3.6 \times 10^6$ /animal; fraction a: $1.4\text{--}4.4 \times 10^6$ /animal; fraction b: $0.3\text{--}0.88 \times 10^6$ /animal; fraction c: $0.045\text{--}0.48 \times 10^6$ /animal; BM: 0.2×10^6 /animal.

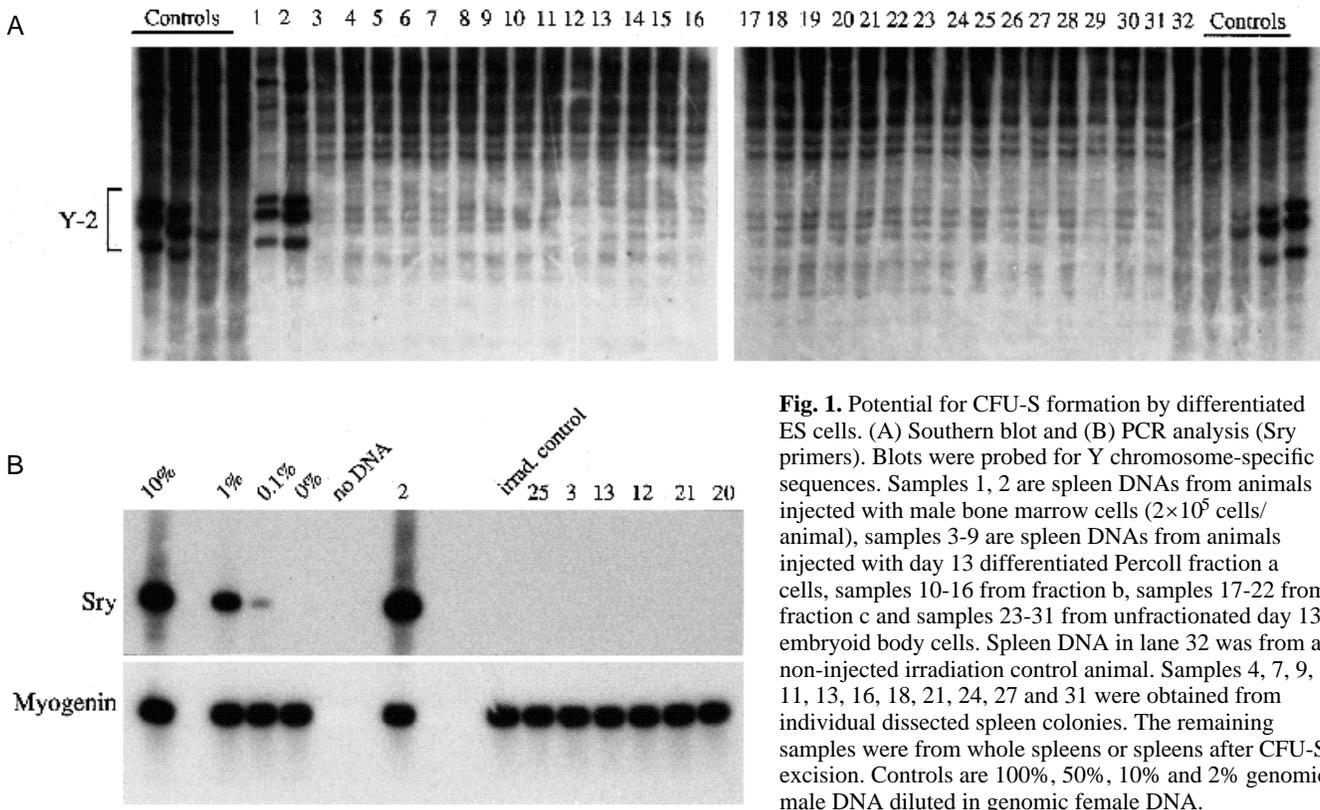


Fig. 1. Potential for CFU-S formation by differentiated ES cells. (A) Southern blot and (B) PCR analysis (Sry primers). Blots were probed for Y chromosome-specific sequences. Samples 1, 2 are spleen DNAs from animals injected with male bone marrow cells (2×10^5 cells/animal), samples 3-9 are spleen DNAs from animals injected with day 13 differentiated Percoll fraction a cells, samples 10-16 from fraction b, samples 17-22 from fraction c and samples 23-31 from unfractionated day 13 embryoid body cells. Spleen DNA in lane 32 was from a non-injected irradiation control animal. Samples 4, 7, 9, 11, 13, 16, 18, 21, 24, 27 and 31 were obtained from individual dissected spleen colonies. The remaining samples were from whole spleens or spleens after CFU-S excision. Controls are 100%, 50%, 10% and 2% genomic male DNA diluted in genomic female DNA.

hours of birth (Capel et al., 1989). Neonatal mice provide a fetal liver hematopoietic environment (Rossant et al., 1986) which might bridge a developmental gap between an embryonic hematopoietic cell and an adult environment. Since only 50% of the neonates we injected were female, in addition to the Sry genetic marker from the donor ES cells, we also used the HSV1-TK marker gene, which we transfected into the donor ES cells. We analyzed the peripheral blood DNA of numerous W^v/W^v female recipients for PCR generated Sry-specific signal. In three out of six injected animals at 2.5 months of age, we observed positive signal around the 0.1% level (Fig. 2A, lanes 4, 5 and 6). Using PCR analysis specific for the TK donor marker in other W^v/W^v recipients, positive signal of 0.1% or greater was seen in the peripheral blood DNA in six out of fourteen mice analyzed at 2-3 months post-injection (Fig. 2B). When compared to DNA standards (using mixes of donor ES cell DNA and normal mouse DNA in a quantitative PCR assay) and myogenin internal controls, we found that the TK signal varied between mice and produced a maximum of 4.4% contribution (Lane 9). The numbers of donor cells necessary to produce these PCR signals (0.1-4.4%) would require at least a 10-fold expansion of the total number of injected cells.

