Published in final edited form as:

Transfusion. 2011 November ; 51(Suppl 4): 94S–105S. doi:10.1111/j.1537-2995.2011.03372.x.

Human placenta and chorion: potential additional sources of hematopoietic stem cells for transplantation

Alicia Bárcena^a, Marcus O. Muench^{b,c}, Mirhan Kapidzic^a, Matthew Gormley^a, Gabriel A. Goldfien^a, and Susan J. Fisher^a

^aThe Ely and Edythe Broad Center of Regeneration Medicine and Stem Cell Research, Center of Reproductive Sciences, Department of Obstetrics, Gynecology & Reproductive Sciences University of California, San Francisco

^bBlood Systems Research Institute, San Francisco

^cDepartment of Laboratory Medicine and the Liver Center, University of California, San Francisco

Abstract

Background—Hematopoietic stem cell (HSC) transplantation is an essential element of medical therapy, leading to cures of previously incurable disease for hematological and non-hematological pathologies. Many patients do not find matched donors in a timely manner, which has driven efforts to find alternative pools of transplantable HSCs. The use of umbilical cord blood (UCB) as a source of transplantable HSCs began more than two decades ago. However, the use of UCB as a reliable source of HSCs for transplantation still faces crucial challenges: the number of HSCs present in a unit of UCB is usually sufficient for younger children but not for adults and the persistent delayed engraftment often seen can result in high rates of infection and mortality.

Study Design and Methods—We propose a new approach to a solution of these problems: a potential increase of the limited number of UCB–HSCs available by harvesting HSCs contained in the placenta and the fetal chorionic membrane available at birth.

Results—We investigated the presence of hematopoietic progenitors/HSC in human placenta and chorion at different gestational ages. The characterization of these cells was performed by flow cytometry and immunolocalization and their functional status was investigated by transplanting them into immunodeficient mice.

Conclusion—HSCs are present in extraembryonic tissues and could be banked in conjunction to the UCB–HSCs. This novel approach could have a large impact on the field of HSC banking and more crucially, on the outcome of patients undergoing this treatment by greatly improving the use of life-saving hematopoietic transplants.

Keywords

Human hematopoietic stem cells; umbilical cord blood; placenta; fetal chorionic membrane

Address correspondence to: Alicia Bárcena, PhD, The Ely and Edythe Broad Center of Regeneration Medicine and Stem Cell Research, Center of Reproductive Sciences, Dept. of Obstetrics, Gynecology & Reproductive Sciences, University of California, San Francisco, 35 Medical Center Way, RMB 902E, Pod A, San Francisco, CA 94143-0665. Phone: (415) 476-1092; Fax: (415) 476-1635; Alicia.Barcelona@ucsf.edu.

Conflict of interest: The authors declare that they have no conflict of interest relevant to the work presented in this manuscript.

Introduction

The production of blood cells is a life-long process that is carried out by HSCs generated and maintained in hematopoietic tissues. HSCs constitute a very small pool of cells that can be functionally defined by their self-renewal properties, their differentiation into all types of mature blood cells and their ability to reconstitute the BM of irradiated hosts ¹. Although HSC transplantation is a critical component in the therapy for the treatment of hematological and non-hematological pathologies, shortages in HSCs from human leukocyte antigen (HLA)-matched donors continues to limit their use and drives efforts to expand existing sources, as well as to identify new sources of HSCs. In many malignant and non-malignant diseases, the most desirable and effective treatment is an autologous HSCs transplant from mononuclear cells isolated from BM aspirates or cytokine-mobilized peripheral blood. The second most desirable situation is to find an HLA-matched donor of HSCs (family-related or unrelated), from whom HSCs can be harvested from the BM to perform an allogeneic transplant. It has been estimated that 50-70% of patients in need for a HSC transplant do not find a suitable adult donor. Therefore, clinicians and the scientific community have turned their attention to new sources of HSCs and only recently, UCB has become an alternative source of transplantable HSCs. The presence of hematopoietic progenitors in UCB, capable to produce hematopoietic colonies *in vitro* was first documented 30 years ago by Prindull *et al* ². It was later reported that HSCs isolated from UCB were capable of long-term hematopoietic reconstitution of immunodeficient SCID and NOD-SCID mice, an experimental measure of human hematopoietic stem cells. These investigators reported that the frequency of SCID-reconstituting HSC is higher in UCB compared with adult BM or mobilized adult peripheral blood ³.

