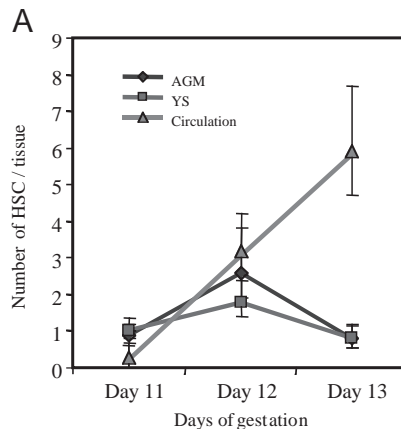CORRIGENDUM

Quantitative developmental anatomy of definitive haematopoietic stem cells/long-term repopulating units (HSC/RUs): role of the aorta-gonad-mesonephros (AGM) region and the yolk sac in colonisation of the mouse embryonic liver

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There is an error in Fig. 1A of this article. The correct version of the figure is printed below.

The authors apologise to readers for this mistake.

Quantitative developmental anatomy of definitive haematopoietic stem cells/long-term repopulating units (HSC/RUs): role of the aorta-gonad-mesonephros (AGM) region and the yolk sac in colonisation of the mouse embryonic liver

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SUMMARY

In the developing mouse embryo the first definitive (transplantable-into-the-adult) haematopoietic stem cells/long-term repopulating units (HSC/RUs) emerge in the AGM region and umbilical vessels on 10-11 days post coitum (d.p.c.). Here, by limiting dilution analysis, we anatomically map the development of definitive HSC/RUs in different embryonic tissues during early colonisation of the liver. We show that by day 12 p.c. the mouse embryo contains about 66 definitive HSC/RUs (53 in the liver, 13 in other tissues), whereas on the previous day the total number of definitive HSC/RUs in the entire conceptus is only about 3. Owing to the length of the cell cycle this dramatic increase in the number of definitive HSC/RUs in only 24 hours is unlikely to be explained purely by cell division. Therefore, extensive maturation of pre-definitive HSCs to a state when they become definitive must take place in the day 11-12 embryo. Here we firstly identify the numbers of HSCs in various organs at 11-13 d.p.c. and secondly, using an organ culture approach, we quantitatively assess the potential of the aorta-gonad-mesonephros (AGM) region and the yolk sac to produce/expand definitive HSC/RUs during days 11-12 of embryogenesis. We show that the capacity of the AGM region to generate definitive HSC/RUs is high on 11 d.p.c. but significantly reduced by 12 d.p.c. Conversely, at 12 d.p.c. the YS acquires the capacity to expand and/or generate definitive HSCs/RUs, whereas it is unable to do so on 11 d.p.c. Thus, the final steps in development of definitive HSC/RUs may occur not only within the AGM region, as was previously thought, but also in the yolk sac microenvironment. Our estimates indicate that the cumulative activity of the AGM region and the yolk sac is sufficient to provide the day 12 liver with a large number of definitive HSC/RUs, suggesting that the large pool of definitive HSC/RUs in day 12 foetal liver is formed predominantly by recruiting 'ready-to-use' definitive HSC/RUs from extra-hepatic sources. In accordance with this we observe growing numbers of definitive HSC/RUs in the circulation during days 11-13 of gestation, suggesting a route via which these HSCs migrate.

Movie available on-line

Key words: Haematopoietic stem cells, AGM region, Yolk sac, Liver, Mouse

INTRODUCTION

Embryonic development of the mammalian haematopoietic system is complex and, in many aspects, poorly understood. Two major sources of haematopoietic activity have been identified in vertebrate embryos. For a long time the yolk sac (YS), where haematopoietic activity is first observed, was assumed to be the primary site of formation of the haematopoietic stem cells (HSCs) that migrate to and colonise the foetal liver and subsequently the bone marrow (Moore and Metcalf, 1970). However, identification of a powerful intra-embryonic HSC activity in avian, amphibian and murine embryos cast doubts on this assumption. In experimentally engineered chick-quail chimeras, haematopoiesis in the adult organism was found to originate from the body but not from the YS of the chimera, suggesting that the YS/embryonic haematopoietic hierarchy is transitory (Dieterlen-Lievre, 1975; Martin et al., 1979). In amphibian embryos the dorsal lateral plate (DLP) mesoderm next to the dorsal aorta contributes mainly to definitive haematopoiesis, whereas the ventral blood island (VBI), which is equivalent to the YS, contributes to both primitive and to some extent definitive haematopoiesis (Chen

and Turpen, 1995). Recent experiments on amphibian embryos, using individual blastomere labelling techniques, have demonstrated that although at the gastrulation stage both DLP and VBI originate from a common ventral bipotential mesoderm layer (Turpen et al., 1997) their precursors are spatially separated at the blastula stage (Ciau-Uitz et al., 2000). Relatively recently an intra body site of definitive haematopoietic activity has been identified in the mouse embryo prior to definitive haematopoiesis in the liver (Godin et al., 1993; Medvinsky et al., 1993; Muller et al., 1994; Cumano et al., 1996; Medvinsky and Dzierzak, 1996; Medvinsky et al., 1996). Before the onset of organogenesis and the establishment of the circulatory system in the mouse embryo, this site, termed the visceral para-aortic splanchnopleura (P-Sp) region, but not the YS, contains multipotent lymphomyeloid progenitors, although at this stage they are incapable of reconstituting adult recipients (Cumano et al., 1996; Cumano et al., 2001). During organogenesis, part of the splanchnopleura transforms into a morphologically distinct composite axial structure consisting of the dorsal aorta, genital ridges and mesonephroi (AGM region). High numbers of spleen-colony forming units (CFU-S) are concentrated in the AGM region prior to the presence of CFU-S activity in the liver (Medvinsky et al., 1993; Medvinsky and Dzierzak, 1996). The first definitive (long-term reconstituting) HSCs appear in the AGM region and umbilical vessels at late 10/early 11 d.p.c. (Muller et al., 1994; Medvinsky and Dzierzak, 1996; de Bruijn et al., 2000). The AGM region is the only tissue in the 10 d.p.c. mouse embryo capable of the autonomous initiation/expansion of definitive HSCs, as demonstrated using an organ culture approach. Slightly later, by 11 d.p.c., HSCs appear both in the liver rudiment and the YS but again only the AGM region is capable of expanding the number of HSCs in organ culture conditions (Medvinsky et al., 1996). These and other features of AGM biology, including the kinetics of CFU-S and HSC development, led to the hypothesis that the AGM region is the primary site of formation of definitive HSCs which then colonise secondary haematopoietic organs, primarily the foetal liver (Dzierzak and Medvinsky, 1995; Dzierzak and Medvinsky, 1998). Recently, more compelling evidence has emerged that indicates HSCs originate in the body of the embryo independently of YS activity. Indeed, as early as day 7 p.c., before circulation is established in the mouse embryo, the P-Sp but not the YS contains ancestors of cells that are capable of long-term, multi-potential repopulation of adult irradiated recipients devoid of NK cells (Cumano et al., 2001). Experiments on human embryos also revealed dramatic differences in the lymphohaematopoietic potential of the dorsal aorta and the YS (Tavian et al., 2001). Upon transplantation into NOD-SCID mice, cultured dorsal aorta cells showed lymphomyeloid reconstitution whereas YS cells were only capable of contributing to the myeloid lineage. Cumulatively, these data from several different research groups point to the embryo body as the site of origin of definitive HSCs.

