Adult Hemoglobins Are Synthesized in Murine Fetal Hepatic Erythropoietic Cells

By Peter M. C. Wong, Siu-Wah Chung, John S. White, Susan M. Reicheld, Margaret Patterson, Bryan J. Clarke, and David H. K. Chui

The hemoglobins present in murine fetal hepatic erythroblasts on days 12–15 of gestation were studied by biochemical and immunocytologic techniques. In addition, fetal hepatic hemopoietic progenitor cells obtained from normal and mutant f/f mouse fetuses on days 11–13 of gestation were cultured in vitro with added erythropoietin and adult spleen cell conditioned medium to form large erythroid colonies. In all instances, adult hemoglobin synthesis was detected in the fetal hepatic erythroblasts and in the erythroid cell cultures in vitro. The tumor promoter, 12-O-tetradecanoylphorbol 13-acetate, enhanced the fetal hepatic erythroid colony growth in vitro, but did not alter the hemoglobin phenotypic expression.

On days 11–15 of gestation, pregnant mice were killed by cervical dislocation. Conceptuses were removed and placed in physiologic saline. The fetal livers were dissected free, and disaggregated into single cell suspension by aspiration through successively smaller hypodermic needles (gauges 21–27).

Hemoglobin Synthesis

³⁵FeCl₃ (New England Nuclear, Boston, MA; specific activity, 2–40 Ci/g) was added to fetal calf serum (Flow Laboratories, McLean, VA) and incubated at room temperature for at least 1 hr. To determine hemoglobin synthesis in circulating embryonic erythrocytes or disaggregated fetal hepatic erythroblasts, 10 μl of equilibrated ³⁵Fe-serum, containing approximately 2 μCi, was added to 2 × 10⁵ cells in 1 ml of medium consisting of Iscove modified Dulbecco's medium (Grand Island Biological Co., Grand Island, NY) and 2% fetal calf serum. The cell suspension was incubated at 37°C for 24 hr. After the incubation, the cells were harvested, washed in normal saline, and lysed in distilled water by freezing and thawing 3 times. The hemoglobins in the hemolysates were later analyzed by polyacrylamide slab gel electrophoresis, followed by autoradiography.

To study hemoglobin synthesis in erythroid cell cultures, 200 μl of a solution consisting of 100 μl of the equilibrated ³⁵Fe-serum, containing approximately 20 μCi, and 100 μl of Iscove modified Dulbecco's medium were added to 1 ml of methylcellulose culture. One to two days after the addition of the radioactive iron tracer, embryoid cells in the methylcellulose cultures were harvested and processed as described above. The total hemoglobin content in some erythroid cultures was determined by the benzidine peroxidase microassay.

Immunocytologic Studies

Male New Zealand white rabbits were immunized by repeated intramuscular injections of a mixture of complete Freund's adjuvant and hemolysates of peripheral blood present in adult mice or murine fetuses of days 13–14 of gestation. One week after the last injection, sera with an antibody titer of 1:8 to 1:32, according to the Ouchterlony double diffusion method, were obtained.

Adult and embryonic hemoglobins were purified by DEAE-cellulose anion-exchange column chromatography. The purity of the isolated adult and embryonic hemoglobins was ascertained by isoelectric focusing. The purified fraction of the embryonic hemoglobins contained HbE1, EI, and EII only. The purified fraction of adult hemoglobins was similarly free of embryonic hemoglobins.

For the purification of anti-HbA antibodies, the rabbit anti-HbA antisera were first absorbed with cyanogen-bromide-activated Sepharose 4B (Pharmacia, Piscataway, NJ) coupled with the purified fraction of HbE, and followed by positive affinity chromatography using Sepharose 4B coupled with purified HbA. The IgG
fraction was isolated by DEAE-cellulose column chromatography and conjugated with fluorescein isothiocyanate. For the purification of anti-HbE antibodies, the rabbit anti-HbE antisera was absorbed with Sepharose 4B coupled with adult hemolysate. The partially purified antisera were precipitated with (NH₄)₂SO₄, and the IgG fraction was isolated by DEAE-cellulose column chromatography. These were conjugated with rhodamine isothiocyanate.