Two decades ago, the first HLA-matched UCB transplant into a 6-year-old boy with Fanconi anemia (a non-malignant hematological disease, often associated with BM failure) took place in the USA. The outcome of this pioneering transplant was successful and since then, several clinical studies demonstrated that UCB could be used as a source of transplantable HSCs ⁴⁻⁶. More than 14,000 UCB transplants have been performed to date worldwide and UCB is now frequently banked for transplantation in hospitals and blood banks in the USA, Europe, Australia and Japan ⁷, with more than 400,000 UCB units currently stored in banks.

The use of UCB-HSCs has revolutionized unrelated stem cell transplants (now comprising 28% worldwide) allowing cures of previously incurable diseases. UCB stem cells are unique in that they allow successful transplantation across HLA barriers. For reasons not yet completely understood, in many of the UCB transplants performed partial HLA-matching between the donor and the recipient, the patients experienced a lower incidence and severity of acute and chronic graft-versus-host disease (GVHD), adding another advantage to the use of UCB for HSC transplantation. In addition, while BM-HSCs donors can be found for 60% of Caucasian patients, only 12% of ethnic minority patients find a match. In striking contrast, UCB transplantation is suitable for >90% of patients, irrespective of ethnic background.

Despite its undoubted benefits, the use of UCB as a reliable and widely employed source of HSCs for transplantation still faces many crucial challenges. The small numbers of HSCs present in a single unit of UCB is the single most important factor impacting engraftment and survival. During the last 4 years, our group has taken a novel approach on the identification and characterization of new sources of transplantable HSCs. We reasoned that if the UCB contains a significant number of HSCs, the placenta, an organ that supports the fetus during development, and the chorionic membrane or chorion, a fetal membrane that surrounds the fetus, might harbor additional stem cells. We review our current knowledge on

the hematopoietic potential of these extraembryonic tissues and the role they might play in the overall context of embryonic, fetal and neonatal hematopoiesis as well as their use for HSC transplantation.

Materials and Methods

Isolation of hematopoietic progenitors from placenta and chorionic membrane

This study was approved by the University of California at San Francisco Committee on Human Research. We used a method that our group devised for isolating human placental cells ⁸ that has been modified to achieve the maximum the recovery of hematopoietic cells ⁹.

Isolation of hematopoietic progenitors from umbilical cord blood (UCB)

Samples of UCB were diluted 1:1 with PBS before centrifugation over Nycoprep 1.077 g/ml (Axis-Shield PLC, Oslo, Norway). The resulting light-density cell suspension of UCB was immediately used for phenotypic analyses, fluorescence in situ hybridization, or cell sorting experiments as described below for placental cells.

Monoclonal antibodies

Isotype-matched control IgG₁, IgG_{2a}, IgG_{2b} and IgG₃ were purchased from BD Biosciences (San Jose, CA) or BioLegend (San Diego, CA), and IgM was obtained from Invitrogen Corporation (Carlsbad, CA). They were conjugated to fluorescein isothiocyanate (FITC), PE or APC as indicated in the figure legends. Propidium iodide (PI) was purchased from Sigma-Aldrich Chemical Co (St. Louis, MO) and used at a final concentration of 1 µg/ml. The antibodies that were used for cell separation and immunofluorescence included anti-CD34-PE (8G12, IgG₁, Invitrogen Co.), anti-CD34-APC (581, IgG₁, Beckman Coulter, Brea, CA), anti-CD45-FITC, -PE, and -APC (HI30, IgG₁, Invitrogen Co.), anti-CD38-PE (HB-7, IgG₁, BD Biosciences), anti-CD56-FITC (C5.9, IgG_{2b}, Exalpha, Inc., Boston, MA), anti-HLA-DR-PE (L243, IgG₁, BD Biosciences), anti-CD33-PE (P67.6, IgG₁, BD Biosciences), anti-CD13-PE (L138, IgG₁, BD Biosciences), anti-CD38-PE (HIT2, IgG₁, BD Biosciences), anti-CD235a-FITC or -PE (11E4B7.6, IgG₁, Beckman Coulter), anti-CD117-PE (YB5.B8, IgG₁, BD Biosciences), anti-CD133-PE or -APC (AC133, IgG₁, Milteny Biotec, Inc., Auburn, CA), anti-CD41-PE (HIP8, IgG₁, BD Biosciences), anti-CD14-FITC, -PE, or -APC (MFP9, IgG_{2b}, BD Biosciences), anti-CD15-FITC (MMA anti-Leu-M1, IgM, BD Biosciences), and anti-CD3-FITC, -PE, or -APC (SK7, anti-Leu-4, IgG₁, BD Biosciences), anti-CD19-FITC (4G7, IgG₁, BD Biosciences), anti-CD20-FITC (2H7, IgG_{2b}, BD Biosciences).