The above data does not, however, rule out a possibility that at later stages the YS is involved in the independent production or expansion of definitive HSCs. Although early YS cells are unable to repopulate adult irradiated recipients upon direct transplantation, when transplanted into the embryo they can contribute to adult haematopoiesis (Weissman et al., 1978; Toles et al., 1989). Analogous results have been achieved by transplantation of YS cells into newborn recipients (Yoder and Hiatt, 1997; Yoder et al., 1997). In addition, by day 8 p.c. both the P-Sp and the YS contain cells that can mature into definitive HSCs by co-culture with an AGM-derived stromal cell line (Matsuoka et al., 2001). Thus at least from day 8 p.c. the YS contains cells (pre-definitive HSCs), which are capable of development into definitive HSCs upon maturation in an embryonic or newborn microenvironment (Medvinsky and Dzierzak, 1999). However, it remains unclear if and when during normal embryo development these cells mature into definitive HSCs and whether they have to migrate to an inductive AGM microenvironment in order to do so.

In our previous papers we focused on the initiation of definitive HSC production in the mouse embryo (Muller et al., 1994; Medvinsky and Dzierzak, 1996). Here we explore the subsequent stage of HSC development from day 11 until day 13 when the number of definitive HSCs increases in the embryo. From day 11 p.c. onwards the number of HSC/RUs increases dramatically in the liver (Morrison et al., 1995; Ema and Nakauchi, 2000) but little is known about their distribution in the rest of the embryo, the routes of their migration and the mechanisms underlying their expansion in the liver. Various types of multipotent, pluripotent and bi-potent myeloid and lymphoid progenitors have been identified during embryogenesis in the YS, AGM region, liver, thymic and splenic rudiments (Moore and Metcalf, 1970; Velardi and Cooper, 1984; Johnson and Barker, 1985; Wong et al., 1986; Eren et al., 1987; Liu and Auerbach, 1991; Cumano et al., 1992; Morrison et al., 1995; Ema et al., 1998; Kawamoto et al., 1998; Nishikawa et al., 1998; Liu et al., 1999; Ohmura et al., 2001; Palis et al., 2001; Traver et al., 2001; Douagi et al., 2002) and different types of progenitor cells are disseminated via the circulation (Moore and Metcalf, 1970; Johnson and Barker, 1985; Rodewald et al., 1994; Delassus and Cumano, 1996). However, as the links within the haematopoietic hierarchy and between tissues are unclear, it is difficult to resolve an entire anatomical picture of the development of definitive HSCs. Although data from some publications has revealed fragments of it (Moore and Metcalf, 1970; Ikuta et al., 1990; Morrison et al., 1995; Berger and Sturm, 1996; Sanchez et al., 1996; de Bruijn et al., 2000; Ema and Nakauchi, 2000; Hsu et al., 2000; de Bruijn et al., 2002; North et al., 2002), a comprehensive quantitative anatomical map of HSC/RUs development during embryogenesis has not been produced. Here, we have attempted to create a temporal and spatial map of development of definitive HSC/RUs in the 11-13 d.p.c. mouse embryo. To this end, the number of HSC/RUs in different embryonic tissues has been estimated using a limiting dilution method (Szilvassy et al., 1990). In addition, the potential of the AGM region and the YS to produce and/or maintain definitive HSC/RUs has been assessed using an organ culture approach (Medvinsky and Dzierzak, 1996).

We have found that expansion of the pool of HSC/RUs within the liver occurs concurrently with increasing numbers of HSC/RUs in the circulation. In addition to the previously reported activity of the AGM region during day 10-11 p.c. (Medvinsky and Dzierzak, 1996; de Bruijn et al., 2000), we report here that a day later, at 12 d.p.c., the YS becomes competent to generate (and/or expand) definitive HSC/RUs. This finding suggests that both the AGM region and the YS produce HSCs that colonise the developing liver in two

subsequent waves, the peaks of which fall on days 10-11 p.c. and day 12 p.c. respectively.

MATERIALS AND METHODS

Animals and cells

CBA/Ca, C57BL/6 and (CBA×C57BL6) F_1 mice were bred in the animal breeding unit of the University of Edinburgh or purchased from Harlan. Cell suspensions were prepared from non-cultured or cultured 11-13 d.p.c. embryonic tissues after incubation with 0.1% collagenase-dispase (Sigma) in PBS at 37°C. Embryonic blood was collected within 2-3 minutes after separation of the YS from the embryo body and kept on ice before transplantation to preserve HSCs. Special care was taken to remove umbilical and vitelline arteries from preparations of the YS and embryonic circulation. (CBA×C57BL6) F_1 mouse male embryos were used as donors for transplantation into (CBA×C57BL6) F_1 female recipients. In some experiments (CBA×C57BL6) F_1 Ly5.1/2 embryos were used for transplantation into (CBA×C57BL6) F_1 Ly5.2 female recipients. Recipient mice were irradiated at 9.5 Gr split into two doses separated by a 3 hour interval in the Cs source at a rate 21.6 rad/minute. The mice received neomycin (0.16 g/100 ml) in acidified drinking water for the first 4 weeks after transplantation.

Organ culture

Tissues were cultured in myelo-cult medium (Stem Cell Technology) supplemented with 10^{-6} M hydrocortisone hemi-succinate (Sigma) on Durapore 0.65 µm filters (Millipore) supported by stainless steel stands (5% CO_2 in air) at the gas-liquid interface as previously described (Medvinsky and Dzierzak, 1996). 12 d.p.c. foetal liver was explanted in small pieces comparable in size to the AGM region. Cultures were set up for 3-5 days and 7-9 days.

Analysis of donor contribution into recipient haematopoietic system

The contribution of donor cells was assessed 6-8 weeks and 3.5-5 months after transplantation as described previously (Medvinsky and Dzierzak, 1996). Briefly, the percentage of male donor cells in the haematopoietic system of female recipients was assessed by comparison with standards of serially diluted male in female DNA (0.1%, 1%, 10%, 100%). Both test DNA and standards were amplified by PCR using primers specific for male Y2B and mouse myogenin sequences. As previously, we have restricted our analysis to those cells (definitive HSCs), which upon transplantation, contributed at a level of 10% or higher in the haematopoietic system of irradiated recipients (Muller et al., 1994; Medvinsky and Dzierzak, 1996).

Transplantations of day 12 and 13 tissues in some cases were carried out using Ly5.1 embryos and multilineage contribution was assessed in recipient mice by assessing co-expression of the Ly5.1 marker and lineage specific markers by antibody staining and subsequent analysis on FACSCalibur (Beckton-Dickinson). For this purpose biotinylated anti-Mac-1, anti-B220 (secondary stained with Strepdavidin-PE; Sigma), PE-conjugated anti-CD3ε and FITC-conjugated anti-Ly5.1 antibodies (Pharmingen) were used. In some cases the bone marrow of reconstituted mice was transplanted into secondary recipients.

Quantitation of HSC/RUs by limiting dilution analysis

Upon transplantation, one definitive HSC is sufficient to differentiate into all lymphoid and myeloid cell types and to contribute over several months at a high level to the haematopoietic system of an irradiated recipient (Lemischka, 1992; Morrison et al., 1997). In order to estimate the number of HSCs/RU in various organs we have adopted a limiting dilution method by transplanting low numbers of HSCs (several dilutions) into irradiated recipients (Szilvassy et al., 1990).

Test cells were co-transplanted intravenously with $2×10^4$ bone marrow cells to ensure short-term survival of the recipient. The number of HSCs in tested tissues was estimated by Poisson statistics based on the proportion of non-repopulated recipients in long-term (longer than 3.5 months) repopulating experiments. Serial dilutions are expressed in embryo equivalents (e.e.). A minimum of 2 and maximum of 12 different dilutions were used for each tissue. For each dilution between 4 and 21 recipients were transplanted in a minimum of two independent replicate experiments. The final numbers of HSCs were estimated by the maximum likelihood method using Genstat 5 package and expressed as the most probable numbers (MPN) (GenStat 5 Release 3 Reference Manual, 1993). The asymmetric error range in parentheses next to MPN and also showing on the graphs is typical for this kind of analysis which involves confidence interval estimation of the Poisson mean.

