

The human placenta is a hematopoietic organ during the embryonic and fetal periods of development[☆]

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ABSTRACT

We studied the potential role of the human placenta as a hematopoietic organ during embryonic and fetal development. Placental samples contained two cell populations—CD34⁺CD45^{low} and CD34⁺CD45^{low}—that were found in chorionic villi and in the chorioamniotic membrane. CD34⁺CD45^{low} cells express many cell surface antigens found on multipotent primitive hematopoietic progenitors and hematopoietic stem cells. CD34⁺CD45^{low} cells contained colony-forming units culture (CFU-C) with myeloid and erythroid potential in clonogenic in vitro assays, and they generated CD56⁺ natural killer cells and CD19⁺CD20⁺sIgM⁺ B cells in polyclonal liquid cultures. CD34⁺CD45^{low} cells mostly comprised erythroid- and myeloid-committed progenitors, while CD34⁻ cells lacked CFU-C. The placenta-derived precursors were fetal in origin, as demonstrated by FISH using repeat-sequence chromosome-specific probes for X and Y. The number of CD34⁺CD45^{low} cells increased with gestational age, but their density (cells per gram of tissue) peaked at 5–8 wk, decreasing more than sevenfold at the onset of the fetal phase (9 wk of gestation). In addition to multipotent progenitors, the placenta contained myeloid- and erythroid-committed progenitors indicative of active in situ hematopoiesis. These data suggest that the human placenta is an important hematopoietic organ, raising the possibility of banking placental hematopoietic stem cells along with cord blood for transplantation.

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Introduction

Human hematopoiesis begins at ~16–18.5 days of development in the yolk sac (Lockett, 1978) and is gradually replaced by sequential intraembryonic sites, such as the aorta-gonad-mesonephros (AGM) region and the embryonic liver (Tavian et al., 1996). Hematopoietic progenitors expressing CD34 and CD45 are first detected in the AGM at day 27 (Tavian et al., 1999), quickly expand, and disappear by day

40. In the current model of hematopoietic development, intraembryonic hematopoietic stem cells (HSCs), presumably produced in the AGM region, establish definitive hematopoiesis (Cumano et al., 2000). However, the AGM region, which contains a limited number of HSCs, is hematopoietic for only a short time, suggesting either that AGM-derived HSCs rapidly and efficiently migrate to the liver or that HSCs are being produced in another, unidentified tissue.

The presence of HSCs in the mouse placenta with potential similar to that of bone marrow (BM) cells was suggested some 40 years ago by Till and McCulloch (1961) and Dancis et al. (1968). In 1979, Melchers showed that the mouse placenta contains hematopoietic progenitors before the fetal liver is hematopoietic (Melchers, 1979). Two groups recently reported that the mid-gestation murine placenta is a richly hematopoietic organ that contains high-proliferative-potential colony-forming cells of fetal origin (Alvarez-Silva et al., 2003) and HSCs that can reconstitute hematopoiesis in adult mice (Ottersbach and Dzierzak, 2005). Furthermore, murine placental hematopoiesis is present before the placental circulation is established, demonstrating that the placenta is not only a niche but also a source of hematopoietic cells that functions at the same time as the AGM (Corbel et al., 2007;

Abbreviations: AGM, aorta-gonad-mesonephros; APC, allophycocyanin; BFU-E, burst-forming unit–erythroid; CFU-C, colony-forming unit culture; Epo, erythropoietin; FACS, fluorescence-activated cell sorter; FL, Flt-3/Flk-2 ligand; PI, propidium iodide; SDM, serum-deprived medium; TPO, thrombopoietin; UCB, umbilical cord blood.

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Rhodes et al., 2008; Zeigler et al., 2006). These findings strongly support the inclusion of the placenta in our current model of site-specific and orchestrated embryonic hematopoiesis.

The hematopoietic potential of the human placenta is currently unknown. CD235a⁺ (glycophorin A) CD34⁺CD45⁻ erythroblast cells, but not CD34⁺CD45⁺ progenitors, have been identified in first and second trimester placentas (Challier et al., 2001, 2005). In recent reports, a pluripotent cell population expressing markers of mesenchymal and embryonic stem cells was described in crude preparations of term placental cells (Fukuchi et al., 2004; Yen et al., 2005) and in association with the amniotic epithelium (Miki et al., 2005). Pluripotent stem cell markers, such as SSEA-3, -4, and TRA 1-60, 1-81, have been detected in the amnion (Miki et al., 2007). The placental mesenchymal populations display adipogenic, osteogenic, neurogenic, hepatogenic and pancreatogenic potential (Chang et al., 2007; Chien et al., 2006; Fukuchi et al., 2004). However, the hematopoietic potential of these placental cell populations has not been explored. In the present study, we searched for hematopoietic progenitors in the human placenta by using flow cytometry and confocal microscopy. We also investigated whether the output of this extraembryonic hematopoietic niche remains constant or changes over time, by estimating the frequency of hematopoietic progenitors in placentas of different gestational ages (from 5.4 to 39.5 weeks). We then evaluated the hematopoietic potential of cells isolated by cell sorting using *in vitro* hematopoietic progenitor assays.

Materials and methods

Isolation of placental hematopoietic progenitors

This study was approved by the University of California–San Francisco Committee on Human Research. We used the basic method that our group devised for isolating human placental cells (Fisher et al., 1989), with a few modifications that significantly improved the recovery of hematopoietic cells. We added a final enzymatic digestion treatment of the placental cell preparations with 181 U/ml collagenase I-A, 0.12 mg/ml DNase I, 0.70 mg/ml hyaluronidase type I-S, and 1 mg/ml BSA in PBS at 37 °C. Digestion continued for 5–60 min until total cellular dissociation was observed. Then the cells were centrifuged over Nycoprep (1.077 g/ml; Greiner Bio-One, Monroe, NC) for 30 min (25 °C) at 600 ×g. Further purification of CD34⁺CD45^{low} or CD34⁺CD45^{low} cells was achieved by staining the cell suspensions with directly conjugated mAbs—anti-CD34-allophycocyanin (APC) (Beckman Coulter, Fullerton, CA) and anti-CD45-PE (Invitrogen/Caltag, Carlsbad, CA)—and sorted using FACSDiva or a FACSAria (Becton Dickinson, San Jose, CA).

Isolation of hematopoietic progenitors from umbilical cord blood and fetal BM

Samples were processed immediately after being obtained, by a method described previously (Bárcena et al., 1999). The resulting light-density cell suspension of fetal BM or UCB was used for phenotypic analyses, FISH, or cell-sorting experiments as described below for placental cells. At least 3 preparations of each type were analyzed.

Reagents

Isotype control antibodies IgG1, IgG2a, IgG2b and IgG3 were purchased from BD Biosciences (San Jose, CA); IgM was obtained from Invitrogen/Pharmingen. mAbs were conjugated to FITC, PE or APC as indicated in the figure legends. Propidium iodide (PI) (Sigma-Aldrich, St. Louis, MO) was used at a final concentration of 1 μg/ml to identify dead cells. Antibodies used for cell separation and immunofluorescence are listed, along with their manufacturers, in Supplemental

Table 2. Cytokines, purchased from R&D Systems (Minneapolis, MN), are listed in Supplemental Table 3.