Immunofluorescence and flow cytometry

Cell surface phenotypic analyses were performed as previously described ¹⁰ using a two-laser FACSCalibur or LSR II cytometer (BD Biosciences) for acquisition of samples and CellQuest (BD Biosciences) or FlowJo, version 9.2, (Tree Star, Inc., Ashland, OR) software for analysis.

Immunolocalization and fluorescence microscopy

Freshly isolated full-term placental tissue was fixed in 3% paraformaldehyde for 90 min, and processed as previously described ¹¹. Sections (8 µm) of the fixed/frozen tissue were stained with mAbs against CD34 (directly conjugated to FITC; clone 581, IgG₁, BD Pharmingen), and CD45 (clones 2B11 + PD7/26, IgG₁, Dako, Glostrup, Denmark). The binding of antibodies that were not direct conjugates was detected using the appropriate species-specific secondary antibody, a rhodamine-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). For controls, some sections

were stained with the secondary antibody alone. Staining with DAPI (Vectashield mounting medium with DAPI, Vector Laboratories, Burlingame, CA) was used to visualize nuclei. Slides were imaged on a Leica DM 5000 microscope equipped with a Leica 350DX camera (Leica Microsystems, Inc., Buffalo Grove, IL).

Human hematopoietic reconstitution of murine BM and spleen

Immunodeficient NOD.CB17-*Prkdc^{scid}/J* (NOD-SCID) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Research was performed with approval of the Committee on Animal Research at the University of California San Francisco. Female mice were transplanted as adults (≥ 8 weeks old) by tail-vein injection using a 28g insulin syringe (BD Biosystems) several hours after 325 cGy γ -irradiation. Isolated placental cells were mixed with 2×10^7 irradiated (3000 cGy) human midgestation BM cells, isolated as previously described¹², were used as carrier cells. Animals were maintained in a barrier facility in microisolator cages under specific-pathogen free conditions.

Analysis of engraftment

Femoral BM and splenocytes were harvested 11 weeks after transplantation. Spleens were passed through nylon sieves (BD Biosystems) to produce a single-cell suspension and light-density splenocytes were isolated by centrifugation over a layer of 1.077 g/ml Lymphoprep (Axis-Shield PLC) medium. Cells were suspended in PBS containing 5% normal mouse serum, 2 μ g/ml anti-mouse CD16/CD32 (clone 93, BioLegend, San Diego, CA) and 0.01% NaN₃ (Sigma Chemical Co., St. Louis, MO).