Our calculations are based on the assumption that HSCs from different tissues and at different stages of development have an equal seeding efficiency. However, this may not be the case and therefore we, as others (Ema and Nakauchi, 2000), introduce an operational term 'repopulating unit' (RU), which is not necessarily related to a single cell and appears next to HSC abbreviation in the text.

In separate experiments we have found that the $2×10^4$ carrier bone marrow cells injected per recipient contain on average **2 (1.6, 2.6)** long-term repopulating HSC/RUs, which is similar to numbers reported by some other groups (Abkowitz et al., 2000). This may explain why when other researchers transplanted 10 times more bone marrow carrier cells ($2×10^5$) (about 20 HSC/RUs) along with day 11 embryonic liver no liver contribution was detected in recipient mice (Ema and Nakauchi, 2000), as the donor HSCs may have been out competed by an excess of HSCs in the carrier bone marrow.

Assessment of content of circulating HSC/RUs in various embryonic tissues

The number of definitive HSC/RUs in the circulation was assessed in transplantion experiments as described above. The number of HSC/RUs in the circulation quoted in Fig. 1A and table 1 represent only a proportion of all circulating HSC/RU i.e. those that were released upon separation of the YS and the embryo body. The relative proportion of circulatory blood cells in various embryonic tissues was estimated by a comparison of the numbers of red blood cells in the tissues with those in the circulation. To this end haemoglobinized cells from the circulation and cells obtained after trypsinization of dissected tissues were stained with 0-Dianisidine and counted under the microscope (Iuchi and Yamamoto, 1983) as a measure of the contamination of tissues with embryonic blood. The likely contribution of circulating HSC/RUs to the number of HSC/RUs recovered from different embryonic tissues was calculated from the following formula:

$$HSC/RU_t = \frac{RBC_t}{RBC_c}\ HSC/RU_c$$

where HSC/RU_t is the estimated total number of circulatory HSC/RUs in the tissue; HSC/RU_c is the number of HSC/RUs in circulation measured by transplantation; RBC_t is the number of red blood cells in the tissue and RBC_c is the number of RBC in the transplanted circulation.

RESULTS

Tissue distribution of definitive HSC/RUs within 11 d.p.c. embryo

According to Poisson statistics the number of HSC/RUs within the AGM region, YS and the liver of the 11 d.p.c. embryo is close to 1 per tissue (Fig. 1). In the present experiments in

Fig. 1. HSC/RUs in tissues of the developing mouse embryos (total number per tissue). (A) Distribution of HSC/RUs in AGM, YS and the circulation of day 11-13 embryos. (B) HSC/RUs in the fetal liver of day 11-13 embryos. Numbers of HSC/RUs in tissues were estimated using a limiting dilution method and presented as described in Materials and Methods. Numbers of recipient mice (RM) and dilutions (D) used were as follows. (A) 11 d.p.c.: (AGM) 27RM, 2D; (YS) 25RM, 3D; (circulation) 16RM, 3D; 12 d.p.c.: (AGM) 25RM, 7D; (YS) 56RM, 13D; (circulation) 43RM, 9D. (B) Liver (11 d.p.c.) 23RM, 2D; (12 d.p.c.) 37RM, 6D; (13 d.p.c.) 55RM, 12D.
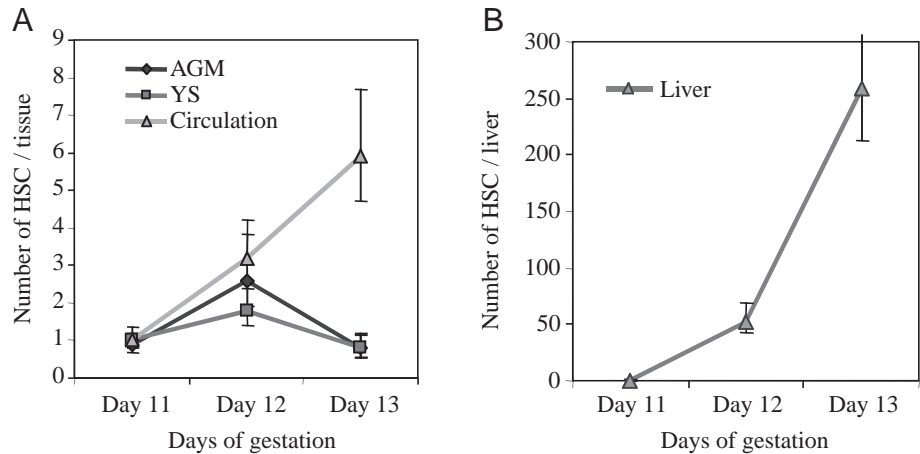
contrast to our previous report (Muller et al., 1994) we are now able to detect some HSC/RUs in 11 d.p.c. embryonic blood, possibly because of improved methods of preservation of embryonic blood cells before transplantation (see Materials and Methods). Bone marrow from primary recipients reconstituted with HSC/RUs from the 11 d.p.c. circulation could be successfully transferred to secondary recipients (data not shown). This finding has revealed a route for the dissemination of HSC/RUs from the AGM region as early as day 11 p.c., which has previously been suggested but never confirmed experimentally. In these present experiments we were not able to reconstitute recipient mice with the body

remnants of day 11 embryos (data not shown) and therefore attribute the previously reported rare cases of reconstitution with body remnants (Muller et al., 1994) to the presence of circulating HSC/RUs and/or the occasional inclusion of umbilical and vitelline vessels into the transplant (de Bruijn et al., 2000).

Tissue distribution of definitive HSC/RUs within 12 d.p.c. embryo

On day 12 p.c. both the AGM region and the YS contain approximately two to three HSC/RUs each, which is higher than on day 11 p.c. (Fig. 1A). At this time the embryonic

Table 1. The comparison of numbers of HSC/RUs found in transplantation experiments by limiting dilution analysis with probable numbers of HSC/RUs in tissues contributed by embryonic circulation*

Embryo age	Tissue	Number of benzidine-positive cells (mean±s.d.)	Estimated circulating number of HSC/RUs/tissue	Actual number** of HSC/RUs/tissue	Number of recipient mice (RM): number of dilutions (D)
Day 12	Bled circulation	$(92\pm11)\times10^4$	–	3.2 (2.6, 4.2)	43:9
	AGM	$(0.8\pm0.0)\times10^4$	0.02	2.7 (1.9, 3.7)	25:7
	YS	$(21\pm4)\times10^4$	0.7	1.8 (1.4, 2.4)	56:13
	Liver	$(131\pm12)\times10^4$	4.4	53 (43, 69)	37:6
	Body (w/o liver)	nd	nd	12.1 (9.1, 19.3)	15:2
	Body (w/o liver, AGM, PB)	$(48\pm6)\times10^4$	1.6	5.8 (4.4, 8.2)	17:3
	Cord	nd	nd	0.8 (0.6, 1.8)	7:2
	Spleen	nd	nd	0	7:1
	Thymus	nd	nd	0	2:1
	Lung	$(1.9\pm0.1)\times10^4$	0.1	0.4 (0.2, 0.6)	17:6
	Limb	nd	nd	0.5 (0.3, 0.9)	7:2
	Heart	$(4.6\pm3.0)\times10^4$	0.2	0	2:1
	Head	$(18\pm3)\times10^4$	0.6	0.5 (0.4, 0.8)	11:3
Day 13	Bled circulation	$(139\pm22)\times10^4$	–	5.9 (4.7, 7.7)	49:12
	AGM	$(1.5\pm0.3)\times10^4$	0.05	0.8 (0.6, 1.2)	17:7
	YS	$(24\pm2)\times10^4$	0.8	0.8 (0.6, 1.2)	21:5
	Liver	$(205\pm20)\times10^4$	NA	260 (212, 320)	55:12
	Body (w/o liver)	$(51\pm11)\times10^4$	1.8	5.6 (4.0, 7.8)	20:3
	Cord	nd	nd	nd	nd
	Spleen	nd	nd	0.2 (0.1, 0.4)	12:4
	Thymus	nd	nd	0	12:3
	Lung	$(2.0\pm1.0)\times10^4$	0.1	0	3:1
	Limb	0.1×10^4	0	1.0 (0.8, 1.6)	11:2
	Heart	$(6.8\pm0.4)\times10^4$	0.2	0	3:1
	Head	$(11\pm3)\times10^4$	0.4	(0.2)***	5:1

