Mouse placenta is a major hematopoietic organ

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Summary

Placenta and yolk sac from 8- to 17-day-old (E8-E17) mouse embryos/fetuses were investigated for the presence of in vitro clonogenic progenitors. At E8-E9, the embryonic body from the umbilicus caudalwards was also analysed. Fetal liver was analysed beginning on E10. At E8, between five and nine somite pairs (sp), placenta, yolk sac and embryonic body yielded no progenitors. The first progenitors appeared at E8.5 at the stage of 15 sp in the yolk sac, 18 sp in the embryonic body, 20 sp in the placenta and only at E12 in the fetal liver (absent at E10, at E11 not determined). Progenitors with a high proliferation potential that could be replated for two months, as well as the whole range of myeloid progenitors, were found at all stages in all organs. However, the earliest of these progenitors (these yielding large, multilineage colonies)

were 2-4 times more frequent in the placenta than in the yolk sac or fetal liver. In the fetal liver, late progenitors were more frequent and the cellularity increased steeply with developmental age. Thus, the fetal liver, which is a recognized site for amplification and commitment, has a very different hematopoietic developmental profile from placenta or yolk sac. Placentas were obtained from GFP transgenic embryos in which only the embryonic contribution expressed the transgene. 80% of the colonies derived from these placental cells were GFP+, and so originated from the fetal component of the placenta. These data point to the placenta as a major hematopoietic organ that is active during most of pregnancy.

Key words: Placenta, Hematopoiesis, Clonogenic progenitors, Mouse

Introduction

The ontogeny of the hematopoietic system in vertebrates is characterized by the sequential activity of distinct hematopoietic organs. Yolk sac, fetal liver, thymus, spleen and bone marrow are the organs identified as carrying out this function in mammalian embryos (Metcalf and Moore, 1971). Of these, only the yolk sac produces its own stem cells (the others are seeded from an extrinsic source). Within the embryo, the region of the aorta has been recognized to produce long-term-reconstituting hematopoietic stem cells (LTR-HSCs) (Medvinsky and Dzierzak, 1996; Cumano et al., 2001) and is presently held responsible for the production of definitive HSCs (Keller et al., 1999). However, the developmental period during which this region produces HSCs is short and its activity becomes extinct when bone marrow colonization has barely initiated. The present consensus is thus that aorta-gonad-mesonephros (AGM) cells home to the liver before colonizing the bone marrow. We have been wondering whether, during fetal life, another site might produce HSCs long enough to be responsible, at least partly, for the direct colonization of the bone marrow.

In the avian embryo, a second appendage (the allantois) was shown to produce hematopoietic progenitors (Caprioli et al., 1998; Caprioli et al., 2001). Because the mouse chorioallantoic placenta develops by fusion of the allantois to the ectoplacental cone, we asked whether the placenta might participate in mouse fetal hematopoiesis and probed it for the presence of

hematopoietic progenitors. The mouse placenta has previously been shown to harbor B-cell progenitors (Melchers, 1979). We initiated our investigation around the stage of fusion of the allantois to the ectoplacental cone, which occurs in embryos with seven somite pairs (sp) or thereabouts [at embryonic days 8-9 (E8-E9)] (Theiler, 1972; Downs, 2002).

Here, we report a developmental analysis comparing the types and frequency of in vitro clonogenic progenitors (Metcalf et al., 1979) detected in the placenta, yolk sac, early embryo and fetal liver, every two days from E8 to E17. We could show that: (1) the placenta was 2-4 times richer in myeloid progenitors than the fetal liver; (2) these progenitors were derived from the fetal, not the maternal, compartment; (3) by E9 and E10, the placenta already harbored progenitors, whereas the liver did not; (4) at each stage studied, high-proliferation-potential colony-forming cells (HPP-CFCs), which were able to give rise to subcolonies of the same type up to 60 days, were several times more frequent in placenta than in fetal liver or yolk sac. HPP-CFCs are defined as multilineage colonies grown in semisolid medium whose diameter exceeds 0.5 mm (Pohlmann et al., 2001).

To confirm the initiation of hematopoiesis, we analysed the expression of several genes that are involved in the emergence of hematopoietic activity, at the 18 and 20 sp stages. The *Runx1* gene encodes the DNA binding subunit of a transcription factor belonging to the core binding factor (CBF) family (Wang et al., 1996). Its expression is rigorously restricted to the sites where

HSCs become committed in the embryo (North et al., 1999; North et al., 2002; Bollerot and Jaffredo, 2003), thus representing a distinct sign of HSC emergence. The *Scl/Tal1* (*Tal1* – Mouse Genome Informatics) gene is required for the development of all hematopoietic lineages (Porcher et al., 1996; Robb et al., 1996). Finally the *Tel/Etv6* gene activity is required for the appearance of hematopoietic stem cell activity in the bone marrow (Wang et al., 1998).