Hematopoietic progenitor assays

To enumerate CFU-C, methylcellulose clonal cultures of sorted CD34⁺CD45^{low} and CD34⁺CD45^{low} placental cells or UCB were prepared at a density of 5×10^2 /35-mm plate (BD Falcon, BD Biosciences) in either Methocult GF H4435 (StemCell Technologies, Inc., Vancouver, BC, Canada) or SDM supplemented with the same concentration of methylcellulose and cytokines present in Methocult (Bárcena et al., 1996). In some experiments, two different batches of characterized FBS (Hyclone) and one batch of FBS (Stem Cell Technologies Inc.) was added to SDM at the concentrations indicated in the figures. Colonies containing erythrocytes were scored as BFU-E after 3 weeks at 37 °C in 5% CO₂ in air. CFU-GM (colony-forming unit granulocyte-macrophage) colonies that expressed CD14 and CD15 were identified by morphology and flow cytometry (Muench and Bárcena, 2001). CFU-mix colonies contained both granulocyte-macrophage and erythroid progenitors.

Lymphoid- and myelo-erythroid-megakaryocyte differentiation in liquid cultures

Sorted CD34⁺CD45^{low} cells were counted, washed in PBS containing 0.5% BSA, and cultured at 5×10^3 to 7.5×10^3 cells/ml in 48-well plates (Costar, Cambridge, MA) in the presence of SDM supplemented with 50 ng/ml SCF, 100 ng/ml Flt-3/Flk-2 ligand (FL), 20 ng/ml interleukin (IL)-15, 20 ng/ml IL-7+20 ng/ml GM-CSF+20 ng/ml IL-3 (lymphoid-permissive conditions (Muench and Bárcena, 2001), or the combination of 50 ng/ml SCF, 100 ng/ml FL, 20 ng/ml IL-15, 20 ng/ml GM-CSF, 20 ng/ml IL-3, 20 ng/ml thrombopoietin (TPO)+10 U/ml erythropoietin (Epo) (myelo-erythroid-megakaryocyte-permissive conditions (Muench and Bárcena, 2004)). Medium was refreshed 2× a week by changing half the volume and adding fresh cytokines. After 21–23 days in culture at 37 °C in 5% CO₂ in air, cells were harvested, washed with PBS supplemented with 0.5% BSA, and stained with directly conjugated mAbs before flow cytometric analyses.

B-cell differentiation cultures

Non-irradiated MS-5 murine BM stromal cells (Itoh et al., 1989) (kindly provided by K.J. Mori, Niigata University, Niigata, Japan) were maintained in α-minimal essential medium (Invitrogen/Gibco) supplemented with 10% characterized FBS (Hyclone), 50 μM 2-mercaptoethanol (Sigma), 50 U/ml of penicillin G and 50 μg/ml of streptomycin sulfate (Invitrogen). Two days before seeding of placental progenitors, MS-5 cells were prepared at a concentration of 10⁴ cells/well in 48-well tissue culture plates (Becton Dickinson) coated with 0.1% gelatin (Sigma). CD34⁺CD45^{low} placental cells were sorted as described above and cultured at a density of 5×10^4 cells/well in 0.4 ml on the MS-5 feeders in Iscove modified Dulbecco medium (Invitrogen/Gibco) supplemented with 5% FBS, 5% human AB serum (Gemini Bio-Products, West Sacramento, CA), 50 ng/ml SCF, 100 ng/ml FL and 20 ng/ml IL-7. The cells were cultured for 3 weeks at 37 °C in 5% CO₂ in air and were fed every week by removing half the medium and replacing it with fresh medium and cytokines. After 3 weeks in culture, cells were harvested using 0.05% trypsin (Invitrogen/Gibco), washed and stained with mAbs against CD19, CD20, IgM and CD45 prior to flow cytometric analyses.

Immunofluorescence and flow cytometry

Cell surface phenotypic analyses were performed as described (Bárcena et al., 1993) using a two-laser FACSCalibur or LSR II cytometer (BD Biosciences) and CellQuest (BD Biosciences) or FlowJo (Tree Star, Inc., Ashland, OR) software.

FISH

CD34⁺⁺CD45^{low} cells were isolated by cell sorting from the placenta of a 19-week male fetus. BM from the same conceptus was used as a positive control. We obtained 9×10^3 CD34⁺⁺CD45^{low} placental cells and 6.6×10^4 CD34⁺⁺CD45^{low} fetal BM cells. Cells (2×10^2 to 4×10^2) were deposited on slides immediately after purification, and FISH was performed with X and Y chromosome-specific probes using methods that we have published (Weier et al., 2005). Results were confirmed by performing identical FISH analyses on total CD45⁺ cells from first and second trimester placentas.

Immunofluorescence and confocal microscopy

Freshly isolated placental tissue from 7 to 17 weeks of gestation was fixed in 3% paraformaldehyde for 90 min, and processed as previously described (Prakobphol et al., 2006). Biopsies were imaged

using a Leica MZ-16 confocal microscope with a DFC camera. Sections (30 μ m) of the fixed/frozen tissue were stained with mAbs against CD34 (directly conjugated to FITC; BD Pharmingen, clone 581), CD45 (Dako, clones 2B11 +PD7/26), or vimentin (Abcam Inc, Cambridge, MA, clone SP20). The binding of antibodies that were not direct conjugates was detected using the appropriate species-specific secondary antibody, either rhodamine-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc, West Grove, PA) or Alexa 594-conjugated goat anti-rabbit (Invitrogen/Molecular Probes). As a control, some sections were stained with the secondary antibody alone. Staining with Hoescht 33542 or TOTO-3 iodide (Invitrogen/Molecular Probes) was used to visualize nuclei. Slides were imaged on a Leica CTR 6500 microscope equipped with UV 488, 546 and 633 lasers when using Hoescht staining or on a Zeiss LSM 5 Pascal when using TOTO-3. Scanned images were processed using the Leica TCS SP5 software package. Z projections were generated in ImageJ software.