*Circulatiing and actual numbers of HSCs/RUs per tissue were assessed and presented as described in the Materials and Methods.

**More than 1e.e. was transplanted per recipient in experiments with only one dilution. Tissues in which no HSC/RUs were detected at least one dilution was more than 1e.e.

***One recipient was found reconstituted. Numbers of HSC/RUs in tissues are estimated using a limiting dilution method and presented as described in Materials and Methods.
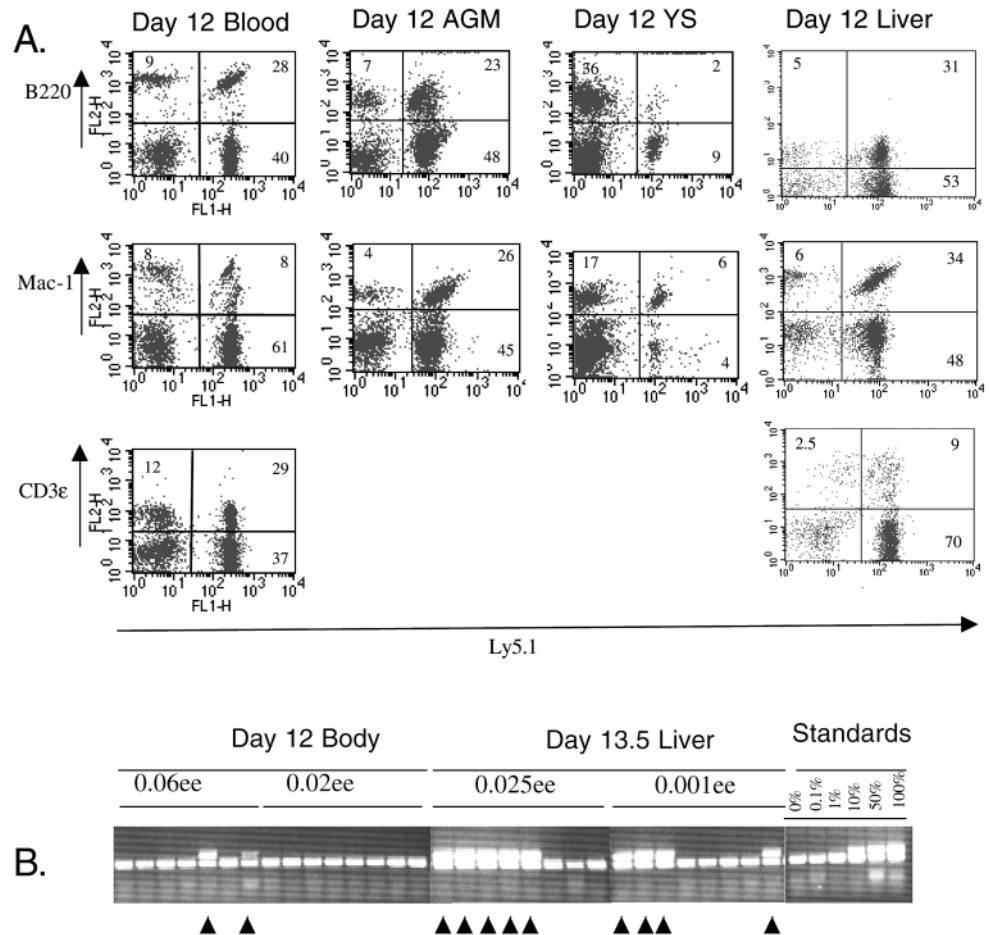
Fig. 2. Multilineage repopulation with
12 d.p.c. embryonic tissues. The
analysis of reconstitution of mice
transplanted with embryonic tissues
was performed 4 months or longer
after transplantation either by FACS
analysis (A) or using Y-specific PCR
as described previously (B)
(Medvinsky and Dzierzak, 1996).
(A) Multilineage reconstitution with
12 d.p.c. cells of embryonic tissues
was confirmed in some recipients by
co-staining for donor Ly5.1 and
lineage-specific B220, Mac-1 and
CD3ε markers. In some reconstituted
mice the percentage of Ly5.1+, Mac-
1+ cells was very low (data not
shown). (B) Y-specific PCR analysis
of blood samples. Each line on the gel
represents an individual mouse. The
level of reconstitution was assessed
with reference to results of PCR using
standard dilutions of male DNA in
female DNA. Arrowheads point to the
mice reconstituted at the level of close
to 10% or more.

circulation also contains about three HSC/RUs. The significant
numbers of HSC/RUs in the circulation suggests intensive
trafficking of HSC/RUs within the embryo. By day 12 p.c. the
number of HSC/RUs in the embryonic liver reaches **53 (43,69)**
thus increasing approximately 50-fold from day 11 p.c.
Multilineage contribution to recipient mice was confirmed by
analysis of selected mice (Fig. 2).

Estimates of possible HSC/RUs numbers derived from the
circulation in these organs showed that HSC/RUs numbers
within both the AGM region and the YS are significantly above
the numbers of HSC/RUs attributable to circulating blood
present in these tissues (Table 1). To directly test the number of
circulating HSC/RUs contained within the AGM region we
flushed out 12 d.p.c. dorsal aorta (Table 2). As expected the
flushed out samples of embryonic blood contained fewer
HSC/RUs than remained in the dorsal aorta [**0.3 (0, 1.7)** and **1.6
(1.2, 2.4)** respectively]. Therefore, either both the AGM region
and the YS on day 12 p.c. are involved in the specific production
of HSC/RUs, **or** circulating HSC/RUs have been selectively
retained in these tissues. This issue has been more closely
examined in organ culture experiments described below.

In contrast to day 11 p.c., on day 12 p.c. HSC/RUs were
consistently detected in the body of the embryo. The number
of HSC/RUs in the body of the 12 d.p.c. embryo without the
liver was estimated to be **12.1 (8.1, 19.3)**. When in addition to
the liver, the AGM region and blood were also removed the
total number of HSC/RUs in all remaining tissues was **5.8 (4.4,**

8.2) (Table 1). From the amount of blood in body transplants
(Table 1) we estimate that about **1.6** HSC/RUs in the embryo
body belong to the pool of circulating HSC/RUs. Therefore, it
may be that apart from the AGM region and the YS a few
HSC/RUs are harboured in other tissues of the body. Amongst
individually tested tissues (thymus, spleen, lung, forelimbs,
heart and head) transplanted separately, the lungs consistently
reconstituted irradiated recipients. They contained **0.4 (0.2,
0.6)** HSC/RUs, which is above the expected **0.06** HSC/RUs that
would be brought there by the circulation (Table 1). Forelimbs
also contained about **0.5 (0.3, 0.9)** HSCs. Some untested
tissues may contain solitary HSC/RUs as well.