Materials and methods

Mice

C57BL/6 Ly-5.2 mice and C57BL/6 Ly-5.2 mice transgenic for an enhanced green fluorescent protein (GFP) cDNA (Okabe et al., 1997) were used. GFP+ animals are easily recognized because newborns, being hairless, fluoresce under ultraviolet (UV) light. When GFP/+ heterozygote males were crossed to wild-type females, the fetal component of the placenta in the heterozygote progeny (hereafter designated GFP+) carried the transgene, whereas the maternal component was wild type. It was possible to diagnose GFP+ embryos immediately after retrieval by direct examination under an stereomicroscope equipped with UV light. The GFP+ fetal component of the placenta was clearly distinguished from the maternal GFP– component in these embryos (Fig. 1). We are indebted to M. Okabe for allowing us to use the GFP+ strain and to S. Ezine for supplying the founder animals. Animals were bred in our animal care facilities.

Embryos and cell preparations

Mature females were caged with breeding males and the day of vaginal plug was considered as day 0 of embryonic development (E0). Pregnant females between E8 and E17 were killed by cervical dislocation. Conceptuses were opened under a stereomicroscope, somite pairs were counted in E8-9 embryos and the following tissues or organs were dissected: at E8-E10, placenta, yolk sac and caudal half of the embryo; from E10 to E17, placenta (free of umbilical cord), yolk sac and liver. The tissues were weighed and dissociated by drawing through a 16 G needle before incubation with 0.1% pancreatin (Sigma, St Louis) in PBS for 15 minutes at 37°C. Dispersed cells were then drawn successively through 21 G, 25 G and 26 G needles into a syringe, deposited into a polystyrene tube and pelleted at 500 *g* for 10 minutes. Single cell suspensions were counted and viability was tested via trypan blue exclusion. Aliquots of the cell suspensions were diluted 1:100 in a solution containing 3% acetic acid and 0.01% methylene blue in PBS, and nucleated cells were counted in a hemocytometer. The caudal halves of E8 and E9 embryonic bodies were also dissected and treated according to this procedure.

CFC and HPP-CFC assays

For analysis of progenitor activity in each organ, 10^4 cells pooled from between four and six embryos were plated per well in LabTek chambers (four wells) in 500 µl MethoCult M3434 methylcellulose media (Stem Cell Technologies, Vancouver, BC, Canada). The commercial medium contains methylcellulose in IMDM [15% fetal calf serum (FCS), 1% bovine serum albumin (BSA), 10^{-4} M 2-mercaptoethanol, 2 mM glutamine, 3 U ml^{-1} recombinant human (rh) erythropoietin, 10 ng ml^{-1} rh interleukin 6 (IL-6), 10 ng ml^{-1} recombinant mouse (rm) IL-3, 50 ng ml^{-1} rm Stem Cell Factor (SCF), 10 µg ml^{-1} rh insulin and 200 µg ml^{-1} human transferrin (iron saturated)]. After 14 days of culture at 37°C in a humid atmosphere containing 5% CO_2, colonies were identified based on their morphology under an inverted microscope and scored. Each value was obtained from the means of three or four independent assays. Individual colonies were plucked using fine-drawn Pasteur pipettes and May-Grünwald-Giemsa stained. In some experiments, the cells were obtained from GFP+ embryos in which only the fetal contribution to the placenta was transgenic.

To check for the presence of long-term HPP-CFCs, first passage colonies with a diameter exceeding 0.5 mm were plucked from eight wells after 20-25 days of culture, pooled, washed with PBS. Cells were counted and 10^4 cells were replated per well in MethoCult M3434 medium. The colonies were replated three times according to the same protocol and scored 60 days after initial seeding.

Statistical analysis

Mann-Whitney *U* non-parametric analysis was performed using Instat (GraphPad Software, San Diego, CA).

RNA extraction and RT-PCR

Total RNA was extracted from cells obtained from tissues with GenElute Mammalian Total RNA Kit (Sigma). For reverse-transcription polymerase chain reaction (RT-PCR), first-strand cDNA was synthesized with the use of anchored oligo(dT)$_{23}$ and enhanced AMV reverse transcriptase (Sigma) from 0.5 µg total RNA. Specific cDNA was amplified with Taq DNA polymerase (PCR Master Mix, Promega) using pairs of oligonucleotide primers as follows:

HPRT, 5′-CACAGGACTAGAACACCTGC-3′ and 5′-GCTGGTG-AAAAGGACCTCT-3′ (Keller et al., 1993);

Runx1, 5′-CCAGCAAGCTGAGGAGCGGCG-3′ and 5′-CGGAT-TTGTAAAGACGGTGA-3′ (Lacaud et al., 2002);

Tel/Etv6, 5′-CGTGTGAGTCTCAGGCTTGT-3′ and 5′-GCTGTA-AAGGAAGAGACCCG-3′ (GenBank Accession Number: AW557856); and

Scl, 5′-ATGGAGATTTCTGATGGTCCTCAC-3′ and 5′-AAGT-GTGCTTGGGTGTTGGCTC-3′ (Lacaud et al., 2002).