In order to establish whether repopulation is long term, as persistent reconstitution is a definitive characteristic of adult and fetal hematopoietic stem cells (Jordan and Lemischka, 1990), we performed sequential testing of the peripheral blood DNA of recipient animals over a period of 6.5 months. While a few mice initially possessed ES cell donor-derived PCR signal in peripheral blood until 1.5 months posttransplantation, other mice became positive after about 3.5

months (Fig. 3). Most interestingly, some of these mice remained positive after 4-6.5 months, suggesting reconstitution by hematopoietic precursor/stem cells. The level of ES cell donor-derived signal varied between recipients ranging from 0.1-4% of total circulating nucleated cells.

Since we were routinely able to detect the low level presence of donor ES cells in recipient animals, we wanted to establish the frequency of repopulation and determine whether the length of time of ES cell differentiation had an effect on donor repopulation. Of 39 W^v/W^v newborn recipients analysed by PCR for donor ES cell markers, 19 were donor positive in peripheral blood DNA (Table 2) resulting in approximately a 50% frequency of repopulation. All days from day 5 to day 22 of *in vitro* ES cell differentiation produced repopulating activity (see Table 2). However, days 11 through 13 gave the peak of repopulating activity with 10 out of 14 animals positive (71%). We were unable to determine if day 5 is the earliest time point for this activity since earlier days of differentiation often contain undifferentiated ES cells leading to tumorigenesis. Large lung and ovarian tumours were consistently found at about 6 weeks after injection of undifferentiated or day 3 differentiated ES cells. However, no tumours were observed in any animals when day 5 and later differentiated ES cells were injected, demonstrating that the limited repopulation is a normal characteristic of the ES cell-derived precursors.

Tissue and cell lineage distribution of ES-derived hematopoietic cells

The hematopoietic organs of recipient animals were examined for the presence of ES cell-derived donor cells.