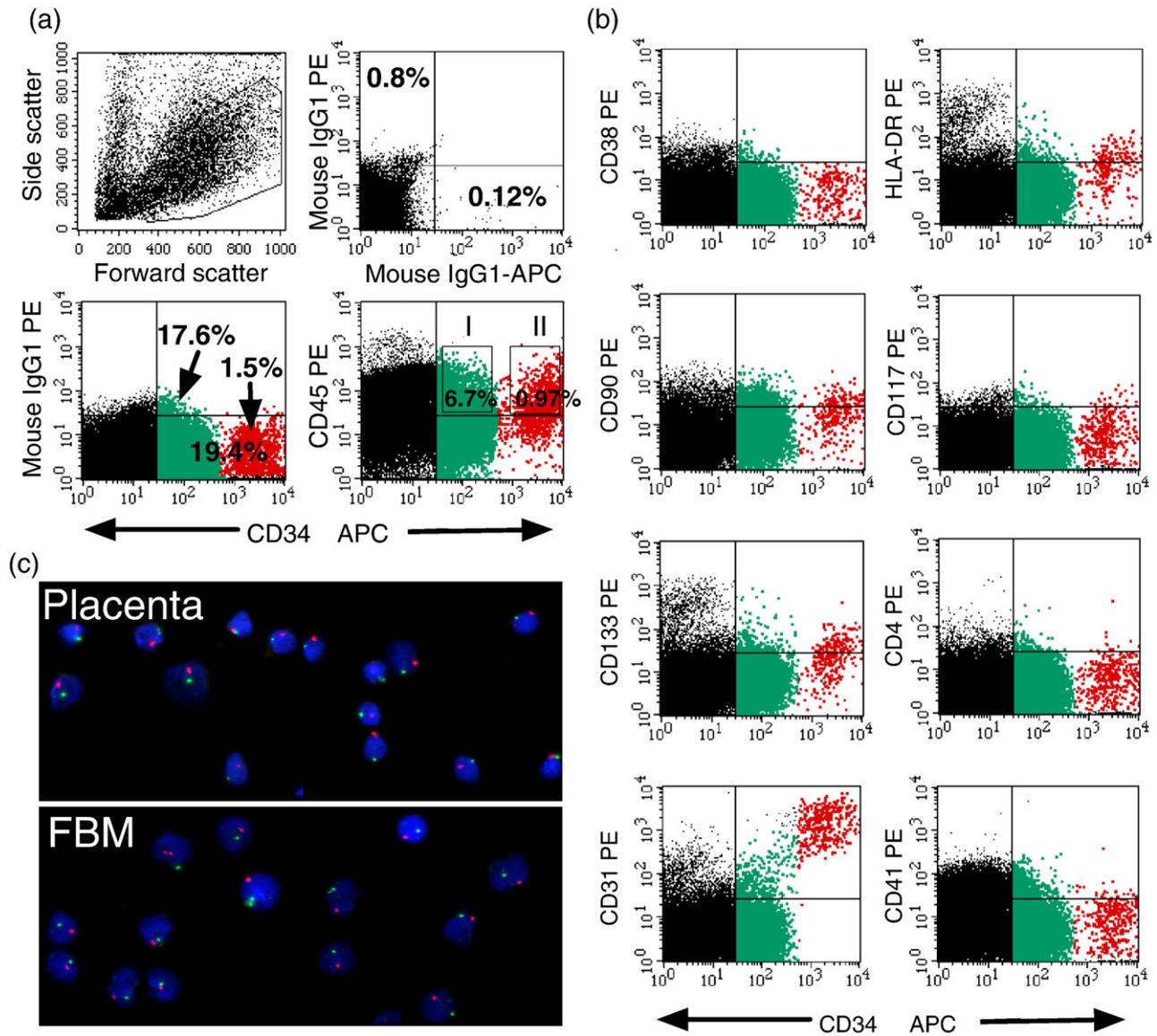


Fig. 1. Phenotypic profile and fetal origin of placental hematopoietic progenitors. In panels a and b, freshly isolated light-density cell suspension from a 10-week placenta was stained with the indicated mAbs and PI, to identify dead cells. (a) The forward versus side-scatter profile of 10^5 cells and the electronic gate used to include all hematopoietic cells. Another gate was set to exclude PI⁺ cells (not shown). Isotype-matched negative controls are included. The gates set for cell sorting experiments used in panel c and in Figs. 6 and 7 are shown in the CD34-APC versus CD45-PE dot plot (I for CD34⁺CD45^{low}; II for CD34⁺⁺CD45^{low}). (b) Staining of the same cells with mAbs against markers expressed by hematopoietic progenitors. Multicolor analysis was performed to highlight the CD34⁺⁺CD45^{low} (red) and CD34⁺CD45^{low} (green) cells. Results are representative of 12 experiments. (c) Sorted CD34⁺⁺CD45^{low} cells (using gate II in panel a) from placenta and from fetal BM (FBM) from the same 19-week specimen were hybridized with fluorescent probes specific for the X (green) or Y (red) chromosomes. Cell nuclei were stained with 4',6'-diamidino-2-phenylindole (blue). Photomicrographs of the slides at 40 \times magnification were taken with a Nikon E-800 microscope/camera.

Results

The placenta contains hematopoietic progenitors of fetal origin

Placental samples from 5.4 to 39.5 weeks of pregnancy were analyzed for cells expressing both CD34 and CD45 hematopoietic progenitor cell surface markers (Lansdorp et al., 1990). Initially, we focused on these cells so that we could discriminate between the hematopoietic population and endothelial cells with a CD34⁺CD45⁻ phenotype. Two populations with phenotypic characteristics of hematopoietic progenitors were observed at the following frequencies and ranges among the light density fraction of placental cells: CD34⁺CD45^{low} (2.86–20.91%) and CD34⁺⁺CD45^{low} (0.03–1.2%) ($n=59$) (Fig. 1a). Expression of additional antigens displayed by multipotent progenitors and HSCs was investigated (see Supplemental Table 1). CD34⁺⁺CD45^{low} and CD34⁺CD45^{low} cells were largely CD38⁻ (Fig. 1b). Otherwise, placental cells were phenotypically similar to the equivalent populations found in fetal liver and BM; some expressed HLA-DR, CD133, low levels of CD117 (c-kit), CD90 (Thy-1), and high levels of CD31 (PECAM-1). Neither CD34⁺⁺CD45^{low} nor CD34⁺CD45^{low} cells expressed detectable levels of CD4, CD41 (Fig. 1b), CD95 or CXCR4 (data not shown). Both populations expressed CD13 and CD33 (Fig. 5b) and CD44 (data not shown).

The low to negative expression of CD38 by placental hematopoietic progenitors was very intriguing, since CD34⁺⁺CD38⁻ cells are considered to be highly enriched in HSC. CD38 is a type II transmembrane glycoprotein, and an ectoenzyme (ADP-ribosyl cyclase), which expression is upregulated during differentiation of hematopoietic progenitors (Deaglio et al., 2001). We investigated whether the low CD38 expression could be due to the enzymatic digestion of the placental samples or it is intrinsic to placental tissue. Two approaches were taken to determine the effect of enzymatic digestion on the CD38 antigen. In one set of experiments, two first trimester and two second trimester placentas were processed by two methods: half of the tissue was dissociated using the usual enzymatic method (using collagenase I and trypsin) and the other half was processed avoiding trypsin and instead, using commercially available non-enzymatic dissociation

buffer. The resulting cells were stained with anti-CD38 mAbs and subjected to flow cytometry. No notable change in the mean intensity of CD38 expression or in the percentage of CD38⁺ cells was observed between the two dissociation methods. In a second set of experiments, fetal liver tissue, which expresses high levels of CD38 and contains abundant hematopoietic progenitors (Muench et al., 1994), was also dissociated using enzymatic and non-enzymatic methods. As with the results using placenta, no difference in CD38 expression by CD45⁺ cells was observed (data not shown). Therefore, we conclude that the CD38 antigen is relatively resistant to digestion by trypsin and the lack of CD38 expression by hematopoietic progenitors in the placenta is a tissue-specific characteristic of these cells.

To investigate the fetal vs. maternal origin of the placental hematopoietic progenitors, CD34⁺⁺CD45^{low} cells were sorted from a placenta at 19 wk of gestation and fetal BM from the same male specimen, as a fetal internal positive control. Slides were prepared immediately after the cell purification, and FISH experiments were performed using X (green) and Y (red) chromosome-specific probes. As shown in Fig. 1c, all the placental hematopoietic progenitors displayed both X and Y fluorescent chromosomes in the same manner as the fetal BM cells, demonstrating their fetal origin. Results were confirmed by performing identical FISH analyses on total CD45⁺ cells from first and second trimester placentas.

Hematopoietic progenitors are present in chorionic villi and fetal membranes

The finding of phenotypically identifiable hematopoietic progenitors in chorionic villi throughout gestation, along with recent reports of pluripotent stem cell markers in the amnion (Miki et al., 2007), led us to explore the presence of hematopoietic progenitors in the chorioamniotic membranes. Fetal membranes were carefully dissected from the villi of 7 placentas (6.3–15.3 wk), and both tissues were analyzed by flow cytometry (Fig. 2). Chorionic villi and fetal membranes contained similar percentages of CD34⁺⁺CD45^{low} cells and CD34⁺CD45^{low} cells, indicating their broad distribution. The number of CD34⁺⁺CD45^{low} cells isolated per gram of tissue from both