Samples were denatured at 94°C for 5 minutes, followed by 35 amplification rounds, each consisting of 94°C for 30 seconds (denaturing), 60°C for 1 minute (annealing) and 72°C for 3 minutes (extension). Products were separated on a 1.2% agarose gel, stained with ethidium bromide and photographed.

Results

Cells dissociated from the various organs gave rise to colonies in the methylcellulose-based, growth-factor-enriched medium. In order to determine whether the clonogenic precursors were from maternal or fetal origin, cells were obtained from fetuses resulting from the cross between heterozygous GFP+ males and GFP– females, in which the fetus and the fetal component of the placenta is GFP+ (Fig. 1). 80% of the placental colonies were GFP+ (Fig. 2) when examined under UV light. No mixed colonies were observed; that is, either all the cells were GFP+ or all the cells were GFP–, in agreement with the established fact that each colony derives from a single progenitor. Most progenitors observed in methylcellulose assays were thus derived from the fetal component of the placenta. All colonies derived from yolk sac, embryonic caudal halves or liver cell suspensions were GFP+. Most culture series were performed using GFP+ embryos, except a few cases in which breeding problems precluded the obtainment of these embryos.

### Frequency of short-term progenitors at E8-17

Three different types of progenitors – CFU-GEMMs (colony-forming units, granulocytes, erythrocytes, monocytes, macrophages), BFU-Es (burst-forming units, erythroid) and CFU-GMs (colony-forming units, granulocytes, macrophages) – diagnosed from the colony phenotype, were scored separately on day 14 of culture (Table 1, Fig. 3). CFU-GEMMs exceeding 0.5 mm in diameter (HPP-CFCs) were selected for serial replating. The E8-E9 donor embryos were staged according to the number of somite pairs. None of the tissues
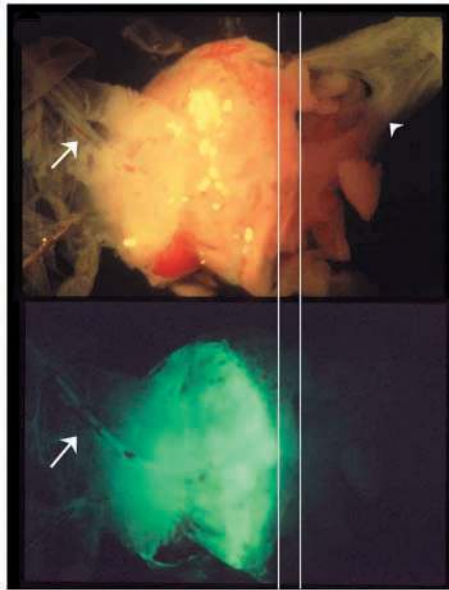
Fig. 1. E15 Placenta. Bright field, top; UV fluorescence, bottom. The fetal component is GFP+, the maternal component GFP−. Notice the zone of invasion by fetal blood vessels (weakly fluorescent between the white lines). Arrows point to the umbilical cord, the arrowhead to the zone of attachment to the uterus.

Table 1. Colony formation from early tissues

	Cell source	CFU-GEMM	BFU-E	CFU-GM
E8				
5 sp	P	0	0	0
	YS	0	0	0
	CH	0	0	0
9 sp	P	0	0	0
	YS	0	0	0
	CH	0	0	0
E8.5				
15 sp	P	0	0	0
	YS	2.5±0.6	1.75±0.5	0
	CH	0	0	0
18 sp	P	0.5±0.5	0	0.5±0.5
	YS	3.0±1.5	2.5±0.5	0
	CH	3.5±1.5	0.5±1.0	2.5±2.0
E9				
20 sp	P	4.5±1.0	1.0±0.5	2.5±1.0
	YS	5.0±1.0	4.0±1.0	2.0±0.5
	CH	6.0±1.5	1.0±1.5	5.0±1.0

Table shows number of GFP+ colonies/10^4 cells.

10^4 cells were cultured in the presence of growth factors (SCF, IL3, IL6, EPO). Colonies were scored at 14 days of culture.