We took various approaches to the identification and characterization of these cells. The first analysis was performed by PCR on DNA from various hematopoietic tissues, from macrophage and mast cell cultures and from splenic T or B cells after mitogenic expansion in culture. As shown in Fig. 4A, when the tissues from four W^v/W^v mice were examined, the bone marrow and peripheral blood from two animals

were positive, thymus from another, and spleen and bone marrow from the fourth mouse. When non-adherent bone marrow cells from 5 W^v/W^v mice (PCR positive in peripheral blood) were expanded in mast cell cultures, no donor signal was observed (data not shown). In another five W^v/W^v mice positive in peripheral blood, peritoneal macrophages were cultured. Again, no donor signal was observed (data not shown). However, when previously untested SCID mice were examined (Fig. 4B), we found one in four mice positive for donor signal in mitogen activated T cells cultured from splenocytes and one in four animals positive in mitogen stimulated splenic B cells. Thus, ES-derived cells can be found in most hematopoietic tissues of injected mice and include mature hematopoietic cells of the B and T lymphoid lineages but not macrophage and mast cell lineages.

We also performed FACS analysis on cells from SCID recipients using an antibody specific for the donor histocompatibility type and antibodies specific for hematopoietic cell markers B220, Thy-1, CD4 and CD8. As shown in Fig. 5A, the peripheral blood of a SCID animal was analyzed for CD4 and CD8 positive cells 41 days after injection of day 13 differentiated ES cells. We observed 2.2% of mononuclear cells positive for CD4 and 1.0% positive for CD8. These percentages correspond very well with the 1.9% CD4 and 0.9% CD8 cells found in a control SCID littermate 41 days after a transplant of normal 129 bone marrow cells. Control SCID mice that did not receive cells were negative for both markers at 41 days. Another set of SCID recipient mice was analyzed 8 weeks after injection of Percoll fraction c, day 7 differentiated ES cells (Fig. 5B). In this set of animals, we utilized the H-2 difference between SCID recipient mice ($H-2K^d$) and donor ES cells ($H-2K^b$) to detect donor cells. When using antibodies to $H-2K^b$ and