Table 2. Distribution of HSC/RUs within day 12 AGM

	Number of HSC/RUs per tissue	Number of recipient mice (RM):number of dilutions (D)
Aorta (after flushing)	1.6 (1.2, 2.4)	10:2
Flushed out of the aorta	0.3 (0, 1.7)	11:2

The dorsal aorta was dissected and contents flushed out using a mouth
micropipette. The dorsal aorta was dissociated and transplanted into irradiated
recipients in 2 dilutions. Flushed out samples of circulating blood were also
transplanted (as two different dilutions) into a separate group of recipients.
Numbers of HSC/RUs in tissues were estimated using a limiting dilution
method and presented as described in Materials and Methods. These results
support the idea that the majority of HSC/RUs in the AGM do not belong to
the pool of circulatory HSC/RUs as shown in Table 1.

Tissue distribution of definitive HSC/RUs in 13 d.p.c. embryo

By day 13 p.c. of development the number of circulating HSC/RUs in the embryonic vasculature remains high **5.9 (4.7, 7.7)** and the number of HSC/RUs in the liver continues to grow, reaching **260 (212, 320)** (Fig. 1). The number of HSC/RUs decreases in both the AGM region and the YS to **0.8 (0.6, 1.2)** and **0.8 (0.6, 1.2)** HSC/RU per tissue respectively (Fig. 1, Table 1). The total number of HSC/RUs in the body outside the liver remains stable compared to 12 d.p.c. body (Table 1) and HSC/RUs are no longer detectable in the lungs. Forelimb transplants reconstituted three out of five recipient mice in two dilutions. Since the amount of blood in distal limbs was extremely low, freely circulating HSC/RUs are unlikely to account for this (Table 1). HSC/RUs found in 12-13 d.p.c. limbs may reflect early colonisation of developing long bones with HSC/RUs. Further analysis of the limbs of 14 d.p.c. embryos will be required to test the reliability of this conclusion.

Analysis of the HSC potential of 11 d.p.c. embryonic tissues using an organ culture approach

In the day 11 p.c. embryo five sites contain HSC/Rus: the AGM region, the YS, the liver, blood and umbilical vessels (Muller et al., 1994; Medvinsky and Dzierzak, 1996; de Bruijn et al., 2000). It has been shown using an organ culture approach that the AGM region is the only one of these tissues capable of expanding or generating HSC/RUs at this age (Medvinsky and Dzierzak, 1996; de Bruijn et al., 2000). Here we have quantitatively reassessed the potential of 11 d.p.c. embryonic tissues to produce HSC/RUs, by quantifying the number of HSC/RUs that are produced and maintained in organ culture. After 3-5 days in culture, the number of HSC/RUs in 11 d.p.c. AGM region increased from **0.9 (0.7, 1.1)** to **12 (10.0, 17.6)**

($P<0.05$) (Fig. 3A) followed by a drop in the numbers of HSC/RUs after 7-9 days in culture.

As was shown previously, 11 d.p.c. YS is incapable of expanding the initial numbers of explanted HSC/RUs. Each explant of 11 d.p.c. YS contains approximately one HSC before and after 3 days in culture (Fig. 3A). However, in contrast to the AGM region, 11 d.p.c. YS explants are not able to maintain HSC/RUs for a longer time in culture. Similarly, in 11 d.p.c. liver about 1 HSC can be detected after 3 but not after 7 days in culture.

Analysis of HSC potential of 12 d.p.c. embryonic tissues by organ culture

When 12 d.p.c. AGM region was tested, we found that after 3-5 days in vitro it contained the same number of HSC/RUs as it initially contained in the embryo (Fig. 3B). A slight increase in the numbers of HSC/RUs was observed after 7-9 days in culture. 12 d.p.c AGMs are larger than 11 d.p.c. AGMs and therefore culture conditions could be suboptimal. To optimise the culture we reduced the size of the explants by subdissection of 12 d.p.c. AGMs and found no signs of HSC expansion in these cultures either (data not shown). In addition, we found that the microenvironment of the 12 d.p.c. AGM is highly supportive of long-term (up to 4 weeks) maintenance of HSC/RUs (Kumaravelu et al., unpublished observation). Thus, we infer that the ability of the AGM region to expand (and/or generate) definitive HSC/RUs is significantly attenuated on day 12 p.c., as compared to 11 d.p.c., concurrent with progressive specification of the AGM region into gonads and mesonephric derivatives.

In contrast to 11 d.p.c. YS, 12 d.p.c. YS explants acquire the capacity to increase the numbers of HSC/RUs during culture. Before culture 12 d.p.c. YS contains **1.8 (1.4, 2.4)** HSC/RUs but after 3 days in culture it contains **6.8 (5.0, 9.8)** HSC/RUs
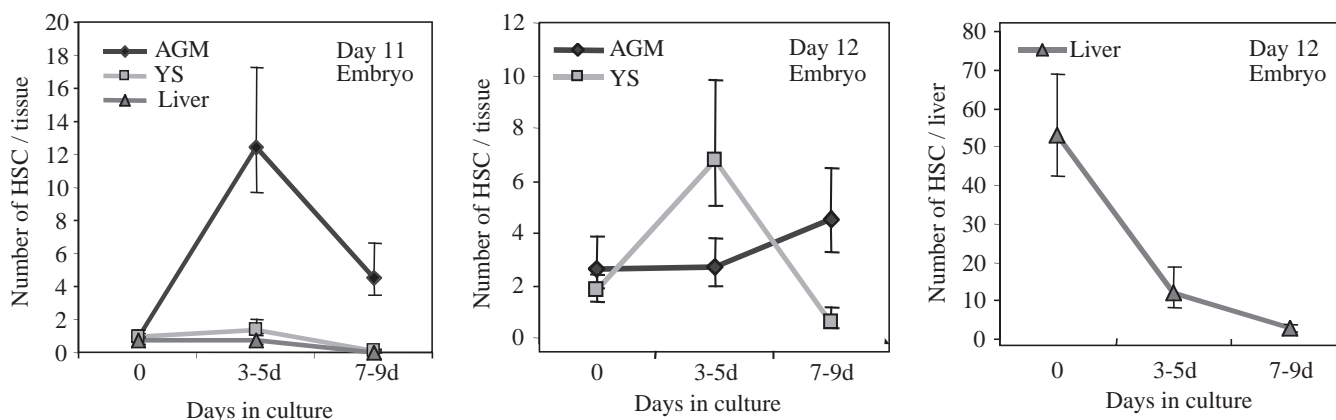