The monospecificity of the antibodies that were conjugated with fluorescent dyes was ascertained by solid-phase radioimmunoassay. Briefly, 100 ng of purified HbA or HbE was placed in the wells of a polystyrene plate flat-bottomed microtiter plate (Fisher, Toronto, Ontario). Fifty microliters of purified antibodies at different dilutions was later added and incubated for 1 hr at room temperature. Subsequently, 50 μl of 125I-labeled protein-A (New England Nuclear; specific activity 10 μCi/μg), containing approximately 20,000 cpm, were added for an additional hour. The amount of anti-HbE antibodies, the rabbit anti-HbE antisera were absorbed to a polyvinyl chloride flat-bottomed microtiter plate (Fisher, Toronto, Ontario). Fifty microliters of purified antibodies at different dilutions was later added and incubated for 1 hr at room temperature. Subsequently, 50 μl of 125I-labeled protein-A (New England Nuclear; specific activity 10 μCi/μg), containing approximately 20,000 cpm, were added for an additional hour. The amount of protein-A attached to the rabbit-antibody–mouse-hemoglobin complexes was detected by the radioactivity measured in a Beckman gamma counter.

Smears of disaggregated fetal hepatic erythroblasts were prepared by cytocentrifugation. These were air-dried and fixed in acetone/methanol. They were stained with both anti-HbA antibodies at 1:8 dilution and anti-HbE antibodies at 1:10 dilution for 1 hr at room temperature. After washing, the slides were examined in a Zeiss microscope equipped for reflected light fluorescence microscopy.

Erythroid Cell Cultures

Erythroid cell cultures in methylcellulose were done as previously described. Sheep plasma erythropoietin, step 3 (Connaught Laboratory, Toronto, Ontario; specific activity 2–4 U/mg of protein) was added to these cultures, usually at a final concentration of 1–2 U/ml. Adult spleen cell conditioned medium was prepared from C57BL/6J mice and was added to account for 20% of the erythroid cultures.

Six to eight days later, the large erythroid colonies in the cultures were counted according to previously published criteria, and the erythroblasts in the cultures were harvested for hemoglobin analysis. Occasionally, individual erythroid colonies were picked from the cultures for smear preparation and cytologic examination.

Separation of Hemoglobins by Isoelectric Focusing

A sensitive microtechnique for separating hemoglobins by isoelectric focusing was developed. The gel consists of 3.6% acrylamide, 0.15% bisacrylamide, 7.5% (w/v) of Ampholine, pH 7.0–9.0 (LKB), 1.9% of Ampholine, pH 3.5–10.0, and 4.7% (v/v) glycerol (BDH Chemical Co., Toronto, Ontario). It was polymerized with riboflavin (0.0007%), ammonium persulfate (0.014%), and Temed (0.008%) under fluorescent light for 1 hr. All chemicals were obtained from BioRad Chemical Company (Richmond, CA) unless otherwise specified. The thin-layer microgel of approximately 0.4 mm in thickness was made to cover a 43 × 70 mm glass coverslip.

The anolyte was 0.1 M H₃PO₄, and the catholyte was 0.4 M NaOH. Prefocusing was carried out at 2 W constant power for 30 min, using the Pharmacia Power Pack (ECPS 3000/150). After the samples were applied, focusing was started at 2 W for 15 min and continued for another 30 min at 4 W. The temperature was maintained at 0 ± 2°C during the focusing procedure.

At the end of the run, the gel was fixed in 12.5% trichloroacetic acid (Fisher) and 4% sulfosalicylic acid (Sigma, St. Louis, MO) for 10 min. It was then stained for 20 min with 2% (w/v) benzidine dihydrochloride (Sigma) in 0.5 M acetic acid containing 0.015% (v/v) H₂O₂. After staining, the gel was immersed in 0.5 M ammonium acetate, subsequently air-dried, and scanned by a Beckman densitometer (model CDS-200).

12-O-Tetradecanoylphorbol 13-Acetate (TPA)

TPA was purchased from Sigma Chemical Co. It was dissolved in acetone at concentrations of 1.6 × 10⁶ M to 1.6 × 10⁸ M. These were further diluted with Iscove modified Dulbecco medium to concentrations of 1.6 × 10⁴ M to 1.6 × 10⁶ M. To each milliliter of methylcellulose culture, 100 μl of these TPA solutions was added to make up the final concentration of 1.6 × 10⁴ M to 1.6 × 10⁶ M TPA. The final concentration of acetone in such erythroid cultures was 0.1% (v/v).