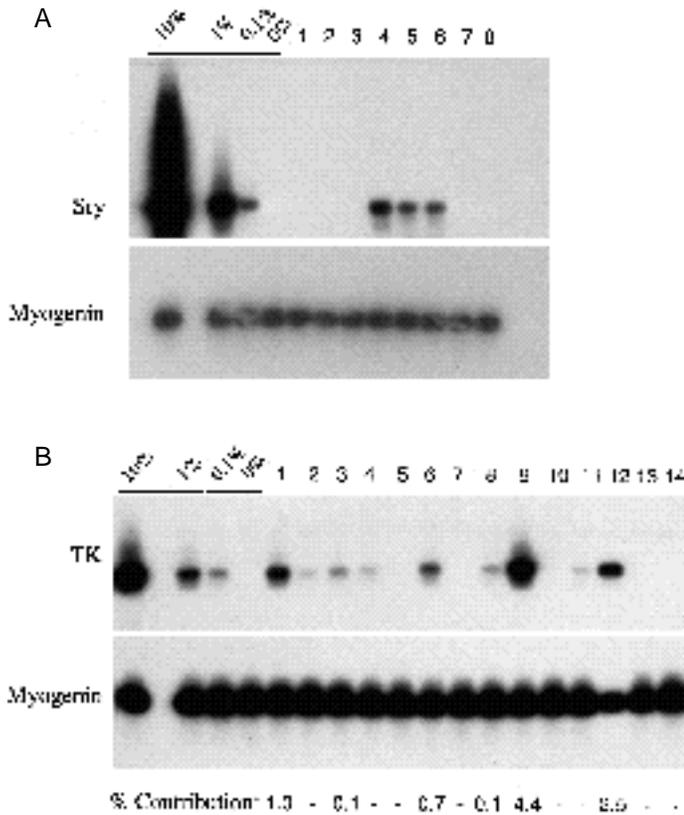


Fig. 2. Analysis of the peripheral blood from injected W^v/W^v animals. (A) PCR analysis (Sry primers and myogenin primers) of peripheral blood DNA at 2.5 months posttransplantation. Animals were injected as newborns with the following AB-1 ES cells. lane 1: no cells; lane 2: day 12, fraction b; lane 3: day 12, fraction c; lane 4: day 12, fraction c; lane 5: day 16, fraction b; lane 6: day 14, fraction a; lane 7: day 14, fraction b; lane 8: no cells. The percentages of contributing male signal are as follows. lane 4: 0.2%; lane 5: 0.1%; lane 6: 0.1%. (B) PCR analysis (HSV1-TK and myogenin primers) of peripheral blood DNA 2-3 months posttransplantation. Mice were injected as newborns with the following disaggregated AB-1.6 embryoid body cells. lane 1: day 22, fraction a; lane 2: day 18, fraction a; lane 3: day 18, fraction b; lane 4: day 18, fraction c; lane 5: day 5, unfractionated; lane 6: day 5, fraction a; lane 7: day 5, fraction b; lane 8: day 5, fraction c; lane 9: day 12, fraction c; lane 10: day 16, unfractionated; lane 11: day 16, fraction b; lane 12: day 16, fraction b; lane 13: day 16, fraction c; lane 14: day 9, fraction c. Each sample was analysed by PCR at least three times. Autoradiogram of a Southern blot after gel electrophoresis is shown. The contribution standards were derived by diluting genomic male into genomic female DNA. The percentage contribution of ES cell-derived signal in the peripheral blood was quantitated by PhosphorImager and are as indicated. The 20 animals analyzed here are included in the 39 W^v/W^v mice in Table 2.

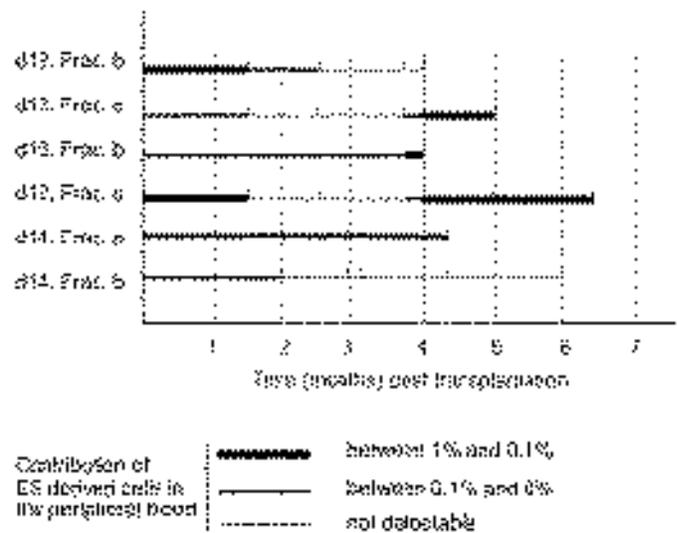


Fig. 3. Sequential repopulation analysis of the peripheral blood by ES cell-derived cells as a function of time posttransplantation. Peripheral blood DNA samples were either analysed by Sry/myogenin- or HSV1-TK-/myogenin-specific PCR. The thickness of the bars represent the ES cell contribution (AB-1 or AB-1.6) in the peripheral blood of injected W^v/W^v mice. The short vertical lines along the top of each line designate the sampling point.

Table 2. Effect of age on repopulation with disaggregated embryoid body cells.

Age of embryoid bodies	Mice positive/total
d5-d10	1/7
d11-d13	10/14
d14-d16	5/12
d17-d22	3/6

W^v/W^v mice were injected with ES cells and peripheral blood DNA was analysed by PCR. Injected cell numbers ranged from 0.8×10^5 to 3.6×10^6 . Animals were tested at different time points after injection, starting at 1 month up to 6.5 months and were included as positive if they showed at one or more time points a positive Sry and/or HSV1 TK PCR product.