Fig. 3. Potential of day-11 and day-12 embryonic haematopoietic tissues to produce/expand definitive HSC/RUs as assessed by an organ culture approach. (A) Cultured day-11 tissues, (B) cultured day-12 AGM region and YS, (C) cultured day-12 liver. Note that among day-11 tissues (A) only the AGM region was able to expand HSC/RUs and among day-12 tissues (B) only the YS was able to expand HSC/RUs, suggesting two consecutive waves of HSC generation/expansion and liver colonisation, firstly from the AGM and secondly, the next day, from the YS. 12 d.p.c. embryonic liver was not capable of maintaining HSCs: a dramatic decrease in number of HSC/RUs was observed after 3 days in culture (see Discussion). Numbers of HSC/RUs in tissues are estimated using a limiting dilution method and presented as described in Materials and Methods. Numbers of recipient mice (RM) and the dilutions (D) used were as follows. Day 11 tissues (A): (AGM, uncultured) 27RM, 2D; (AGM, 3-5 days in culture) 41RM, 8D; (AGM, 7-9 days in culture) 14RM, 3D; (YS uncultured) 25RM, 3D; (YS 3-5 days in culture) 20RM, 6D; (YS 7-9 days in culture) 15RM,3D; (liver, uncultured) 23RM, 2D; (liver, 3-5 days in culture) 29RM, 9D; (liver, 7-9 days in culture) 14RM, 4D. Day 12 tissues (B): (AGM, uncultured) 25RM, 7D; (AGM, 3-5 days in culture) 31RM, 7D; (AGM, 7-9 days in culture) 14RM, 3D; (uncultured YS) 39RM, 8D; (YS, 3-5 days in culture) 17RM, 4D; (YS, 7-9 days in culture) 11RM, 2D; (liver, uncultured) 37RM, 5D. Day 12 liver (C): (3-5 days in culture) 14RM, 3D; (7-9 days in culture) 24RM; 6D.

per explant (*P*<0.05) (Fig. 3B). However, like the 11 d.p.c. YS and in contrast to the AGM region, 12 d.p.c. YS was not able to maintain HSC/RUs in long-term cultures; after 7 days in culture the number of HSC/RUs dropped to less than 1, being **0.6 (0.4, 1.2)** HSC/RUs per YS. This may possibly reflect the transitory nature of haematopoietic activity in the YS in vivo.

Explants of 12 d.p.c., foetal liver were not able to maintain the initial number of HSC/RUs in culture, which could be explained either by suboptimal culture conditions for this tissue or by immaturity of day 12 liver microenvironment (Fig. 3C).

DISCUSSION

Shortly before the onset of organogenesis the embryo starts to generate a transitory population of embryonic haematopoietic cells that serve its immediate needs. These first haemopoietic cells, consisting mainly of committed myeloid progenitors, CFU-S and primitive erythroid cells, appear in the embryonic circulation in growing numbers and then colonise the initially haematopoietically inactive embryonic liver (Moore and Metcalf, 1970; Johnson and Barker, 1985; Medvinsky, 1993; Medvinsky et al., 1996). Definitive HSCs, which give rise to the adult haematopoietic hierarchy, develop slightly later and gradually form a massive pool in the foetal liver, which becomes the main source of HSCs which subsequently colonise the bone marrow (Dzierzak and Medvinsky, 1995; Morrison et al., 1997; Dzierzak et al., 1998). Owing to the absence of unique markers for definitive HSCs at present, direct monitoring of their origin and movements during development, as well as definition of the developmental boundaries of the haematopoietic system is not possible and can only be inferred indirectly from HSC functional assays. Some markers have been successfully used to narrow down the anatomical location of definitive HSCs but their expression is not restricted purely to HSCs (North et al., 1999; Manaia et al., 2000; Ma et al., 2002). In addition, HSC migration is not necessarily restricted to the vascular network and their location in tissues may not be accompanied, as has been shown for the AGM region, by active haematopoiesis (Medvinsky et al., 1996; Godin et al., 1999). Although some progress has been achieved, in contrast to the development of solid organs, an anatomical and histological description of development of the definitive haematopoietic system is lacking. Here, using a quantitative approach we analyse the early development of definitive HSC/RUs, the 'germinal layer' of the definitive haematopoietic system.

Early development of definitive HSC/RUs in the mouse embryo involves at least two key stages; (i) initiation of definitive HSC/RUs (late day 10-early day 11 p.c.) and (ii) expansion of the pool of definitive HSC/RUs. In addition, some tissues may be involved in the maintenance of HSC/RUs. Since HSCs may rapidly change their location during embryogenesis, detection of HSCs in tissues does not identify whether these tissues are capable of generating, expanding, or maintaining them or whether these tissues merely transiently contain them. In order to try and distinguish between these possibilities, we and others previously developed an organ culture approach which enables isolated tissues to be individually tested to reveal their HSC activity (Medvinsky and Dzierzak, 1996; Cumano et al., 2001). For example, the number of HSC/RUs within the AGM region on days 10-11 p.c. is no higher than

Table 3. Total increase in HSC/RUs numbers in the developing mouse embryo

	Day 10	Day 11	Day 12
AGM region	~0	~0.9	~2.7
Yolk sac	0	~1.1	~1.8
Circulation	0	~0	~3.1
Liver	0	~0.7	~53
Body (w/o AGM, circulation, liver)	0	0	~5.8
Total	0	~3	~66

within the YS and the liver. However, in contrast to other embryonic tissues at this age, the AGM region is capable of autonomously initiating and expanding HSC/RUs in vitro suggesting that the initial pool of definitive HSC/RUs is generated within the AGM region and these then colonise the liver (Morrison et al., 1995; Medvinsky and Dzierzak, 1996; Ema and Nakauchi, 2000).

Here, using an improved protocol we have been able to detect rare HSC/RUs in day 11 circulation (compare with our previous report) (Muller et al., 1994). This reveals a previously proposed, but never experimentally shown, route by which 11 d.p.c. AGM derived HSCs can colonise the liver. During days 12-13 p.c. the number of HSC/RUs in the circulation rises (approximately 6 HSC/RUs were detected on day 13 p.c.) consistent with the more intense colonisation of the foetal liver with HSCs from extra-hepatic sources during this period. It is worth bearing in mind that HSC/RUs present in the circulation at the moment of embryo dissection are likely to represent only a small proportion of total HSC/RUs present in the circulation over the entire day of gestation.

Our present calculations indicate that the total number of HSC/RUs within the developing embryo increases dramatically from about 3 (day 11 p.c.) to 66 (day 12 p.c.) mainly due to accumulation of HSC/RUs in the liver (Table 3). This is in accordance with previously published numbers of HSC/RUs in day 12 liver (Ema and Nakauchi, 2000). Owing to the length of the cell cycle it is unlikely that such an increase in HSC number occurs entirely from amplification of a few HSC/RUs that initially colonized the liver. If the possibility of de novo/primary formation of HSC/RUs in the liver is ruled out then this increase must be the result of a massive immigration of HSC/RUs from extra-liver source(s). Our quantitative data are in accord with this hypothesis as explained below.

In the 11 d.p.c. embryo the number of HSC/RUs within the liver is low and can be easily explained by colonisation from the AGM region. In fact, one 11 d.p.c. AGM region can produce as many as twelve HSC/RUs after 3 days in culture; and we assume that in vivo this process may be significantly more efficient. By 12 d.p.c. the HSC productivity of the AGM region decreases as assessed by the organ culture test. However measurement of HSC/RUs numbers in uncultured AGMs showed that the number of HSC/RUs in the AGM region is still higher than would be expected in a non-haematopoietic tissue. Conversely, by 12 d.p.c. the YS showed noticeable HSC productivity in vitro. It was capable of expanding the number of HSC/RUs from about 1.8 to 6.8 during a 3-day culture period.