Mouse cells were identified by labeling with phycoerythrin (PE)-cyanine dye 7 (PE-Cy7) murine CD45 (clone 30-F11, BD Biosciences) and TER-119 (clone TER-119, BioLegend) and subsequent flow cytometric analyses. Human leukocytes were identified by staining with allophycocyanin (APC)-H7 (clone 2D1, BD Biosciences). Human cells were further stained for the following fluorescein isothiocyanate (FITC)-labeled mAbs: CD15 (clone VIMC6, Invitrogen Co.), CD34 (clone 581, Invitrogen Co.), CD41 (clone VIP11, Invitrogen Co.), CD56 (clone C5.9, Exalpha Biologicals Inc.), and CD59 (clone MEM-43, Invitrogen Co.). Phycoerythrin (PE) mAbs used were: CD19 (clone SJ25-C1, Invitrogen Co.), CD33 (clone P67.6, BD Biosciences), CD38 (clone HB7, BD Biosciences), CD42b (clone MB45, Invitrogen Co.), and CD235a (clone 11E4B7.6, Beckman-Coulter Inc.). The following APC-labeled mAbs were used: CD14 (clone Tük4, Invitrogen Co.), CD34 (clone 581, Invitrogen Co.), CD71 (clone M-A712, BD Biosciences), and CD133 (clone AC133, Miltenyi Biotec).

After samples were stained with saturating levels of mAbs and washed, the cells were suspended in PBS containing 0.3% bovine serum albumin (Roche Diagnostic Corporation, Indianapolis, IN), 2 μ g/ml PI to label dead cells and analyzed using an LSR II flow cytometer (BD Biosystems). Data were analyzed using FlowJo software, version 9.2 (Tree Star, Inc.).

Results and Discussion

Hematopoiesis during human embryonic and fetal development

In mammals, embryonic/fetal hematopoiesis is a nomadic process, where different anatomical sites become active at different stages of development. In contrast, normal postnatal hematopoiesis occurs almost exclusively in the BM, which supports life-long maintenance of a self-renewing HSC population that differentiates into all the blood lineages. It is generally accepted that an extra-embryonic structure, the yolk sac, provides the first niche for embryonic hematopoiesis. For decades, numerous reports have supported or refuted the “definitive hematopoiesis” potential of this early wave of progenitors¹³, and

currently it is accepted that the yolk sac hematopoietic progenitors have a limited (“embryonic”) hematopoietic potential. Accumulating evidence points to another hematopoietic site, the intra-embryonic aorta-gonad-mesonephros (AGM), as the source of definitive or adult hematopoiesis in the mouse ^{14,15}. In humans, the evidence supporting a role for the AGM in establishing definitive hematopoiesis is powerful, albeit indirect. At three weeks, before the circulation is established, intra-embryonic sites contain multilineage (myeloid and lymphoid) progenitors. In contrast, yolk sac components are erythro-myeloid progenitors with no lymphopoietic potential ¹⁶. These results suggest that the second wave of progenitors developing in the AGM is very likely able to establish definitive hematopoiesis, which will continue during the embryonic and fetal periods in the liver, BM and thymus.

The model of hematopoietic development described above turned out to be incomplete. Transplantable hematopoietic cells in the mouse placenta were reported ~40 yrs ago by the groups of Till and McCullough ¹⁷ and later on, by Dancis *et al.* ¹⁸. The ability of placental hematopoietic progenitors to reconstitute recipients was shown to be similar to that of BM cells. Importantly, the activity of the placenta-derived progenitors did not depend on the organ's blood supply ¹⁹, suggesting the existence of an endogenous HSCs population. A decade later, Melchers reported that the mouse fetal placenta contains hematopoietic progenitors, in particular B-cell precursors, before the fetal liver is hematopoietic, ²⁰. Twenty years later, Dieterlen-Lievre's group showed that the avian allantois (equivalent to the mammalian placenta) contains hematopoietic progenitor cells ²¹, but their HSC potential was not addressed. Alvarez-Silva *et al.* were the first to perform a detailed investigation of the hematopoietic potential of mouse placental cells ²². These authors compared the colony forming capabilities, in terms of myeloid and erythroid progenitors, of the placenta, yolk sac and embryo at different stages of development. They concluded that the placenta and the embryo proper (presumably the AGM region) simultaneously produce *in vitro* clonogenic, multilineage, hematopoietic progenitors during the mid-gestation period. Additionally, based on self-renewal activity, the placental progenitors appeared to be a more primitive population than the cells that were isolated from other tissues. They were more numerous as well. Two reports further expanded our knowledge of hematopoietic progenitors in the mouse mid-gestation placenta. Gekas *et al.* described a CD34⁺c-kit⁺ pool with a 15-fold higher frequency than the equivalent AGM population. As to function, they showed that a crude preparation of placental cells that were transplanted with competitor BM cells reconstituted irradiated recipients to the same degree as those obtained from the AGM region or fetal liver. Interestingly, the onset of placental, AGM and yolk sac HSC activity, which coincides temporally, precedes HSCs seeding of the fetal liver and the circulation ²³. Another group described a CD34⁺CD31⁺ population in the embryonic vasculature of the placenta; most of these cells co-expressed c-kit, CD41 and CD45 ²⁴. All together, these reports provided evidence of the role of the mouse placenta as a hematopoietic niche that should be included in the site-and stage-specific model of embryonic hematopoiesis.