Each value is the mean±s.d. of four assays (four wells per assay).

P, placenta; YS, yolk sac; CH, caudal halves of embryos; FL, fetal liver; sp, somite pairs.

tested from the stages of 5 sp and 9 sp gave rise to any colonies (Table 1). The liver rudiment was tested starting on E10. The in situ emergence of HSCs within the embryo at E8-E11 is firmly substantiated and the capacity to produce these cells has been ascribed to the periaortic region (Garcia-Porrero et al., 1995; Wood et al., 1997). Several pieces of evidence show that these cells locate to the caudal half of the embryo, from the umbilical level downwards (Cumano et al., 1993; Belaoussoff et al., 1998); for the needs of our comparative study, we included the caudal half (CH) of E8-E10 embryos.

CFU-GEMMs were first obtained from the yolk sac at the
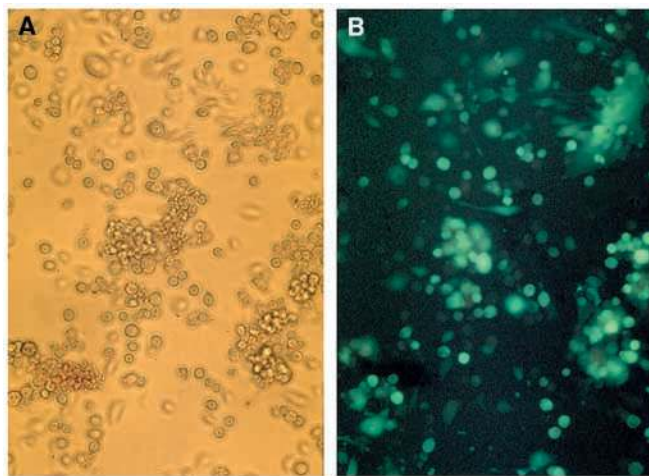
Fig. 2. A 14-day colony from an E15 placenta from the cross between a female C57BL/6 Ly-5.2 and a C57BL/6 Ly-5.2 GFP+ male. (A) Phase contrast. (B) UV fluorescence.

15 sp stage (not tested between the 9 sp and 15 sp stages) and caudal half of the embryo at 18 sp (Table 1). They barely began appearing in placental cell cultures at the 18 sp stage. In the liver, clonogenic progenitors were absent at E10 but were found at E12 (E11 not determined). Concerning frequency, GFU-GEMMs were rare in the 18 sp placenta but increased significantly in number at 20 sp, dropped slightly until E12 and doubled again at E15-17 (Fig. 3). Liver progenitor frequency, although it increased sharply, was at all times at least two times lower than in the placenta (Fig. 3). Indeed, the most striking profile was in the placenta, in which the E10 frequency equaled that in the yolk sac and became about threefold higher than in the two other tissues at E17.

Furthermore, many placental CFU-GEMMs were huge and so qualified more adequately as HPP-CFCs, with a very large core of hemoglobinized cells surrounded by dense clusters of granulocytes (Fig. 4A,D). Yolk sac and liver colonies were never that large (Fig. 4B,C). On cytological examination, both yolk sac and placental colonies were very rich in erythroblasts (Fig. 4D,E), yolk sac was significantly enriched in late erythroblasts (Fig. 4E), liver displayed erythroid cells expelling their nucleus (Fig. 4F), and megakaryocytes were especially abundant in placental CFU-GEMMs (not shown).

The same pattern was found in the case of BFU-Es: the frequency showed wide discrepancies between placenta and the two other structures, becoming at least three times higher in the late placenta. Yolk sac BFU-Es displayed two peaks, on E9 and E12, respectively (Table 1, Fig. 3). Placental BFU-Es had a striking phenotype: they were multicentered and highly hemoglobinized, whereas yolk sac and liver BFU-Es displayed only weakly hemoglobinized clusters. Like CFU-GEMMs, BFU-Es were not present in E10 fetal liver.

The frequency of CFU-GMs varied differently from that of other colony types. Placental progenitors peaked at E12, liver

frequency increased steadily during the period studied and yolk sac frequency remained stable (Fig. 3). E10 liver, again, did not contain any CFU-GMs, with the exception of one colony in four independent assays (i.e. 16 wells). Placenta and yolk sac CFU-GMs were dense, medium to large sized, and often actually so large that they seemed to result from the fusion of clusters (data not shown). The time course of colony emergence in the embryo caudal half fitted with previous studies about the para-aortic-splanchnopleura (P-Sp) (Godin et al., 1995).