RESULTS

Hemoglobins in Fetal Hepatic Erythroblasts

Biochemical studies. Fetal hepatic erythroblasts on day 14 of gestation were labeled with radioactive iron for 24 hr, and the hemolysate was subjected to polyacrylamide gel electrophoresis followed by autoradiography. Figure 1 illustrates that only adult hemoglobin synthesis was observed. No embryonic hemoglobins could be identified with confidence, even when the gels were purposely overloaded with the hemolysate or overexposed. Similar results were obtained when fetal hepatic erythroblasts on day 13 or day 15 of gestation were studied. Studies on hemoglobin synthesis in fetal hepatic erythroblasts prior to day 13 were not performed because, during those days of gestation, almost all of the cells in the circulation are derived from yolk sac blood island nucleated erythrocytes, which actively synthesize primarily embryonic hemoglobins. Instead, immunocytologic studies were done in order to ascertain if embryonic hemoglobins are present in fetal hepatic erythroblasts of earlier gestation.

![Autoradiograph of polyacrylamide slab gel electrophoresis of ⁵⁹Fe-labeled hemoglobins. Lane 1: mixture of hemolysates of day-10 peripheral blood and day-14 fetal hepatic erythroblasts, both labeled with ⁵⁹Fe for 24 hr (the adult minor and embryonic EI hemoglobins comigrate). Lane 2: hemolysate of day-14 fetal hepatic erythroblasts; lane 3: hemolysate of day-10 peripheral blood; lane 4: day-13 fetal hepatic cells cultured for 8 days with 20% adult spleen cell conditioned medium and 2 U erythropoietin/ml; ⁵⁹Fe was added on days 6–8 of culture. Lane 5: hemolysate of day-14 fetal hepatic erythroblasts.](image-url)
Table 1. Solid-Phase Radioimmunoassay to Demonstrate the Monospecificity of Fluorescein-Labeled Rabbit Anti-Mouse HbA Antibodies

<table>
<thead>
<tr>
<th>Dilution of Antibodies</th>
<th>Hb Tested</th>
<th>Fluorescein-Labeled Anti-HbA</th>
<th>Control Rabbit IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/8</td>
<td>HbA</td>
<td>5,250 cpm</td>
<td>620 cpm</td>
</tr>
<tr>
<td></td>
<td>HbE</td>
<td>300 cpm</td>
<td>400 cpm</td>
</tr>
<tr>
<td>1/50</td>
<td>HbA</td>
<td>1,020 cpm</td>
<td>430 cpm</td>
</tr>
<tr>
<td></td>
<td>HbE</td>
<td>230 cpm</td>
<td>220 cpm</td>
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</tbody>
</table>

One hundred nanograms of purified HbA or HbE was placed in microtiter wells and later incubated for 1 hr with 50 µl of antibody or an equivalent amount of normal control rabbit IgG. The rabbit-antibody-mouse-hemoglobin complexes were detected by ¹²⁵I-labeled protein-A (see text).

Table 2. Solid-Phase Radioimmunoassay to Demonstrate the Monospecificity of Rhodamine-Labeled Rabbit Anti-Mouse HbE Antibodies

<table>
<thead>
<tr>
<th>Dilution of Antibodies</th>
<th>Hb Tested</th>
<th>Rhodamine-Labeled Anti-HbE</th>
<th>Control Rabbit IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/10</td>
<td>HbA</td>
<td>740 cpm</td>
<td>800 cpm</td>
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<tr>
<td></td>
<td>HbE</td>
<td>6,290 cpm</td>
<td>890 cpm</td>
</tr>
<tr>
<td>1/20</td>
<td>HbA</td>
<td>360 cpm</td>
<td>790 cpm</td>
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<tr>
<td></td>
<td>HbE</td>
<td>6,140 cpm</td>
<td>680 cpm</td>
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</tbody>
</table>

One hundred nanograms of purified HbA or HbE was placed in microtiter wells and later incubated for 1 hr with 50 µl of antibody or an equivalent amount of normal control rabbit IgG. The rabbit-antibody-mouse-hemoglobin complexes were detected by ¹²⁵I-labeled protein-A (see text).