Thus, at early stages of liver colonisation the high cumulative HSC productivity of the AGM region and the YS may provide the liver with a large part of the 'ready-to-use'
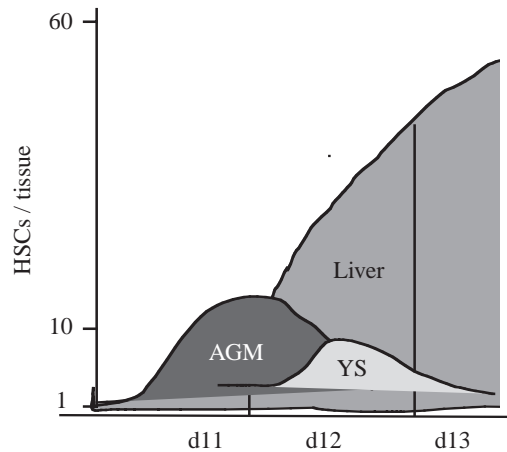
Fig. 4. Schematic representation of colonisation of the embryonic liver with HSC/RUs. The number of HSC/RUs in the liver are as determined in vivo. The number of HSC/RUs in the AGM region and the YS are the numbers that these tissues are able to generate in vitro. In vivo, the high cumulative activity of the AGM region and the YS may provide the liver with a high proportion of the 'ready-to-use' pool of definitive HSC/RUs. The data suggest consecutive colonisation of the embryonic liver with HSC/RUs from the AGM region and the YS.

pool of HSCs (Fig. 4). It is interesting that in the same culture conditions the liver itself was unable to increase or even maintain the initial number of HSCs. This suggests either that by day 12 p.c. the liver is not yet competent to expand HSCs or that the culture conditions used are not fully adequate. It is important to note in relation to this that a cell line derived from day 14 foetal liver is capable of maintaining HSCs over a period of 1month (Moore et al., 1997).

The generation/expansion of definitive HSCs/RUs in 12 p.c. YS culture is likely related to previous observations that from 8-10 d.p.c. the YS contains immature cells that when placed into an embryonic or newborn environment become capable of contributing into adult haematopoiesis (Weissman et al., 1978; Toles et al., 1989; Yoder and Hiatt, 1997; Yoder et al., 1997; Matsuoka et al., 2001). Our data suggest that these early YS cells may not necessarily need processing in the AGM region to become functional definitive HSCs but can mature in situ on day 12 p.c. The controversy over the origin of HSCs has always centred on the issue of whether the P-Sp/AGM region or the YS is the initial site of their generation. However, these data indicate that definitive HSCs may develop independently and asynchronously in these two different sites of the mouse embryo suggesting that the argument over the first source of HSCs is not relevant. However, at present, the possibility of cross-seeding of the YS and the AGM region with definitive HSCs and/or their ancestor cells cannot be excluded. Lineage tracing of YS and AGM haematopoiesis from early stages of development is required to finally resolve this issue.

In summary, we have carried out a comprehensive anatomical mapping of the development of definitive HSC/RUs in the mouse embryo from 11-13 d.p.c. during which time the embryonic liver becomes colonised. We have shown that increasing numbers of HSC/RUs in the liver is accompanied by the appearance of growing numbers of HSC/RUs in the embryonic blood. The data presented here suggests that in

addition to early waves of colonisation with committed and multipotent haematopoietic progenitors (Moore and Metcalf, 1970; Johnson and Barker, 1985; Dzierzak and Medvinsky, 1995) the liver is colonised by two consecutive waves of definitive HSC/RUs. The initial wave of HSC/RUs arrives from the AGM region on day 10 p.c., reaches a maximum by day 11 p.c. and disappears by day 13 p.c. On day 12 p.c. when AGM activity is decreasing, the second wave of colonisation arrives from the YS. This wave marks the embryonic stage when early YS cells mature into definitive HSC/RUs.

A visual demonstration of development of HSC/RUs in 10-12 d.p.c. mouse embryo as it is viewed by the authors is presented as a movie and accompanies the Web version of the article (http://dev.biologists.org/supplemental/).

REFERENCES

Abkowitz, J. L., Golinelli, D., Harrison, D. E. and Guttorp, P. (2000). In vivo kinetics of murine hemopoietic stem cells. *Blood* **96**, 3399-3405.

Berger, C. N. and Sturm, K. S. (1996). Estimation of the number of hematopoietic precursor cells during fetal mouse development by covariance analysis. *Blood* **88**, 2502-2509.

Chen, X. D. and Turpen, J. B. (1995). Intraembryonic origin of hepatic hematopoiesis in Xenopus laevis. *J. Immunol.* **154**, 2557-2567.

Ciau-Uitz, A., Walmsley, M. and Patient, R. (2000). Distinct origins of adult and embryonic blood in Xenopus. *Cell* **102**, 787-796.

Cumano, A., Dieterlen-Lievre, F. and Godin, I. (1996). Lymphoid potential, probed before circulation in mouse, is restricted to caudal intraembryonic splanchnopleura. *Cell* **86**, 907-916.

Cumano, A., Ferraz, J. C., Klaine, M., Di Santo, J. P. and Godin, I. (2001). Intraembryonic, but not yolk sac hematopoietic precursors, isolated before circulation, provide long-term multilineage reconstitution. *Immunity* **15**, 477-485.

Cumano, A., Paige, C. J., Iscove, N. N. and Brady, G. (1992). Bipotential precursors of B cells and macrophages in murine fetal liver. *Nature* **356**, 612-615.

de Bruijn, M. F., Speck, N. A., Peeters, M. C. and Dzierzak, E. (2000). Definitive hematopoietic stem cells first develop within the major arterial regions of the mouse embryo. *EMBO J.* **19**, 2465-2474.

de Bruijn, M. F. T. R., Ma, X., Robin, C., Ottersbach, K., Sanchez, M.-J. and Dzierzak, E. (2002). Hematopoietic Stem Cells Localize to the Endothelial Cell Layer in the Midgestation Mouse Aorta. *Immunity* **16**, 673-683.

Delassus, S. and Cumano, A. (1996). Circulation of hematopoietic progenitors in the mouse embryo. *Immunity* **4**, 97-106.

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

Douagi, I., Colucci, F., Di Santo, J. P. and Cumano, A. (2002). Identification of the earliest prethymic bipotent T/NK progenitor in murine fetal liver. *Blood* **99**, 463-471.

Dzierzak, E. and Medvinsky, A. (1995). Mouse embryonic hematopoiesis. *Trends Genet.* **11**, 359-366.

Dzierzak, E. and Medvinsky, A. (1998). Developmental Origins of Haematopoietic Stem Cells. In *Molecular Biology of B-cell and T-cell Development* (ed. J. G. Monroe and E. V. Rothenberg), pp. 3-25. Totowa, CA: Humana Press.

Dzierzak, E., Medvinsky, A. and de Bruijn, M. (1998). Qualitative and

quantitative aspects of haematopoietic cell development in the mammalian embryo. *Immunol. Today* **19**, 228-236.

Ema, H., Douagi, I., Cumano, A. and Kourilsky, P. (1998). Development of T cell precursor activity in the murine fetal liver. *Eur. J. Immunol.* **28**, 1563-1569.

Ema, H. and Nakauchi, H. (2000). Expansion of hematopoietic stem cells in the developing liver of a mouse embryo. *Blood* **95**, 2284-2288.

Eren, R., Auerbach, R. and Globerson, A. (1987). T cell ontogeny: extrathymic and intrathymic development of embryonic lymphohemopoietic stem cells. *Immunol. Res.* **6**, 279-287.

GenStat 5 Release 3 Reference Manual, G. C. (1993). ed., pp. 796. Oxford: Clarendon Press.

Godin, I., Garcia-Porrero, J. A., Dieterlen-Lievre, F. and Cumano, A. (1999). Stem cell emergence and hemopoietic activity are incompatible in mouse intraembryonic sites. *J. Exp. Med.* **190**, 43-52.

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

Hsu, H. C., Ema, H., Osawa, M., Nakamura, Y., Suda, T. and Nakauchi, H. (2000). Hematopoietic stem cells express Tie-2 receptor in the murine fetal liver. *Blood* **96**, 3757-3762.