All in all, the salient points in this developmental pattern are that: (1) the appearance of clonogenic progenitors in the placenta lagged slightly behind that in the embryo proper; (2) progenitors were 2-4 times more frequent in the placenta than in the liver; (3) placental colonies had, at all times, a more immature phenotype than these from yolk sac or liver. The differences in frequency and immaturity were especially striking at E15 and E17.
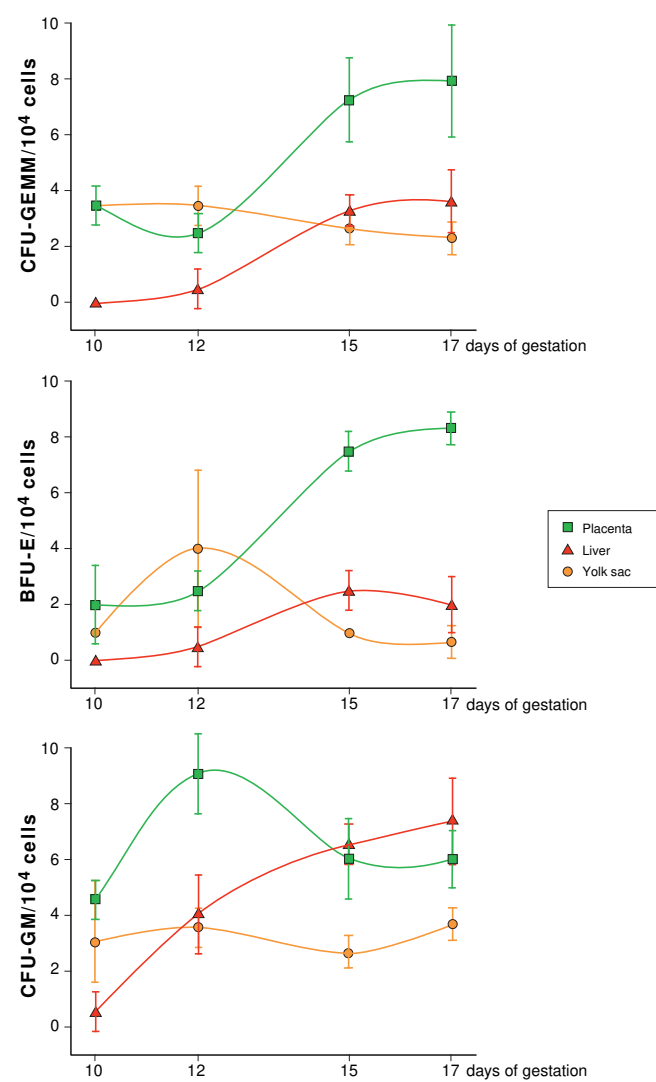
Fig. 3. Developmental frequencies of CFU-GEMMs, BFU-Es and CFU-GMs in E10 to E17 placenta, yolk sac and liver, 14 days after plating. Each point is the mean±s.d. from four independent experiments in which cells from four to six embryos were pooled (four wells in each experiment).

Cellularity of the organs and total number of progenitors in each organ

The total number of nucleated cells nearly doubled in the placenta and yolk sac from E10 to E17. By contrast, the number of cells in the liver increased about 13-fold between E12 and E17 (Table 2). From these counts, it is possible to evaluate the total number of progenitors in each organ at these developmental stages (Table 3).

Three features emerge. At E10, the placenta was ten times richer in progenitors than the yolk sac. The total placental count then grew modestly, reaching a fourfold increase by E17. The yolk sac peaked at E12. By contrast, the liver was void at E10 but increased its progenitor content exponentially between E12 and E17. Together with the different arrays of colony types observed in yolk sac and placenta on one hand (early progenitors) and liver on the other (late progenitors), these changes indicate that, whereas the liver is a site for amplification and differentiation, the placenta and yolk sac provide a microenvironment more favorable to the maintenance (and perhaps emergence, at least in the case of yolk sac) of early progenitors.

Long-term HPP-CFCs

In order to monitor the self-renewal capacity of hematopoietic progenitors in the various organs, we replated the very large multilineage colonies derived from HPP-CFCs every 2 weeks for 2-3 months. These experiments were carried out using E12, E15 or E17 unsorted cell preparations. At each replating, some very large colonies were again obtained. When the colonies were scored at 60 days, 4.5 CFU-GEMMs were obtained from 10^4 replated placental cells. Similar results were observed whether cells were obtained from E12, E15 or E17 embryos (Fig. 5, E15). By contrast, HPP-CFCs were rarely obtained

Table 2. Developmental analysis of the number of nucleated cells

Time	Tissue	Mass (mg)/mouse	Cell content ($\times 10^{-4}$)
E10	Placenta	38.4±9.0	34±9
	Yolk sac	22.5±7.0	4±1.5
	Liver	13.2±5.0	0.65±0.36
E12	Placenta	47.8±6.0	30.7±12
	Yolk sac	28.7±6.0	7.5±2.4
	Liver	34.5±8.0	63±35
E15	Placenta	71.6±9.0	47.7±9.4
	Yolk sac	33.8±5.0	6.9±2.7
	Liver	46.5±10.0	383±105
E17	Placenta	85.5±8.0	57.9±12
	Yolk sac	39.3±5.0	7.4±1.3
	Liver	58.5±12.0	860±303

Tissue mass and cell content are expressed per tissue from one embryo. Each point was calculated from 10 organs.