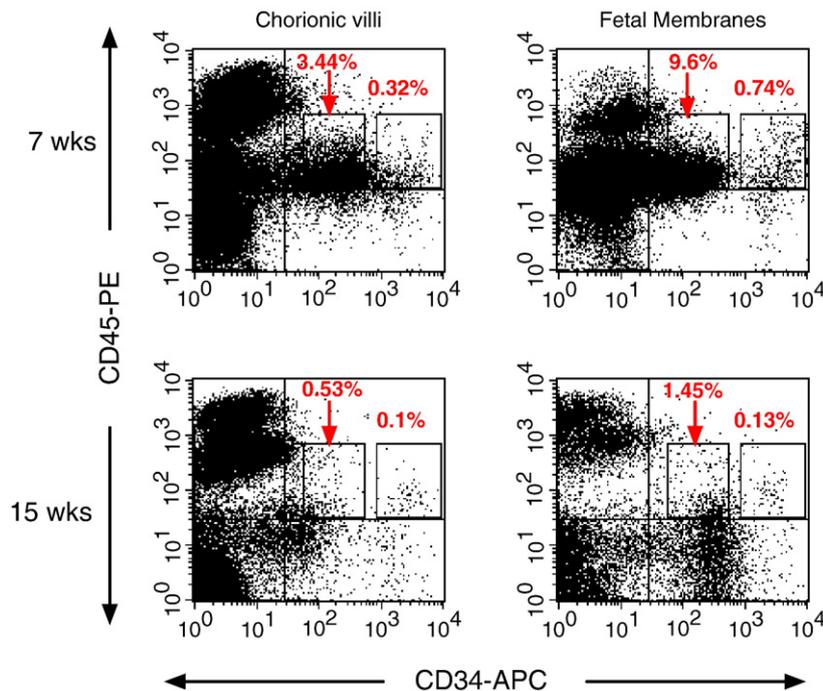


Fig. 2. Hematopoietic progenitors were present in chorionic villi and the chorioamniotic membranes. Freshly isolated light-density cells from villi and fetal membranes (A, pooled 6.3 +6.5 weeks; B, 15.3 weeks) were stained with CD34, CD45 and PI and analyzed (see Fig. 1 legend).

regions was also similar (data not shown). In preliminary experiments, CD34⁺CD45^{low} cells isolated from both the chorioamniotic membrane and chorionic villi generated hematopoietic colonies in standard methylcellulose cultures (data not shown), evidence that these two extraembryonic sites are potentially hematopoietic. The CD45⁺ cells lacking CD34 expression in both regions were primarily CD14⁺ Hofbauer cells (Fig. 5c).

The placenta is richer in hematopoietic progenitors in the embryonic phase of development than in the fetal phase

Variation in the frequency of CD34⁺CD45^{low} cells during gestation (0.03–1.2%) was analyzed on a large set of 59 samples. Although the

total number of CD34⁺CD45^{low} cells steadily increased as the placenta grew (Fig. 3a), the highest numbers per gram of tissue were recovered during the embryonic period (Fig. 3b). The frequency of CD34⁺CD45^{low} cells was about 7 times higher at 5–8 wk ($n=18$) than at 9–12 wk ($n=15$); afterward, the values remained fairly constant ($P<0.001$; unpaired Mann–Whitney t -test). A plot of the values from individual 5- to 12-wk placentas ($n=33$) showed that tissue from the earliest gestational age analyzed (5 weeks) contained the highest density of CD34⁺CD45^{low} cells (Fig. 3c). Similar results were obtained when the frequency of CD34⁺CD45^{low} cells was analyzed at different gestational ages (data not shown).

Immunolocalization of CD34⁺CD45⁺ cells in placenta

To identify and begin characterizing the placental hematopoietic niche, we investigated the physical location of CD34⁺CD45⁺ cells using both conventional immunofluorescence (data not shown) and confocal microscopy (Fig. 4). We observed that cells co-expressing CD34 and CD45 antigens reside in primarily two locations: near placental blood vessels of all sizes (Fig. 4D) and within the mesenchymal compartment of the villous cores (Fig. 4E). The sensitivity of the immunolocalization techniques did not allow us to distinguish between CD34⁺ and CD34⁺ populations, which we can only detect in quantitative manner using a fluorescence-activated cell sorter (FACS). The relatively low frequency of placental hematopoietic progenitors (0.03–1.2%) that was predicted by flow cytometric analyses was confirmed by confocal microscopy; we found 1–3 CD34⁺CD45⁺ cells per tissue section during 7 to 17 wk of gestation. Regardless of their location, these cells were frequently found in close contact with either endothelial CD34⁺CD45⁺ cells (as shown in Figs. 4B and D) or with vimentin-positive mesenchymal cells (Fig. 4E).

The placenta contains erythroid- and myeloid-committed progenitors

If placental hematopoiesis is an active process, we should find progenitors at various stages of differentiation and committed to several lineages. These experiments focused mostly on the CD34⁺CD45^{low} population, which in other hematopoietic tissues such as the fetal liver contains maturing hematopoietic precursors of multiple lineage that can be identified by lineage-specific antigens such as the erythroid markers CD71, EpoR, and CD235a (glycophorin A) (Lammers et al., 2002; Olweus et al., 1996). Active erythropoiesis was indicated by the abundance of CD71 and EpoR expression in CD34⁺CD45^{low} cells (Fig. 5a). Many of these cells did not express the mature erythrocyte marker CD235a (glycophorin A), suggesting that they were early erythroid precursors (Lammers et al., 2002; Olweus et al., 1996). There was also an abundance of CD235a⁺ cells that expressed EpoR and CD71 and variable levels of CD34, suggesting the presence of intermediate-stage (CD34⁺) and late-stage (CD34⁻) erythroid precursors in the placenta.

The presence of myeloid precursors among CD34⁺CD45^{low} cells was also investigated (Fig. 5b). Most CD34⁺ cells expressed CD13, and 5–30% of CD34⁺ cells expressed CD33. The most immature population of CD34⁺CD45^{low} cells also displayed low levels of these antigens, as we have previously seen in fetal liver (Muench et al., 1994). We observed the broad expression of CD13 by placental cells of hematopoietic and nonhematopoietic origin. CD13 is a membrane-bound aminopeptidase that is expressed by endometrial and decidual tissues (Imai et al., 1992). Four-color FACS results indicated that about half of the CD13⁺ cells displayed CD33 and CD14 (Fig. 5c), consistent with a myeloid origin. The remaining CD13⁺CD33⁻CD14⁻ cells, possibly mesenchymal, were nonhematopoietic, since they lacked CD45 expression (data not shown). The CD14 expression on CD13⁺CD33⁺ cells correlates with the loss of CD34 (data not shown), indicating that they are mature myeloid cells. CD14⁺ cells are specialized resident macrophages (Hofbauer cells) in the chorionic villi, present from 4 week of gestation until term (Benischke and Kaufmann, 2000) and