B220 on lymph node cells, the control SCID mouse that received no cells showed only 1.4% double staining cells. However, 6.2% double positive staining cells were found in the SCID animal which received ES cells. Although the ES cells can contribute to the B220 positive population found in lymph nodes they were not as efficient as control 129 bone marrow injected into a littermate, which produced 29.6% double staining cells. Interestingly, when another SCID recipient was examined for ES cell-derived repopulation, double staining cells positive for Thy-1 and H-2K^b (7%) were observed in the bone marrow (data not shown). Thus, the presence of mature lymphocytes derived from donor ES cells suggest that these embryonic cells have the

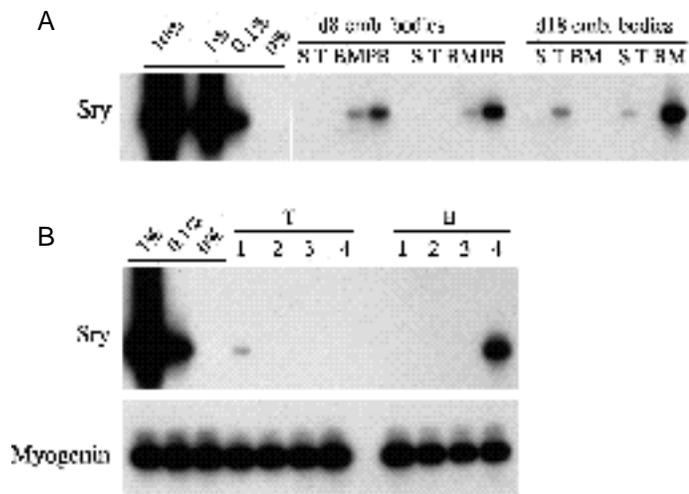


Fig. 4. Repopulation of hematopoietic tissues by ES cell-derived cells. (A) PCR analysis (Sry primers) of hematopoietic tissues from W^v/W^v mice injected with day 8 (D3) and 18 differentiated (AB-1) ES cells. Animals were tested 4 weeks post-injection in spleen (S), thymus (T), bone marrow (BM) and peripheral blood (PB). (B) PCR analysis (Sry and myogenin primers) of cultured splenocytes from injected newborn SCID animals. Splens were harvested 2 - 2.5 months after transplantation of AB-1 embryoid body-derived cells. Newborn SCID mice were injected with the following disaggregated embryoid body cells. mouse 1: day 17, fraction c; mouse 2: day 9, fraction b; mouse 3: day 9, unfractionated; and mouse 4: day 13, fraction c. These animals were not previously tested by PCR for donor signal in peripheral blood DNA. Splenocytes were either grown under B cell or T cell mitogenic growth conditions. Genomic DNA was prepared after mitogen stimulation in culture.

potential to progress through lymphoid differentiation programs.

Finally, as evidence of functional lymphopoietic cell repopulation we tested the peripheral blood of a transplanted adult SCID mouse for circulating antibody. Ouchterlony immunodiffusion analysis demonstrated the presence of donor allotype antibody (Igh^a) in the serum of a mouse transplanted with day 13 differentiated ES cells (Fig. 6, well 3). The combined possibility of this SCID mouse being leaky for immunoglobulin production and cross reactivity of the antiserum was discounted by Ouchterlony analysis with an antiserum specific for the Igh^b SCID allotype. No immunoglobulin of the Igh^b type was detected by this antiserum (Fig. 6, well 10). Thus, differentiated ES cells are capable of producing functional B lymphocytes in vivo.

DISCUSSION

We have examined the potential of normal differentiated ES cells to repopulate the hematopoietic system in vivo after adoptive transfer into neonatal SCID and W^v/W^v and lethally irradiated adult mice. Using donor genetic markers and hematopoietic cell surface markers, we have demonstrated that ES cells are capable of producing cells of the lymphoid compartment but not CFU-S or cells of the macrophage or mast lineages, that ES cells repopulate the peripheral hematopoietic system only to a very limited extent (at best 4-6%), and that low level repopulation can persist for greater than 6.5 months (most recently, for greater than 15 months, unpublished results). This is in direct contrast to expectations from in vitro results demonstrating the production of erythroid, myeloid and lymphoid cells from differentiating ES cells (Wiles and Keller, 1991, Schmitt et al., 1991; Chen et al., 1992) and the complete repopulation of lethally irradiated mice with cells from cultured blastocysts (Hollands, 1988). The demonstration here of only limited in vivo repopulation ability of in vitro differentiated ES cells may be explained in several ways: (i) as a result of injection of embryonic cells into mice with inappropriate fetal/adult microenvironments, (ii) by the absence of embryonic growth factors, (iii) by the fact that the ES cell lines used in these studies possess a limited in vitro differentiation potential despite the fact that our ES cell lines generate germ line chimeric animals with a normal hematopoietic system (M. Lindenbaum, P. P. Pandolfi and F. Grosveld, personal communication) or (iv) that there is a fundamental difference between embryonic and fetal/adult hematopoietic stem cells.