Table 3. Number of progenitors (all types merged) per organ

Embryonic stage	Placenta	Yolk sac	Liver
E10	340	30	0.3
E12	430	80	315
E15	1000	40	4800
E17	1300	50	11,200

from the yolk sac. Liver progenitors did give rise to this type of colony, but the frequency was half that in the placenta. Furthermore, the liver colonies were smaller than the placental colonies. Both parameters thus indicate a progressive extinction of the self-renewal capacity in the liver.
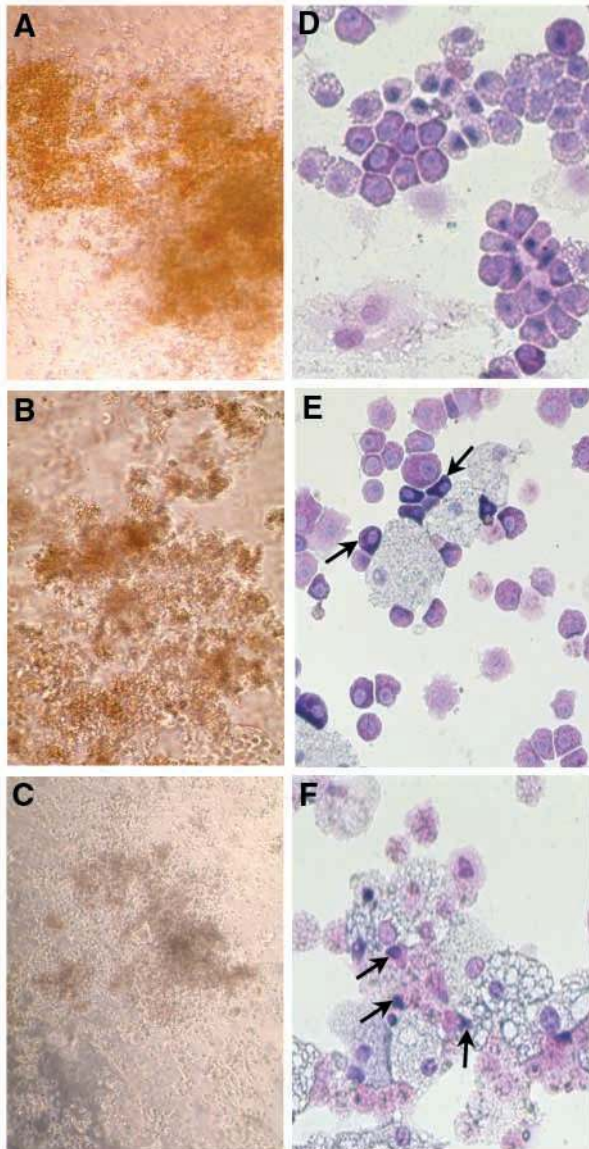
Expression patterns of Runx1, Scl/Tal1 and Tel/Etv6 in E8-E9 embryos

The mRNA expression of these transcription factors was studied in the placenta, the yolk sac and the caudal half of the

Fig. 4. CFU-GEMM colonies obtained from E15 placenta, yolk sac and liver cells after 14 days of culture in methylcellulose-based medium. (A-C) Phase contrast. (D-F) May-Grünwald-Giemsa staining. (A,D) Placenta. (B,E) Yolk sac. (C,F) Liver. Placenta colonies are characterized by a very large, highly hemoglobinized center. Cytology reveals a different degree of erythroid maturity depending on the organs: early erythroblasts predominate in the placenta; late erythroblasts (small, deeply basophilic, arrows in E) are numerous in the yolk sac; fetal liver displays mainly erythrocytes expelling their nucleus (arrows in F).
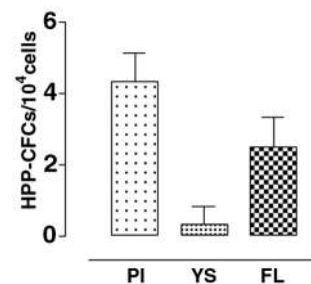
embryo (henceforth designated 'embryo') at the 18 sp and 20 sp stages (E8-9) (Fig. 6). *Runx1* was not detectable in the 18 sp placenta, whereas a clear signal was present in yolk sac preparations and a weak one in the embryo. These data correlate with results of clonogenic assays (Table 1) in which CFU-GEMMs and BFU-Es were obtained at this stage from yolk sac and embryo whereas they were exceptional in placenta. *Runx1* became expressed at the 20 sp stage in the placenta, at the same stage when a significant CFU-GEMM activity was first detected there. *Scl* also appeared in the 20 sp placenta, whereas it was already expressed in the embryo proper at 18 sp. Thus, both *Runx1* and *Scl* became expressed in the embryo slightly earlier than in the placenta, in agreement with the clonogenic progenitor pattern. The *Tel/Etv6* pattern is presently difficult to relate to the other data: signals were detected only in the 18 sp yolk sac and in the 20 sp placenta.