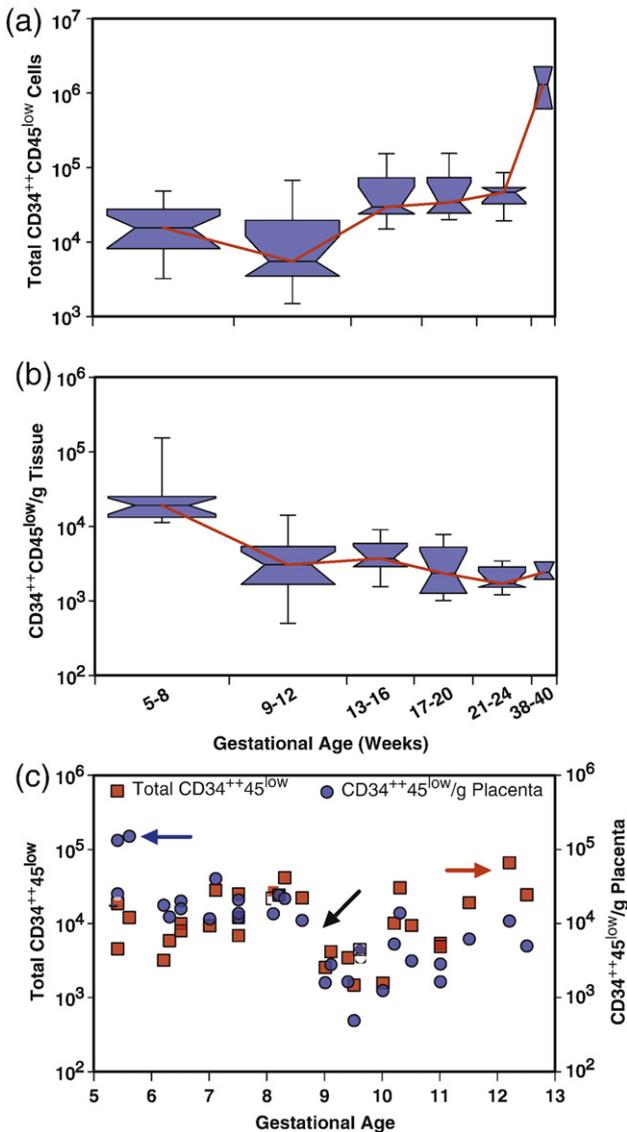


Fig. 3. The hematopoietic compartment of the placenta changes during gestation. (a) Box plot of total CD34⁺CD45^{low} placental cells grouped by gestational age (in weeks). (b) Box plot of the number of CD34⁺CD45^{low} cells per gram of tissue grouped by gestational age. Box plots display the upper and lower quartiles surrounding the median (indicated by the notch), and the span of the whiskers shows the range of the data. Diamonds indicate the position of the mean values. Box width is proportional to sample size (5–8 weeks, $n=18$; 9–12 weeks, $n=15$; 13–16 weeks, $n=9$; 17–20 weeks, $n=7$; 21–24 weeks, $n=7$; 38–40 weeks, $n=3$). (c) Total numbers of CD34⁺CD45^{low} placental cells (red squares) and CD34⁺CD45^{low} placental cells per gram (blue circles) of individual 5- to 12-week tissues ($n=30$). Blue arrow indicates the high number of CD34⁺CD45^{low} cells obtained per gram of 5-week tissues. Black arrow indicates the decrease at 9 weeks in total numbers and cells/gram tissue of CD34⁺CD45^{low} cells. Red arrow indicates the increase in total CD34⁺CD45^{low} placental cells at the end of the first trimester.

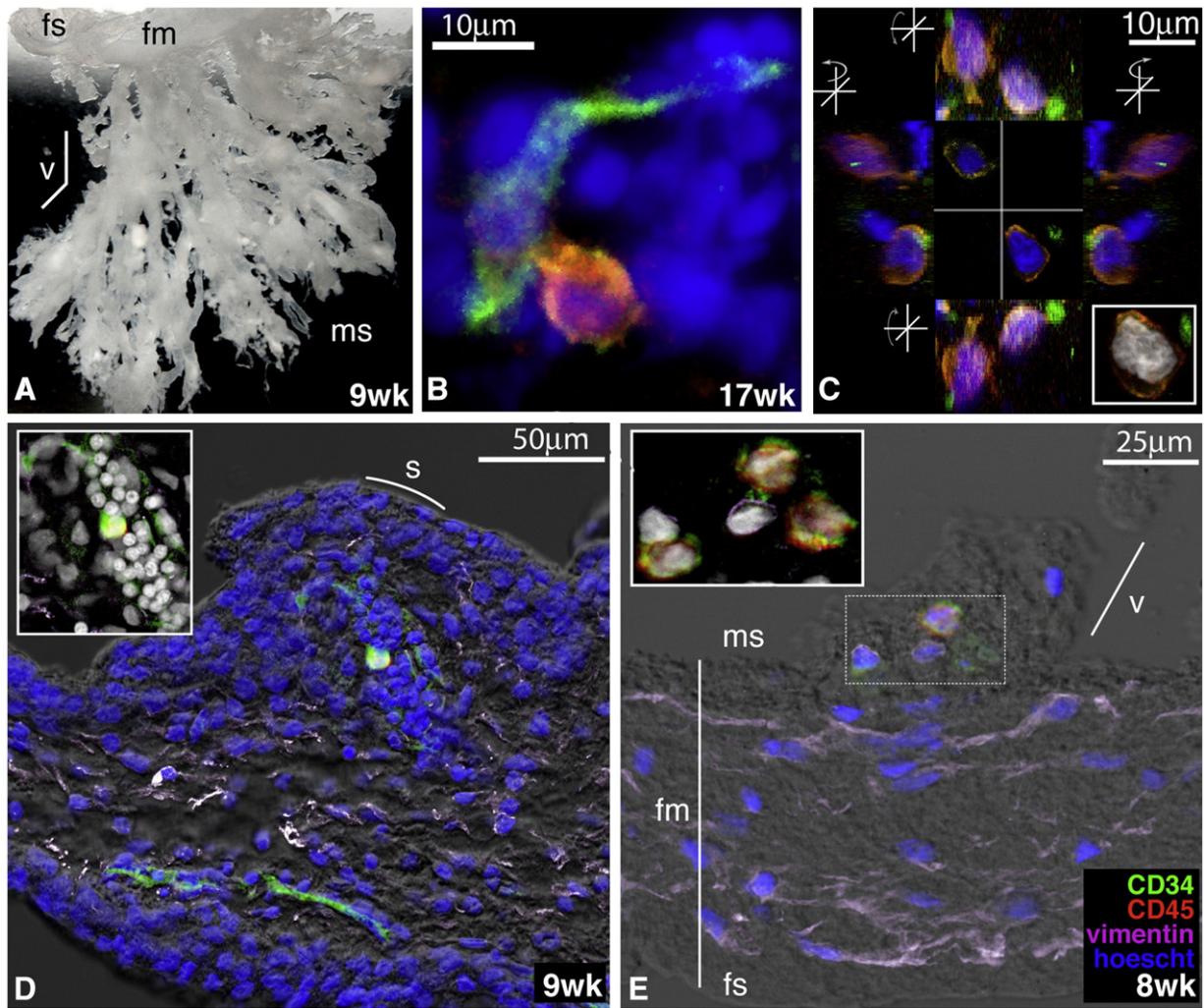


Fig. 4. Localization and morphology of CD34⁺CD45⁺ cells within the human placenta. (A) Chorionic villi biopsied from a 9-week human placenta embedded in gelatin to demonstrate the macroscopic anatomy of these structures (2× magnification). fs, fetal side; fm, fetal membranes; v, villus; ms, maternal side. (B) CD34⁺CD45⁺ cell within the villous mesenchyme in close contact with a CD34⁺CD45⁻ endothelial cell. Tissue sections of the chorionic villi at the gestational ages indicated were stained with anti-CD34 (green) and anti-CD45 (red) mAbs, along with TOTO-3 iodide (blue). Confocal images were obtained by sequential scanning at 63× magnification. (C) Orthogonal views of two CD34⁺CD45⁺ cells, stained as in panel B and from the same 17-week placenta, demonstrating their close associations with CD34⁺CD45⁻ cells. The Z projection (inset) shows the large size and complex morphology of the putative progenitors' nuclei. (D) Longitudinal section of a villus showing a single CD34⁺CD45⁺ cell surrounded by a cluster of CD34⁺CD45⁻ cells. s, syncytium. The tissue section was stained with anti-CD34 (green), anti-CD45 (red), and anti-vimentin (purple) mAbs, Hoesch (blue), and visualized with differential interference contrast (DIC, gray scale). (E) Section of a young villus near the fetal membranes with CD34⁺CD45⁺ cells in the center (stained as in panel D). The Z projection (inset) demonstrates a cluster of CD34⁺CD45⁺ cells that appeared to be tethered to their vimentin-positive neighbors.

they constitute the first mature and most abundant leukocyte population in the placenta. Hofbauer cells (Fig. 5c) expressed high levels of VEGF-R2 and Tie2 (angiopoietin receptor) (Demir et al., 2004; Kayisli et al., 2006) and low levels of CD86; one-third coexpressed the myeloid marker CD15. Most Hofbauer cells expressed low levels of CD80, CD83, DC-SIGN, CD11a, CD11b, and CD11c and moderate to high levels of HLA-DR (data not shown), indicating a mixture of markers expressed on dendritic cells and macrophages.