First, it is likely that the microenvironment in the recipient mice plays an important role in the development of hematopoietic cells with full potential. Our use of adult animals certainly did not provide an environment stimulatory for full and complete engraftment with ES cells, as determined by the lack of CFU-S and long term repopulation. Also, conditions for repopulation of lethally irradiated adult mice require activity of many precursors and mature cells in the hematopoietic hierarchy that may be lacking in the injected ES cell populations. We therefore used hemato/lymphopoietic deficient mice so as to avoid the selective pressure imposed by lethal irradiation. Neonatal

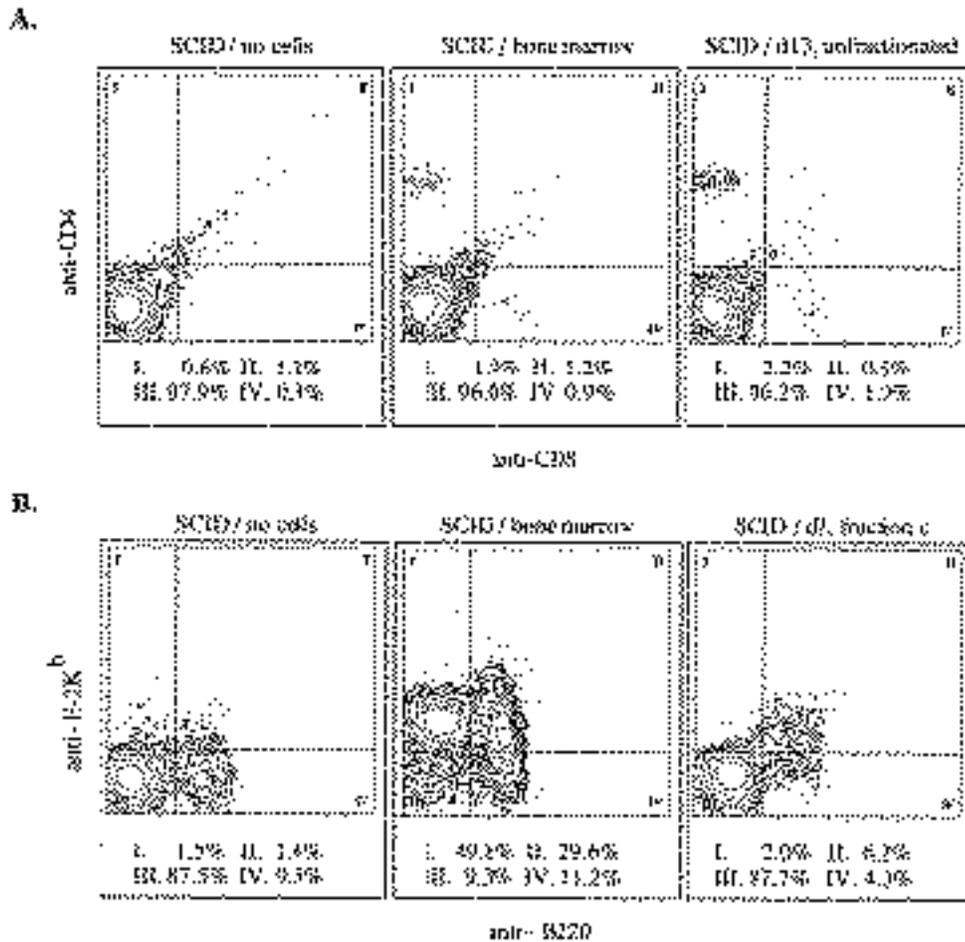
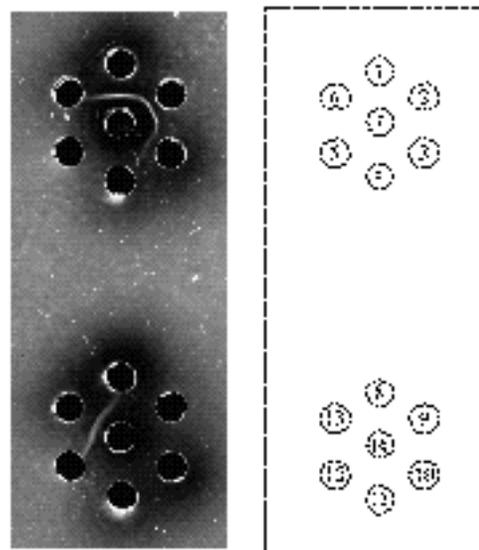