Fig. 2. Immunocytologic studies of disaggregated day-12 fetal hepatic erythroblasts, double labeled with anti-HbA and anti-HbE antibodies. (Top) Immunofluorescence of fluorescein-conjugated anti-HbA. Note that all fetal hepatic erythroblasts and one circulating primitive nucleated erythrocyte (arrow) are stained. (Bottom) Immunofluorescence of rhodamine-conjugated anti-HbE. Note that only the primitive nucleated erythrocyte, characterized by its eccentric and pyknotic nuclei and low nuclear-cytoplasmic ratio, is stained (700×).
HEMOGLOBIN ONTOGENY

Fig. 3. Photomicrograph of a large erythroid colony. Disaggregated fetal hepatic cells on day 13 of gestation were cultured in methylcellulose for 6 days in the presence of erythropoietin (1 U/ml) and 20% adult spleen cell conditioned medium (80×).

Immunocytologic studies. The monospecificity of the rabbit anti-mouse adult and embryonic hemoglobin antibodies conjugated with fluorescent dyes was confirmed by solid-phase radioimmunoassay (Tables 1 and 2). Fetal livers of day 12 of gestation were disaggregated into single-cell suspensions. Smears made and double stained with anti-HbA antibodies conjugated with fluorescein and anti-HbE antibodies conjugated with rhodamine (Fig. 2). The circulating yolk sac blood islands derived erythrocytes, characterized by their small pyknotic nuclei and low nuclear cytoplasmic ratio, were stained by both anti-HbA and anti-HbE antibodies, indicating that both adult and embryonic hemoglobins are present, as has been previously described.7 On the other hand, the fetal hepatic erythroblasts were stained with anti-HbA but not with anti-HbE antibodies, indicating that only adult hemoglobins were detected in these erythroblasts.

Erythroid Colonies in Culture

Fetal hepatic hemopoietic progenitor cells of day 13 of gestation were cultured in methylcellulose with erythropoietin and spleen cell conditioned medium. Large erythroid colonies, each consisting of hundreds of well hemoglobinized erythroblasts, were observed from the sixth to the eighth day of culture (Fig. 3). In some of these large erythroid colonies, granulocytic cells, with their characteristic segmented nuclei, were present. Occasionally, large cells with multilobulated nuclei, morphologically resembling megakaryocytes, were also seen. Cultures with erythropoietin alone produced erythroid colonies much smaller in size when compared to cultures in which both erythropoietin and spleen cell conditioned medium were added.

Hemoglobin Synthesis

Day 13 normal fetal hepatic cells were cultured with erythropoietin and spleen cell conditioned medium. After 6 days in vitro, 59Fe was added to the cultures. The erythroblasts in the cultures were harvested 2 days later. The hemoglobins labeled by radioactive iron were identified by electrophoresis, followed by autoradiography. Figure 1 shows that synthesis of adult major and minor hemoglobins, but not embryonic hemoglobin, was detected in these cultures. Similar results were observed when fetal hepatic cells from day-13 mutant f/f fetuses were cultured (Fig. 4).

In order to determine if erythropoietin plays a role in altering the hemoglobin phenotypic expression, day-13 fetal hepatic cells were cultured in the presence of various doses of erythropoietin for 8 days. The hemoglobins synthesized were labeled with 59Fe during the last 24 hr of culture.

In the cultures with spleen cell conditioned medium, but in which exogenous erythropoietin was not added, cellular colonies were observed, but the number of well hemoglobinized erythroblasts was comparatively few. On the autoradiograph, a very faint band corresponding to the adult major hemoglobin was seen (Fig. 5).

In the cultures in which erythropoietin was present at the concentrations of 1 U and 10 U/ml, synthesis of adult major and minor hemoglobins was observed. No embryonic hemoglobin synthesis could be detected.
Fig. 4. Autoradiograph of polyacrylamide gel electrophoresis of $^{55}$Fe-labeled hemoglobins. Lane 1: hemolysate of day-13 mutant 5/f fetal hepatic cells cultured for 5 days with erythropoietin, spleen cell conditioned medium, and $10^{-6}$ M TPA; $^{55}$Fe was added during the last 48 hr of culture. Lane 2: same as in lane 1, except that TPA was not added to the culture. Lane 3: hemolysate of day-10 peripheral blood. Lane 4: hemolysate of day-13 fetal hepatic erythroblasts.