The presence of other leukocyte subsets in the placenta was investigated by examining the expression of CD3 (displayed by T lymphocytes) and CD56, a marker of natural killer (NK) cells. In cells isolated from a placenta at 22 weeks of gestation (Fig. 5d), the CD45⁺ population contained no detectable T lymphocytes and very few NK cells. The lack of T cells was consistently observed ($n=7$; 12.5–39.5 weeks), and the number of NK cells ranged from 0.05 to 3.5% ($n=9$; 10–39.5 weeks). From our data we conclude that most of the mature hematopoietic cells expressing high levels of CD45 in the human placenta are Hofbauer cells and that very few NK cells are present.

We used the presence of mature lymphocyte populations in our placental preparations as an indicator of the level of blood contamination, since blood is a source of circulating hematopoietic precursors. In preliminary studies, we determined that extensive washes of placental tissue prior to its enzymatic digestion were critical in removing blood contamination. Flow cytometric analyses of the cells recovered from the washes showed the presence of T and NK lymphocytes at the frequencies expected in fetal blood (Muench et al., 2001). Moreover, the presence of hematopoietic precursors within the placental tissue was further confirmed by confocal microscopy (Fig. 4). CD34⁺⁺CD45^{low} cells were identified within the mesenchymal cores of villi, sometimes associated with endothelial cells, but these cells were not observed in the intravascular spaces.

CD34⁺⁺CD45^{low} and CD34⁺CD45^{low} placental cells possess multilineage hematopoietic potential

In vitro clonogenic hematopoietic progenitor assays carried out with sorted CD34⁺⁺CD45^{low} and CD34⁺CD45^{low} cells indicated that

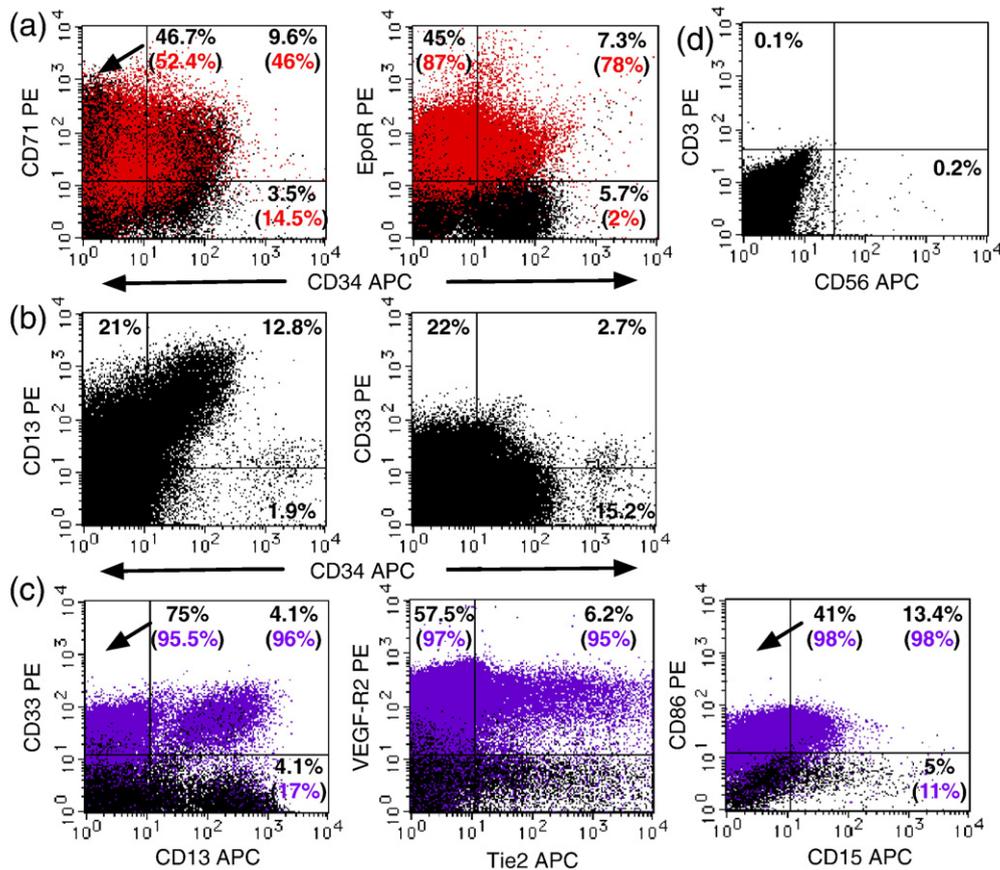


Fig. 5. Erythroid and myeloid progenitors together with NK cells were present in the placenta throughout gestation. (a) A freshly isolated light-density cell suspension from a 21-week placenta was stained for erythroid markers and analyzed (see Fig. 1 legend). Red indicates cells stained with an anti-CD235a-FITC (glycophorin A) mAb. (b) Analysis of a 12-week light-density placental cell suspension using mAbs that recognized myeloid progenitors in combination with an anti-CD34 mAb. (c) Four-color analysis of antigen expression on mature myeloid CD14⁺ cells (purple) (same cells as in panel b). (d) Analysis of CD3 and CD56 expression on 22-week placental cells. In all experiments, $1\text{--}2 \times 10^5$ PI⁺ cells were analyzed. Results are representative of 5 experiments.

both populations generated mostly myeloid progenitor colonies (CFU-GM), with a small number of mixed lineage colonies containing both myeloid and erythroid cells (Fig. 6a). No pure burst-forming unit-erythroid (BFU-E) colonies were observed ($n=7$). Lineage composition of the colonies was examined morphologically and by FACS of individually plucked colonies stained with mAbs against CD45, CD14 and CD15 (data not shown). Our results showed that both CD34⁺CD45^{low} and CD34⁺CD45^{low} placental cells are multipotent hematopoietic progenitors that generated both myeloid and erythroid colonies.

The survival and growth of HSCs and progenitors is regulated by cytokines and other environmental factors. Hematopoietic precursors respond differently to these growth regulators at different stages of human ontogeny. To begin to understand if placental hematopoietic precursors are similarly regulated as other fetal hematopoietic cells, we first investigated the serum dependency of CD34⁺CD45^{low} cells (Fig. 6b). Serum did not significantly affect the production of CFU-mix colonies. The generation of CFU-GM colonies was higher when serum was added to serum-deprived medium (SDM), but still lower than that in Methocult GF H4435, which has a very high serum content (30%). Similar results were obtained with three different batches of FBS from two different companies; all were able to support the *in vitro* growth and differentiation of sorted CD34⁺CD45^{low} cells from umbilical cord blood (UCB), indicating that placental hematopoietic precursors have growth requirements different from those of similar populations of cells isolated from fetal liver (Golfier et al., 1999) and UCB.