Fig. 5. Cell surface characteristics of donor ES cell-derived hematopoietic cells. (A) Surface antigen phenotype of lymphocytes from SCID mice after injection of disaggregated embryoid body cells. Newborn SCID mice were injected with day 13 differentiated, unfractionated CCE ES cells (0.5×10^6) or 129 BM cells (0.5×10^6). Peripheral blood lymphocytes were analysed 1.5 months after transplantation. (B) Day 7 differentiated AB-1 Percoll fraction c cells (0.28×10^6) or 129 BM cells (1×10^6) were injected into newborn SCID mice. Lymph node cells were analysed 2 months after transplantation. Non-injected (no cell) control SCID mice (age matched or littermates) are shown. The percentage of positive stained cells in each quadrant is indicated.

animals were injected to take advantage of the fetal liver microenvironment still present at this stage. However, ES cells still did not fully or completely engraft, although our detection procedures for donor engraftment (more sensitive than the conventional marker systems used previously) allowed us to detect up to 4-6% ES-derived cells in the hematopoietic system of recipient mice. Low levels of engraftment may be due to an inefficiency in the neonatal injection procedure (bone marrow controls ranged from 0.1% to 30% repopulation), limitations related to the severity of the W-locus genetic defect (the W^v mutation is of moderate severity; Fleischman et al., 1982) or differences in haplotype or in cell number injected (Bix et al., 1991).

However, it is interesting to note that when yolk sac cells were injected transplacentally into embryos or fetuses (Toles et al., 1989; Weissman et al., 1977) high levels of repopulation with embryonic cells were observed. Thus, it is possible that neonatal stage animals cannot promote full ES-derived hematopoietic engraftment and it may be necessary

Fig. 6. Donor ES cell-derived immunoglobulin in peripheral blood. Ouchterlony immunodiffusion analysis was performed on serum from a SCID animal transplanted with day 13 differentiated Ficoll fractionated D3 ES cells. Reactivity with anti-Ig^a (well 7) or anti-Ig^b (well 14) antiserum was tested on SCID serum samples and various control sera; wells 1 and 8 contained serum from a normal uninjected 129 mouse (Ig^a control), wells 2 and 9 contained 1/5 diluted serum from a 129 mouse, wells 3 and 10 contained serum from the SCID animal transplanted with day 13 differentiated ES cells, wells 4 and 11 contained serum from an age matched non-injected SCID animal, wells 5 and 12 were PBS controls and wells 6 and 13 contained serum from a normal uninjected C57Bl/10 animal (Ig^b control).



to transplacentally inject ES cells into embryos to observe improvements in repopulation.

The importance of growth factors in some transplantation protocols has been demonstrated by the recent xenografting of human bone marrow into SCID mice (Lapidot et al., 1992; Kyoizumi et al., 1992). Initially, repopulation frequencies were low and averaged about 1%, but when human hematopoietic tissues were implanted or hematopoietic growth factors were injected in addition to the cells, a dramatic increase in stimulation of multilineage hematopoiesis was observed. Hence, cytokine injection may influence in vivo repopulation with ES cells, although addition of cytokines early in the in vitro ES cell differentiation systems of others (Wiles and Keller, 1991; Burkert et al., 1991) had only marginal effects. Since the hematopoietic cells are of the embryonic stage of development, they may require as yet unknown cytokine(s) produced only in the embryo. Thus, our protocol for the repopulation of mice with normal, untransformed differentiated ES cells may provide a functional assay system for the isolation and testing of novel embryonic hematopoietic growth factors.