(Fig. 5). These results indicate that erythropoietin is not capable of activating embryonic hemoglobin synthesis in erythroid colonies derived from fetal hepatic hemopoietic progenitor cells.

**Hemoglobin Content**

A sensitive microtechnique of isoelectric focusing for hemoglobin separation was developed in order to analyze the types of hemoglobins present in erythroid cell cultures in vitro. Preliminary experiments showed that as little as 0.3 μg of hemoglobin in each band could be clearly detected by this isoelectric focusing procedure. The identity of each of the hemoglobin bands in the isoelectric focusing was ascertained by analysis of isolated adult and embryonic hemoglobins eluted from the polyacrylamide slab gel electrophoresis of day-14 fetal peripheral blood hemolysate (Fig. 6).

Fig. 5. Autoradiograph of polyacrylamide slab gel electrophoresis of $^{55}$Fe-labeled hemoglobins. Lane 1: hemolysate of day-14 fetal hepatic erythroblasts; lane 2: day-13 fetal hepatic cells cultured for 8 days with 20% adult spleen cell conditioned medium with $^{55}$Fe added during the last 24 hr of culture; lane 3: same experiment as in lane 2 except that erythropoietin, 1 U/ml, was added to the culture in addition to the spleen cell conditioned medium; lane 4: same experiment as in lane 2 except that erythropoietin, 10 U/ml, was added to the culture in addition to the spleen cell conditioned medium; lane 5: hemolysate of day-10 peripheral blood.

Fig. 6. Isoelectric focusing of hemoglobins. Hemolysate of day-14 peripheral blood cells was separated by polyacrylamide slab gel electrophoresis. Each hemoglobin band was then eluted and run on isoelectric focusing, pH 7–9. Lane 1: eluate from the band containing adult minor and embryonic El hemoglobins. Note that there are contaminating adult major hemoglobin and embryonic El hemoglobins. Lane 2: eluate from adult major hemoglobin band. Lane 3: eluate from the embryonic ElI hemoglobin band. Note that there is some contaminating embryonic El hemoglobin. In a separate experiment, the hemoglobin band visualized between the embryonic El and adult major hemoglobins in the isoelectric focusing is equivalent to the adult leading minor band on the polyacrylamide gel electrophoresis.
Day-13 fetal hepatic cells were cultured in methylcellulose for 8 days with erythropoietin and spleen cell conditioned medium. The hemolysate of the erythroblasts in the cultures was analyzed by isoelectric focusing, and adult hemoglobins were detected (Fig. 7). No embryonic hemoglobins could be detected with confidence, even when the isoelectric focusing was overloaded with five times the optimal amount of the hemolysate (Fig. 7). Similar results were obtained in erythroid cultures of days 11 and 12 fetal hepatic cells (data not shown).

**Effect of TPA on Erythroid Culture and Hemoglobin Synthesis**

The tumor promoter, 12-O-tetradecanoylphorbol 13-acetate, was added to the fetal hepatic erythroid cultures in the presence of both erythropoietin and spleen cell conditioned medium. As shown in Fig. 8, fetal hepatic cells obtained from embryos of day 11 or day 12 of gestation had a high plating efficiency for large erythroid colony growth. TPA appeared to have little effect on these erythroid cultures in terms of the colony number or the total hemoglobin content in these cultures (Figs. 8 and 9). On the other hand, there were 945 large erythroid colonies when 10^9 fetal hepatic cells of day 13 of gestation were cultured for 6–8 days in vitro. Addition of TPA at concentrations of 1.6 \times 10^{-7} M and 1.6 \times 10^{-8} M increased the large erythroid colony counts to 1,800 and 1,730, respectively (Fig. 8), significantly more when compared to the control cultures or the cultures with only acetone added (p < 0.001). Moreover, Fig. 9 shows that the total hemoglobin...
bin content in cultures of day-13 fetal hepatic cells with TPA at similar concentrations was significantly higher than those cultures to which TPA was not added (p < 0.001).