Discussion

The placenta has not been regarded previously as taking part in fetal blood formation (Keller et al., 1999). The paper by Melchers does point to a hemogenic role of the placenta (Melchers, 1979). This author found a peak in the number of lymphoid progenitors (plaque forming cells) in E12 placenta, whereas the liver displayed a similar peak at 3 days after birth. With the technology we used, at all stages studied from E12 to E17, placental cell preparations were 2-4 times richer than the liver in the earliest myeloid progenitors (CFU-GEMMs and BFU-Es). At E10, the placenta already displayed a high CFU-GEMM frequency, whereas the liver was devoid of these progenitors, in agreement with earlier work establishing that the liver rudiment becomes colonized by extrinsic HSCs at E10-11 between the stages of 28 sp and 32 sp (Johnson and Jones, 1973; Johnson and Moore, 1975; Houssaint, 1981). The stage at which myeloid progenitors were first found in the caudal embryo (18 sp) or in the yolk sac (15 sp) in the present work is in agreement with the stage at which B lymphoid progenitors were detected in these structures by Godin et al. (Godin et al., 1995) (in whose work, the stage designated as 15 sp was actually represented by a pool of 20-30 embryos in the 13-18 sp range). Interestingly, the yolk sac maintained a hematopoietic potential during the whole period studied here,

Fig. 5. Long-term HPP-CFC assay. HPP-CFCs were taken from MethoCult M3434 cultures every 20 days. 10^4 cells from pooled colonies were replated at each passage. HPP-CFCs were scored after a total 60 days in culture. Results are expressed as the mean of three independent observations performed in quadruplicate. This graph was established using E15 organs; similar results were yielded by E12 and E17 organs. FL, fetal liver; Pl, placenta; YS, yolk sac.
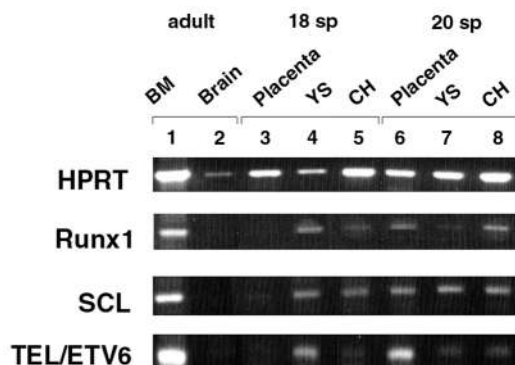
Fig. 6. Expression of transcription factors Runx1, Scl and Tel/Etv6 in E8-E9 embryos. RT-PCR was performed on total RNA extracted from different tissues as indicated. BM, bone marrow; CH, embryo caudal halves.

although at a very much lower level than the placenta. A few endodermal cords from the yolk sac penetrate into the basis of the placenta (Bruns et al., 1985). However at all stages we found it possible to dissect away yolk sac from placenta without any bleeding. It thus appears very unlikely that blood vessels from the yolk sac enter the placenta. The delayed earliest appearance of progenitors in the liver is in agreement with the known influx of progenitors from the yolk sac, the AGM and maybe the placenta.

It was important to verify that the progenitors were derived from the fetal part of the placenta. Melchers used different major histocompatibility haplotypes to distinguish maternal from fetal cells and concluded that the B lymphoid progenitors detected were indeed of fetal origin (Melchers, 1979). Using a green fluorescent protein gene as a marker, we extend this finding to myeloid progenitors.