The multipotency of placental CD34⁺CD45^{low} cells was also further investigated in liquid cultures (Muench and Bárcena, 2001; Muench et al., 2000). Freshly isolated CD34⁺CD45^{low} cells did not express lymphoid cell surface markers, such as CD19, CD20, sIgM or CD56 (data not shown). Sorted progenitors from 3 individual placentas ranging from 12 to 19 weeks of gestation generated 1–6% CD56⁺ NK cells that coexpressed CD45 (data not shown) in 21-day cultures supported by lymphoid-permissive defined culture conditions (in the absence of serum and stroma and in the presence of a cocktail of recombinant cytokines) and a minor population of CD10⁺CD19⁺ cells (0.2–0.8%), indicating B-cell differentiation (Fig. 7a). These cells were CD20[−] or surface-bound IgM[−] (data not shown), suggesting that they were pre-B cells that had not fully matured. Cultured CD34⁺CD45^{low} cells also generated megakaryocytes, erythrocytes and myeloid cells when cultured for 3 weeks in defined myelo-erythroid-permissive conditions (Fig. 7b) (Bárcena et al., 1999; Muench and Bárcena, 2001). CD41 is a member of the integrin family expressed by megakaryocytes and platelets (Duperay et al., 1989). As shown in Fig. 1, freshly isolated CD34⁺CD45^{low} cells were CD41[−], which was acquired by 2–7% of their progeny. Differentiation of CD34⁺CD45^{low} cells along the erythroid lineage was indicated by expression of CD235a on 50–75% of their progeny. Complete monocytic differentiation was indicated by 1–15% of CD14 on CD33⁺ cells.

To fully support the differentiation of placental CD34⁺CD45^{low} cells into B cells, co-culture with the murine stromal cell line MS-5 was performed as previously described (De Smedt et al., 2002).

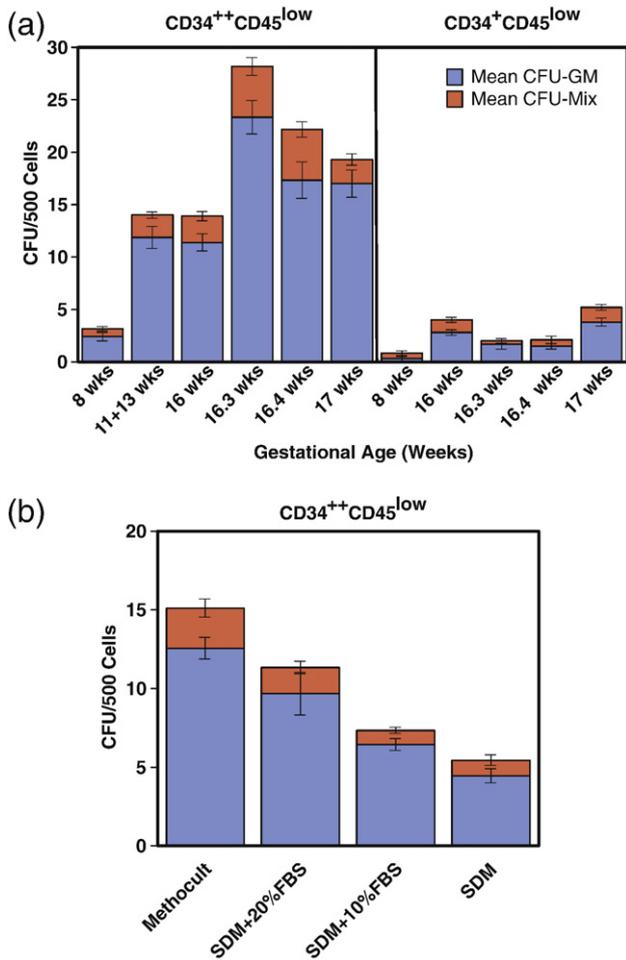


Fig. 6. Colony-forming activity of placental hematopoietic progenitors. (a) Sorted populations from the placentas at the indicated gestational ages were plated in Methocult and scored at 3.5 weeks of culture. Results are shown as the mean \pm SEM of 8–11 replicates for 4 experiments. CFU-GM, myeloid colonies. CFU-Mix, colonies containing both myeloid and erythroid cells. (b) Effects of FBS on colony-forming activity of CD34⁺⁺CD45^{low} sorted cells from a 19-week placenta. Cells were grown in commercial Methocult or in SDM supplemented with FBS.

After 2–4 weeks in culture, the cells were harvested and phenotypically characterized. As shown in Fig. 7c, most of the progeny of CD34⁺⁺CD45^{low} cells were fully differentiated B cells expressing CD19, CD20 and surface IgM.

Discussion

We report that as early as 5 wk of gestation the human placenta contains hematopoietic progenitors at various stages of development, including multipotent and lineage-committed cells, demonstrating that there is ongoing erythropoiesis and myelopoiesis in the human placenta. Thus, it appears that this organ adds to the output of other embryonic and fetal hematopoietic sources, such as the AGM and the liver. The density of these cells sharply declines at the commencement of the fetal phase of development, likely because other fetal sites begin to function as hematopoietic tissues.

Placental hematopoietic progenitors share some phenotypic characteristics with their counterparts in the AGM, fetal liver, and fetal BM (Muench et al., 1994; Tavian et al., 1996, 1999), but they also display unique phenotypic features. CD34⁺⁺CD45^{low} cells from first and second trimester placentas expressed CD133, CD90, CD117, CD33, and HLA-DR. However, placental progenitors, regardless of gestational age, were largely CD38⁻, a characteristic of the very scarce population of HSCs. This is in contrast to the majority of progenitors in fetal,

perinatal and adult hematopoietic tissues that express CD38 (Issaad et al., 1993; Muench et al., 1994; Terstappen et al., 1991). The absence of CD38 expression was not due to enzymatic digestion; placental populations that were obtained enzymatically (dissociated with trypsin) or nonenzymatically (dissociated with commercial cell dissociation buffers) yielded hematopoietic cells that lacked CD38 (data not shown). Our data provide evidence for placental cells with many of the phenotypic characteristics of fetal HSCs but with some variability in expression levels of stem cell markers is apparent.

The lack of CD38 expression by placental hematopoietic progenitors could be due to tissue-specific regulation of CD38 gene expression. CD38 was detected on only ~25% of CD45⁺ mature cells isolated from second trimester placentas, highlighting the observation that this organ contains cells that have an unusual phenotype. We previously reported that the in vitro expression of CD38 by hematopoietic progenitors sorted from fetal liver is dependent on all-trans-retinoic acid (ATRA) and 9-cis-retinoic acid (Muench et al., 1999), cellular products derived from retinol (vitamin A) metabolism that are found in serum. Retinoids are potent regulators of hematopoiesis, inhibiting growth of early progenitors and enhancing differentiation at many points in the pathway (Tocci et al., 1996; van Bockstaele et al., 1993). We hypothesize that the lack of CD38 expression by human placenta-derived cells is a consequence of