Hematopoiesis in the human placenta and chorion during development

In order to search for and functionally study cell populations with potential HSC activity contained in extraembryonic tissues, it is relevant to define the set of proteins that are expressed on their cell surface. The antigenic profile of embryonic/fetal/neonatal and adult cells share some basic phenotypic features that can be employed for their detection and further isolation. It is currently widely accepted that the hematopoietic progenitors in a population with a CD34⁺⁺CD38⁻ cell surface phenotype, which are primitive and multipotent, include HSCs. This subpopulation is enriched for long-term culture initiating cells (LTC-IC) and high-proliferative potential colony-forming cells (HPP-CFC). CD34⁺⁺CD38⁻ cells were originally described in adult BM ^{25,26}, and later on studied in fetal

liver²⁷ and UCB²⁸, although cells from the latter source express low levels of CD38. It is important to note that this subpopulation provides long-term (≥ 12 weeks) multilineage BM reconstitution of immunodeficient NOD/SCID mice, while the more mature CD34⁺⁺CD38⁺ subset, which sustains short-term reconstitution, has a more limited potential²⁹. In addition, a number of other cell-surface antigens are useful tools for enriching and defining human cell populations with HSC activity. Examples include CD90, which is expressed by fetal and adult HSCs that reconstitute *scid* mice engrafted with human bones (SCID-hu mice)³⁰, CD45, which is expressed at low levels by HSCs³¹, and expression of the c-kit receptor (CD117), which is detected in association with human HSCs and progenitors enriched in LTC-IC³². CD133 is also reported to be expressed by UCB cells highly enriched in LTC-IC³³. The presence of these and other markers on cellular subsets that display high levels of CD34 expression allows for the isolation of highly pure populations that can be assayed *in vitro* and *in vivo* for HSC function. Finally, we note that CD34⁻ cells might also contain a small number of immature hematopoietic progenitors, even more primitive than CD34⁺⁺CD38⁻ cells. In the adult mouse, the long-term hematopoietic repopulating activity resides in the CD34^{-low}lin⁻ subset³⁴ and similar results have been obtained using human bone marrow CD34⁻lin⁻ cells in a fetal sheep competitive HSC engraftment model³⁵. Currently, no data are available on the ability of CD34⁻ HSCs to reconstitute human patients after transplantation.

The hematopoietic potential of the human placenta has recently become apparent, since we and others have shown that this organ contains hematopoietic progenitors/stem cells among CD34⁺⁺CD45^{low} cells throughout gestation that produce multilineage progeny and long-term engraftment when transplanted into immunodeficient mice³⁶⁻³⁸. These observations prompt a number of questions regarding the role of the placenta in embryonic and fetal hematopoiesis. For example, what kind of progenitors and HSCs are generated in this location? Are they similar to those found in the yolk sac, displaying a primitive and limited hematopoietic potential? Are these cells another part of the second wave of hematopoiesis? What is their relationship to circulating progenitors? If they are multipotent, adult-like progenitors, are they transplantable, *i.e.*, useful for treating human diseases? Are they present in the placenta and chorion at birth?