Ikuta, K., Kina, T., MacNeil, I., Uchida, N., Peault, B., Chien, Y. H. and Weissman, I. L. (1990). A developmental switch in thymic lymphocyte maturation potential occurs at the level of hematopoietic stem cells. *Cell* **62**, 863-874.

Iuchi, I. and Yamamoto, M. (1983). Erythropoiesis in the developing rainbow trout, Salmo gairdneri irideus: histochemical and immunochemical detection of erythropoietic organs. *J. Exp. Zool.* **226**, 409-417.

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.

Kawamoto, H., Ohmura, K. and Katsura, Y. (1998). Presence of progenitors restricted to T, B, or myeloid lineage, but absence of multipotent stem cells, in the murine fetal thymus. *J. Immunol.* **161**, 3799-3802.

Lemischka, I. R. (1992). The haematopoietic stem cell and its clonal progeny: mechanisms regulating the hierarchy of primitive haematopoietic cells. *Cancer Surv.* **15**, 3-18.

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.

Liu, L. Q., Ilaria, R., Jr, Kingsley, P. D., Iwama, A., van Etten, R. A., Palis, J. and Zhang, D. E. (1999). A novel ubiquitin-specific protease, UBP43, cloned from leukemia fusion protein AML1-ETO-expressing mice, functions in hematopoietic cell differentiation. *Mol. Cell. Biol.* **19**, 3029-3038.

Ma, X., de Bruijn, M., Robin, C., Peeters, M., Kong, A. S. J., de Wit, T., Snoijs, C. and Dzierzak, E. (2002). Expression of the Ly-6A (Sca-1) lacZ transgene in mouse haematopoietic stem cells and embryos. *Br. J. Haematol.* **116**, 401-408.

Manaia, A., Lemarchandel, V., Klaine, M., Max-Audit, I., Romeo, P., Dieterlen-Lievre, F. and Godin, I. (2000). Lmo2 and GATA-3 associated expression in intraembryonic hemogenic sites. *Development* **127**, 643-653.

Martin, C., Lassila, O., Nurmi, T., Eskola, J., Dieterlen-Lievre, F. and Toivanen, P. (1979). Intraembryonic origin of lymphoid stem cells in the chicken: studies with sex chromosome and IgG allotype markers in histocompatible yolk sac-embryo chimaeras. *Scand. J. Immunol.* **10**, 333-338.

Matsuoka, S., Tsuji, K., Hisakawa, H., Xu, M., Ebihara, Y., Ishii, T., Sugiyama, D., Manabe, A., Tanaka, R., Ikeda, Y. et al. (2001). Generation of definitive hematopoietic stem cells from murine early yolk sac and paraaortic splanchnopleures by aorta-gonad-mesonephros region-derived stromal cells. *Blood* **98**, 6-12.

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

Medvinsky, A. and Dzierzak, E. (1996). Definitive hematopoiesis is autonomously initiated by the AGM region. *Cell* **86**, 897-906.

Medvinsky, A. and Dzierzak, E. (1999). Development of the hematopoietic stem cell: can we describe it? [letter]. *Blood* **94**, 3613-3614.

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

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

Moore, K. A., Ema, H. and Lemischka, I. R. (1997). In vitro maintenance of highly purified, transplantable hematopoietic stem cells. *Blood* **89**, 4337-4347.

Moore, M. A. and Metcalf, D. (1970). Ontogeny of the haemopoietic 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.

Morrison, S. J., Wright, D. E., Cheshier, S. H. and Weissman, I. L. (1997). Hematopoietic stem cells: challenges to expectations. *Curr. Opin. Immunol.* **9**, 216-221.

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

Nishikawa, S. I., Nishikawa, S., Kawamoto, H., Yoshida, H., Kizumoto, M., Kataoka, H. and Katsura, Y. (1998). In vitro generation of lymphohematopoietic cells from endothelial cells purified from murine embryos. *Immunity* **8**, 761-769.

North, T., Gu, T. L., Stacy, T., Wang, Q., Howard, L., Binder, M., Marin-Padilla, M. and Speck, N. A. (1999). Cbfa2 is required for the formation of intra-aortic hematopoietic clusters. *Development* **126**, 2563-2575.

North, T. C., de Bruijn, M. F. T. R., Stacy, T., Talebian, L., Lind, L., Robin, C., Binder, M., Dzierzak, E. and Speck, N. A. (2002). Runx1 expression marks long-term repopulating hematopoietic stem cells in the midgestation mouse embryo. *Immunity* **16**, 661-672.

Ohmura, K., Kawamoto, H., Lu, M., Ikawa, T., Ozaki, S., Nakao, K. and Katsura, Y. (2001). Immature multipotent hemopoietic progenitors lacking long-term bone marrow-reconstituting activity in the aorta-gonad-mesonephros region of murine day 10 fetuses. *J. Immunol.* **166**, 3290-3296.

Palis, J., Chan, R. J., Koniski, A., Patel, R., Starr, M. and Yoder, M. C. (2001). Spatial and temporal emergence of high proliferative potential hematopoietic precursors during murine embryogenesis. *Proc. Natl. Acad. Sci. USA* **98**, 4528-4533.

Rodewald, H. R., Kretzschmar, K., Takeda, S., Hohl, C. and Dessing, M. (1994). Identification of pro-thymocytes in murine fetal blood: T lineage commitment can precede thymus colonization. *EMBO J.* **13**, 4229-4240.

Sanchez, M. J., Holmes, A., Miles, C. and Dzierzak, E. (1996). Characterization of the first definitive hematopoietic stem cells in the AGM and liver of the mouse embryo. *Immunity* **5**, 513-525.

Szilvassy, S. J., Humphries, R. K., Lansdorp, P. M., Eaves, A. C. and Eaves, C. J. (1990). Quantitative assay for totipotent reconstituting hematopoietic stem cells by a competitive repopulation strategy. *Proc. Natl. Acad. Sci. USA* **87**, 8736-8740.

Tavian, M., Robin, C., Coulombel, L. and Peault, B. (2001). The human embryo, but not its yolk sac, generates lympho-myeloid stem cells: mapping multipotent hematopoietic cell fate in intraembryonic mesoderm. *Immunity* **15**, 487-495.

Toles, J. F., Chui, D. H., 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.

Traver, D., Miyamoto, T., Christensen, J., Iwasaki-Arai, J., Akashi, K. and Weissman, I. L. (2001). Fetal liver myelopoiesis occurs through distinct, prospectively isolatable progenitor subsets. *Blood* **98**, 627-635.

Turpen, J. B., Kelley, C. M., Mead, P. E. and Zon, L. I. (1997). Bipotential primitive-definitive hematopoietic progenitors in the vertebrate embryo. *Immunity* **7**, 325-334.

Velardi, A. and Cooper, M. D. (1984). An immunofluorescence analysis of the ontogeny of myeloid, T, and B lineage cells in mouse hemopoietic tissues. *J. Immunol.* **133**, 672-677.

Weissman, I., Pappaioannou, V. and Gardner, R. (1978). Fetal haematopoietic origins of the adult hematolymphoid system. In *Cold Spring Harbor Meeting on Differentiation of Normal and Neoplastic Hematopoietic Cells,* (ed. B. Clarkson P. A. Marks and J. E. Till), pp. 33-47. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

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

Yoder, M. C. and Hiatt, K. (1997). Engraftment of embryonic hematopoietic cells in conditioned newborn recipients. *Blood* **89**, 2176-2183.

Yoder, M. C., Hiatt, K., Dutt, P., Mukherjee, P., Bodine, D. M. and Orlic, D. (1997). Characterization of definitive lymphohematopoietic stem cells in the day 9 murine yolk sac. *Immunity* **7**, 335-344.