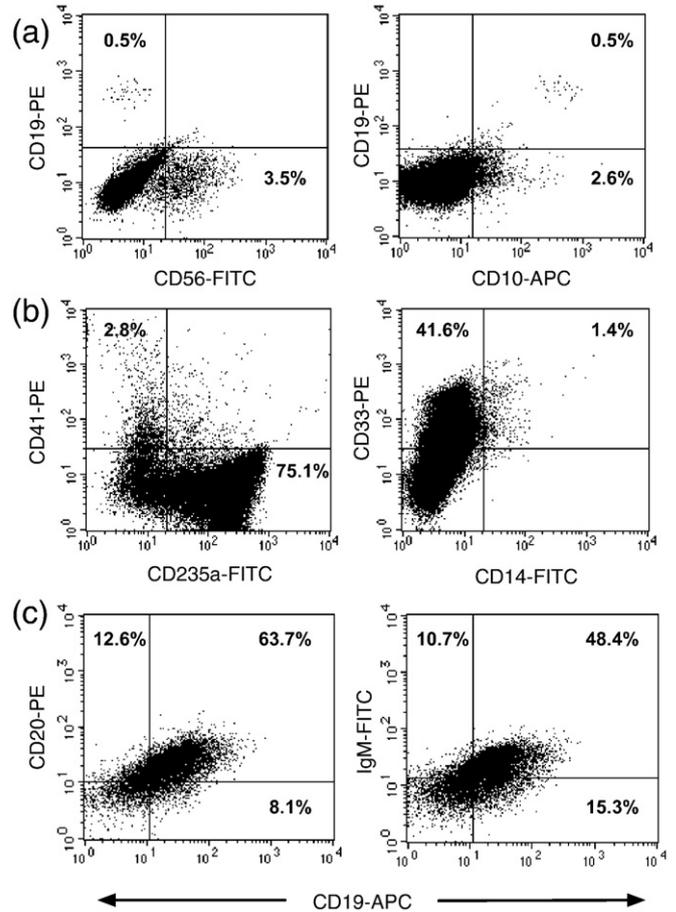


Fig. 7. CD34⁺⁺CD45^{low} placental cells have multilineage hematopoietic potential. CD34⁺⁺CD45^{low} cells sorted from a 19-week placenta were cultured at 7.5×10^3 cells/well in 48-well plates in SDM supplemented with SCF+FL+IL-7+IL-15+GM-CSF+IL-3 (a) or SCF+FL+Epo+GM-CSF+TPO (b). Viable cells (5×10^4) were analyzed after 23 days in culture (see Fig. 1 legend). Results are representative of 3 experiments. (c) CD34⁺⁺CD45^{low} cells sorted from a 12-week placenta were cultured at 5×10^4 cells/well in 48-well plates with MS-5 murine BM stromal cells as feeders. After 3 weeks in culture, cells were detached, washed and stained with the indicated mAbs. The results of an analysis using a gate that contained viable CD45⁺FITC cells (left) and CD45⁺PE cells (right) are shown.

regulatory mechanisms that control retinoid availability and reduce embryonic/fetal exposure to ATRA. For instance, maternal retinoid-binding protein does not cross the placenta and is not synthesized by embryonic/fetal tissues. Therefore, it is likely that the delivery and maintenance of vitamin A at levels that are required for normal development is controlled by a less efficient mechanism, such as retinoid binding to lipoproteins (Quadro et al., 2004).

Fetal liver hematopoietic progenitors produce higher numbers of CFU-C and expand more vigorously in liquid cultures when grown in SDM than in medium supplemented with FBS (Golfier et al., 2000). The growth of placental CD34⁺CD45^{low} cells, however, was highly serum dependent. Another potentially significant functional difference between fetal liver and placental hematopoietic progenitors is that neither CD34⁺CD45^{low} nor CD34⁺CD45^{low} cells isolated from first or second trimester placentas produced BFU-E in standard methylcellulose cultures that support the generation of erythroid colonies, but placental precursors were capable of vigorous erythropoiesis in liquid cultures. These findings suggest that placental hematopoietic precursors are functionally different from their fetal liver or BM counterparts. Alternatively, the placental hematopoietic niche might be unique when compared with other intra-embryonic and intra-fetal hematopoietic environments. The uniqueness of the placental environment could have lasting effects on the growth properties of resident HSCs and progenitors, as these cells do not respond to growth signals in culture in the same way as fetal liver or BM cells. In addition to the unique nature of the hematopoietic niche provided by the placenta, multiparametric (6–7 colors) analyses of first and second trimester placentas have highlighted the considerable heterogeneity of CD34⁺CD45^{low} and CD34⁺CD45^{low} placental cells. This finding suggests that, in addition to multipotent hematopoietic progenitors, these populations could contain several other kinds of progenitors and mature cells (A.B., M.O.M., M.K. and S.J.F., manuscript in preparation), which might partially account for their lower clonogenicity.

One essential characteristic of primitive, pluripotent hematopoietic progenitors and HSCs is their ability to generate cells that contribute to all the blood lineages. This is particularly pivotal for our study, since it is not known if human placental progenitors are embryonic (with exclusive myelo-erythroid potential) or if they are capable of establishing definitive hematopoiesis (myelo-erythroid and lymphoid potential). Our data show that CD34⁺CD45^{low} placental cells generate multilineage progeny, including lymphoid CD56⁺ and CD10⁺CD19⁺ cells under defined culture conditions, and fully mature B cells expressing surface IgM in co-culture with the MS-5 cell line. These data indicate that human placental progenitors can be categorized as definitive progenitors. While we have evidence of the myeloid-erythroid potential of CD34⁺CD45^{low} cells, their lymphoid potential is still under investigation.

Since the intrinsic vasculature of the human placenta develops at a very early stage—~3 weeks of gestation (Hamilton and Boyd, 1970)—it is extremely difficult to determine whether the multipotent progenitors and putative HSCs found in the placenta emerge in situ or immigrate via the fetal circulation. The dearth of T and NK cells in the placental isolates indicates that careful dissection and washing of the chorionic villi prevented contamination with fetal or maternal blood, since these lymphocyte populations are readily detected in fetal blood by 9 weeks (Muench et al., 2001). Our immunolocalization studies show that small numbers of CD34⁺CD45⁺ cells reside in several areas of the chorionic villi, within mesenchymal cores and in association with the vasculature. Reports that the mouse placenta is hematopoietic before vascularization and fusion of the allantois to the chorion (Corbel et al., 2007; Zeigler et al., 2006), and that the placenta produces HSCs in the absence of circulation in *Ncx1* knockout mice whose hearts fail to beat (Rhodes et al., 2008), support the theory that the placenta is not only a niche but also a source of hematopoietic cells. However, the relative importance of the contribution from this site—timing and numbers of cells generated—is not known. The

hematopoietic activity of the mouse placenta manifests before the liver, occurs simultaneously with the AGM, and peaks around mid gestation (Alvarez-Silva et al., 2003). Although the placental contribution to the fetal HSC pool is transient, the HSC output of the placenta is thought to be 15-fold higher than that of the AGM (Gekas et al., 2005). It is thus possible that the placenta contributes more cells than the AGM to the colonization of the liver rudiment.

Another important question is why the placenta is involved in hematopoiesis. We speculate that the large size of the placenta relative to the embryo during early gestation might provide a convenient hematopoietic niche at time in development when there is a great need for hematopoietic activity to supply expanding circulatory system. Placental hematopoiesis could also generate erythrocytes near the site where oxygen is transported by trophoblasts from maternal blood. There may also be immunological and physiological reasons for this layout: the early in situ production of the specialized placental macrophages (Hofbauer cells) that populate the mesenchymal cores of the chorionic villi could help protect the embryo/fetus from microbial infections while training the developing immune cells to recognize a subset of the pathogens that will be encountered later in life.

To our knowledge, this is the first report providing evidence of hematopoiesis in the human placenta that raises interesting possibilities about the potential clinical utility of placentas—which are routinely discarded after terminations early in pregnancy or live births—as a possible source of additional human progenitors with hematopoietic (and nonhematopoietic) potential. Further functional studies are required to better define the placental hematopoietic niche, its role in the embryo/fetus as a source of HSCs, and the full capabilities of placental hematopoietic progenitors as discerned by long-term growth and engraftment assays

Conflict of interest

The authors have declared that no conflict of interest exists.

Note added in proof

Following acceptance of this article for publication, we showed that CD34⁺CD45^{low} cells isolated from midgestation human placentas and transplanted into immunodeficient mice (NOD/SCID) reconstituted erythroid, myeloid, lymphoid and progenitor cells after 11 weeks, while CD34⁺CD45^{low} cells did not reconstitute, further evincing the presence of hematopoietic stem cells in this organ.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2008.11.017.

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