Alternatively, the possibility exists that the ES cells used in these studies may have limited differentiation potential since ES cells have been known to change some characteristics when cultured for extended periods of time. In these experiments we have used low passage ES cells which many investigators have demonstrated to produce in vitro hematopoietic colonies as well as germline chimeric mice. Most importantly, the AB1 cells in most of our studies have been used by others in our laboratory for production of germline chimeras. These animals are completely normal in their hematopoietic system, particularly in the generation of macrophages and mast cells (which we find lacking in adoptive transfer recipients reported here).

Finally, the limited repopulation levels we have observed may be a true characteristic of an embryonic class of hematopoietic precursor. In short term repopulation assays, ES cells do not form classical CFU-S. Interestingly, the potential for early embryonic yolk sac cells to form CFU-S is controversial (Samoylina et al., 1990). At best, the frequency of such embryonic precursors is low in comparison to the frequencies in fetal liver and bone marrow (Moore and Metcalf, 1970; Perah and Feldman, 1977). Therefore, it is not clear whether ES cells should be expected to form CFU-S.

In our long term experiments, the low levels of ES-derived cells in the recipient mice are positive for T and B lymphoid surface markers (CD4, CD8, Thy-1 and B220), produce immunoglobulin and can be expanded under lymphocyte mitogenic conditions. This developmental potential of normal differentiated ES cells for the lymphoid lineages in vivo appears consistent with the in vitro lymphoid potential of yolk sac cells demonstrated by others (Liu and Auerbach, 1991; Ogawa et al., 1988). Thus, the long term persistence of ES-derived hematopoietic cells in the recipient animals suggests the presence of an early lymphoid precursor in the injected ES cell population, although we cannot exclude the possibility that more differentiated and long lived lymphoid cells are present in this population. However, in contrast to results of in vitro cultures and blood island formation of ES cells, we find no evidence of

macrophage and mast cell lineages in vivo. This is interesting in the light of experiments defining the origins of hematopoiesis in the avian system (Dieterlen-Lievre, 1975) where yolk sac hematopoiesis is limited and supplies only the developing embryo (not fetal liver or adult bone marrow) with hematopoietic cells. ES cells may provide only such transient hematopoietic activity and thus, may not be able to contribute to hematopoiesis after adoptive transfer in vivo.

While this is the first time that normal differentiated ES cells have been demonstrated to contribute to low level, lymphoid restricted hematopoiesis in vivo without generation of tumours, their limited repopulation potential do not make them an easily accessible source of pluripotential hematopoietic cells. For the rapid examination of ES cells that have undergone targeted mutagenesis events in hematopoietic-specific genes, the method of Nagy et al. (1990) and Forrester et al. (1991) is the most advantageous. The further use of ES cells in the study of the embryonic hematopoietic system will be aided by examination and transplantation of embryonic tissues and comparisons of repopulating activities. Such experiments are underway utilizing various genetic and clonal markers and may provide insight into the limited low levels of repopulation of embryonic cells.

We thank F. Grosveld and M. Lindenbaum for useful discussions and critical comments, M. Lindenbaum, B. Daly and B. Capel for help with early transplant experiments, C. Atkins for excellent flow cytometry, C. Miles for initial help with the PCR analysis, R. Rossi for assistance in tissue culture and Animal Services for providing newborn litters. Our appreciation also to D. Dresser for Igh allotype-specific antiserum and P. Koopman and J. Gubbay for PCR probes. Also, we thank I.R. Lemischka and F. Grosveld for critical comments on the manuscript. This work is supported by an EMBO longterm fellowship (A. M. M.) and the Medical Research Council, UK.

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