Hemoglobin analysis by either autoradiography of ⁵⁹Fe-labeled cultures or isoelectric focusing of culture hemolysates demonstrates that only adult hemoglobins were synthesized, and no embryonic hemoglobin synthesis could be detected with confidence (Figs. 10–12). These results indicate that TPA augments day-13 fetal hepatic erythroid cell growth, but does not alter the type of hemoglobins synthesized in culture.

**DISCUSSION**

It has been generally accepted, on the basis of previous biochemical investigations, that murine fetal hepatic erythroblasts synthesize adult hemoglobins, but not embryonic hemoglobins. The present investigation utilizes both biochemical and immunocytologic methods and confirms that adult hemoglobins are produced in fetal hepatic erythroblasts. It is possible that embryonic hemoglobins may be synthesized in these erythroid cells in such a minute amount that they could not be detected by the present techniques. The sensitivities of the autoradiography for detecting ⁵⁹Fe-labeled hemoglobins and the immunocytologic method have not been definitively determined. Nevertheless, in all the studies of this investigation, results have been corroborated by at least two different experimental approaches and are consistent with previously published data in the mouse. Recently, it was reported that embryonic hemoglobins are present in neonatal
hemoglobin and spleen. The apparent difference between the findings in the mouse and the hamster may be due to species differences. Nevertheless, it is important to further document the presence of embryonic hemoglobins in hamster neonatal hepatic erythroblasts at the cellular level.

Hemopoietic progenitor cells are present in mouse fetal livers during early gestation. When cultured in vitro with erythropoietin and spleen cell conditioned medium, these progenitor cells give rise to many large erythroid colonies. In some of these erythroid colonies, there are granulocytic cells and possibly megakaryocytes, indicating that the progenitor cells of these colonies with mixed cellularity are multipotential.

It was previously reported that adult hemoglobin synthesis was detected in short-term cultures of fetal hepatic erythroid cells. In the present study, fetal hepatic hemopoietic progenitor cells were cultured in vitro to give rise to large erythroid and mixed cellular colonies. The hemoglobins formed in the erythroid colonies in vitro were studied by two biochemical techniques. Newly synthesized hemoglobins were labeled with radioactive iron and analyzed by polyacrylamide slab gel electrophoresis and autoradiography. In addition, all the hemoglobins present in the erythroid cells harvested from the cultures were analyzed by isoelectric focusing and the hemoglobin bands visualized by the sensitive benzidine-peroxide staining technique. In all instances, only adult hemoglobins were observed in the fetal hepatic erythroid cell cultures. No embryonic hemoglobins could be detected with confidence, even when the isoelectric focusing gels were overloaded with the hemolysate of the erythroblasts harvested from the cultures in vitro. Recently, the synthesis of embryonic hemoglobins in cultures of early embryonic erythroid progenitor cells prior to day 9 of gestation was reported, thus ruling out the remote possibility that the in vitro cultures would only allow the expression of adult hemoglobin synthesis.

Genetic mutant / μ mice have severe hypochromic microcytic anemia, as well as unbalanced globin chain synthesis, during their fetal development. The present investigation shows that the erythroid cultures of the fetal hepatic cells from these anemic mouse embryos produced adult hemoglobins.

The tumor promoter, 12-0-tetradecanoylphorbol 13-acetate, has been reported to be able to augment erythroid colony growth derived from day-13 fetal hepatic cells. These observations were confirmed in the present study. Moreover, TPA did not alter the hemoglobin phenotypic expression in the fetal hepatic erythroid cell cultures.

During murine embryogenesis, the circulating primitive erythrocytes derived from the yolk sac blood islands produce both embryonic and adult hemoglobins. Beginning on day 9 of gestation, there are hemopoietic progenitor cells in the yolk sac as well as in the embryonic circulation, which can give rise to erythroblasts synthesizing adult hemoglobins. The results of the present investigation suggest that the fetal hepatic erythroblasts as well as the hemopoietic progenitor cells in the fetal liver, from day 11 of gestation on, are committed to adult hemoglobin synthesis and have lost the capability of producing embryonic hemoglobins. These results lend support to the hypothesis that circulating hemopoietic progenitor...
cells seed the developing fetal liver to initiate erythropoiesis in that organ.

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