The results we report here do not give clues about whether the progenitors found in the placenta originate in situ or colonize the appendage. Although the yolk sac produces its own stem cells, the fetal liver and bone marrow are known to become seeded by extrinsic stem cells, a sine qua non condition for their hematopoietic activity to switch on (Metcalf and Moore, 1971; Jotereau and Le Douarin, 1978; Johnson and Moore, 1975; Houssaint, 1981; Pardanaud et al., 1989). Although progenitors emerging in the yolk sac might contribute to the seeding of definitive hemopoietic organs (in a more or less transitory fashion, depending on species) (Weissman et al., 1978; Beaupain et al., 1979; Kau and Turpen, 1983; Pardanaud et al., 1989; Yoder et al., 1997; Palis et al., 1999), the secondary wave of progenitors, which becomes committed in the embryo, has been recognized as a source of definitive hematopoiesis, in bird (Dieterlen-Lièvre, 1975), amphibian (Turpen et al., 1981; Ciau-Uitz et al., 2000), mouse (Godin et al., 1993; Godin et al., 1995; Cumano et al., 1996; Medvinsky and Dzierzak, 1996) and human (Tavian et al., 1996) embryos.

The P-SP/AGM (Godin et al., 1993; Medvinsky et al., 1993; Cumano et al., 1996; Medvinsky and Dzierzak, 1996) is accepted as the region where these intraembryonic HSCs become committed (Garcia-Porrero et al., 1995; Garcia-Porrero et al., 1998; Wood et al., 1997; Jaffredo et al., 1998; Jaffredo et al., 2000; de Bruijn et al., 2002; North et al., 2002;

Oberlin et al., 2002). The AGM is at present considered to be responsible for the production of HSCs colonizing the fetal liver. In this region, the hemogenic activity begins around the 15 sp stage in the P-Sp (Godin et al., 1995) and continues in the AGM, where it peaks around the stage of 40 sp and thereafter declines (Medvinsky et al., 1993). It is intriguing that the emergence of progenitors in the placenta follows shortly after the emergence of progenitors in the embryonic aorta, vitelline and umbilical arteries. The progenitor producing activity of the placenta might represent a spatial extension of the activity ongoing in these arteries, which has previously been described by several authors (de Bruijn et al., 2000; North et al., 2002; Tavian et al., 1999).

The appearance of placental progenitors and the first expression of the transcription factors Scl/tal1 and Runx1 are simultaneous. The products of these genes are required for definitive HSC commitment (Porcher et al., 1996; North et al., 1999; North et al., 2002), suggesting that this event might occur in the placenta. The third gene whose expression was analyzed, *Tel/Etv6*, was selected for study because of the mutant phenotype: no $Tel/Etv6^{-/-}$ progenitors colonize the bone marrow of chimeras, whereas these progenitors thrive in the yolk sac and fetal liver (Wang et al., 1998). A more detailed expression pattern over a longer period of development than was analyzed in the present work will be needed to understand how placental hematopoiesis is initiated.

A possible erythropoietic function of the mouse allantois, which is known to contribute blood vessels to the placenta (de Bruijn et al., 2002), was previously explored without success (Downs and Harmann, 1997; Downs et al., 1998). Vasculogenesis, a process often associated with the production of blood progenitors, occurred in the early rudiment cultured in vitro (Downs et al., 1998; Drake and Fleming, 2000) but no definite signs of erythropoiesis could be ascertained (Downs et al., 1998). However, in these experiments, the allantoises were explanted from head-fold-stage embryos, cultured until the 13-15 sp stages and scanned for benzidine-stained erythrocytes. In the present investigation, clonogenic progenitors [detected by their colony-forming potential in the presence of appropriate cytokines (i.e. left for a further 14 days to differentiate)] were first found among cells dissected from 18-20 sp stage embryos.

Because of the highly migratory nature of HSCs and progenitors, the riddle of their origin is difficult to solve once the circulation is established. Interestingly, the frequencies and types of progenitors found in the present investigation are very different from one organ to the other. Such differences have previously been described between human yolk sac and embryo (Huyhn et al., 1995), and argue against a substantial non-directional traffic of progenitors between hematopoietic sites. Interestingly, in this respect, the E15-17 yolk sac still harbors some CFU-GEMMs, suggesting that its hemogenic activity might be sustained longer than previously thought. Among the three organs under scrutiny here, the fetal liver differs from the placenta by having lower frequencies of CFU-GEMMs and HPP-CFCs, a much greater abundance of late progenitors such as CFU-GMs and CFU-Es, and a higher cellularity. Thus, the role of placental hematopoiesis is clearly different from that of the liver, where hematopoietic cells multiply and differentiate.

In conclusion, the presence of abundant early clonogenic progenitors point to the placenta as a major hematopoietic

organ of the fetus. Human cord blood progenitors at birth (Mayani and Lansdorp, 1998) are strikingly similar in frequency and potential to the progenitors detected here in the mouse placenta. Together with the anatomical and developmental relationships between umbilical cord and placenta, these similarities make it likely that cord blood progenitors come from the placenta.

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