Our characterization of placental hematopoietic progenitors and HSCs began with phenotypic analyses of freshly isolated placental cellular preparations. These studies showed the presence of two populations of hematopoietic progenitors in the light density fraction of placental cells: CD34⁺CD45^{low} and CD34⁺⁺CD45^{low}. Placental CD34⁺⁺CD45^{low} cells share some characteristics with their counterpart intraembryonic populations in fetal liver and BM, since they express HLA-DR, CD133, CD117, CD90, and CD31 and lack CD38 expression³⁶. In contrast with the more immature CD34⁺⁺CD45^{low} cell population, CD34⁺CD45^{low} cells expressed many cell surface markers indicative of erythroid- and myeloid-lineage commitment. These findings, in conjunction with the observation that CD34⁺CD45^{low} cells have a very limited clonogenic potential in CFU-C assays led us to conclude that these cells represent the progeny of CD34⁺⁺CD45^{low} cells and contain a more mature repertoire of hematopoietic progenitors³⁶. It is important to mention that we documented the fetal origin of placental hematopoietic progenitors by FISH³⁶.

We reasoned that since the placenta and the chorionic membrane have a common developmental origin, we could additionally search for hematopoietic progenitors in the fetal membranes. The main focus of our National Blood Foundation grant was to study the presence of HSCs in the fetal chorionic membrane and to investigate their functional characteristics in comparison with other extraembryonic and intraembryonic candidate HSCs. Figure 1 shows a diagrammatic representation of the fetus *in utero* at term in conjunction with the extraembryonic structures provided by the fetal membranes (being the

chorionic membrane the outermost layer and the amnion the closest to the fetus), the placenta and the umbilical cord. Initial flow cytometric studies of cellular preparations from whole fetal membranes (containing both chorion and amnion), showed a distribution of cell populations displaying hematopoietic progenitor/HSC phenotype³⁶. To determine whether hematopoietic progenitors were contained in either the chorion or the amnion, we dissected and dissociated the two membranes separately. Our results indicated the exclusive presence of precursors in the chorion (data not shown). This finding allowed us to focus our attention on the chorion. It also diminished the potential variability of our samples, since the chorion and the amnion are physically separated membranes during the first trimester and, as the second trimester progresses and the fetus and the amnion increase in size, the two membranes come in close contact.

Comparative studies of hematopoietic progenitors and HSCs in extraembryonic tissues vs. intraembryonic hematopoietic organs (i.e. from the fetal liver) showed that CD34⁺⁺CD45^{low} cells from both the chorionic membrane or the villi of the placenta were largely CD38⁻, suggesting their very primitive, immature in nature (Fig.2) and that CD38 expression was found on CD34⁺ cells, particularly in the chorionic membrane. In comparison, most of the CD34^{++/+} cells in the fetal liver are CD38⁺ cells, although in all the tissues analyzed at different gestational ages, primitive CD34⁺⁺CD38⁻ cells shared the characteristic of expressing low levels of CD45. This feature allowed us to sort CD34⁺CD45^{low} and CD34⁺⁺CD45^{low} cells and thus discriminate between hematopoietic precursors (expressing both CD34 and CD45) and endothelial cells, which express CD34 but lack CD45 and are an abundant cell type in the highly vascularized placenta (Fig.2). The diminished expression of CD38 on hematopoietic progenitors in extraembryonic tissues might reflect differential gene expression in different hematopoietic sites and it is currently being investigated.

Primitive hematopoietic progenitors are found in both the chorionic membrane and the placenta at all gestational ages, from first to third trimester (Fig.3). Consistent with our previous observations in placenta³⁶, the chorionic membrane was richest in hematopoietic progenitors early in gestation, and the frequency of these cells was maintained from the second trimester throughout the remainder of gestation. An important finding that arose from these studies was that these cells are also present in full term tissues, which makes them readily available to harvest at birth.

One crucial question regarding the placenta's hematopoietic potential is whether this organ is a hematopoietic niche per se or whether HSCs that originate in other sites colonize the placenta when the circulation is established. Two reports specifically examined this issue in the mouse placenta. In this species, the chorionic-allantoic placenta forms by fusion of the allantois (mesodermal precursors of the umbilical cord) with the chorionic plate. Both groups independently demonstrated that the pre-fusion allantois³⁹ or in combination with the chorion⁴⁰ have hematopoietic potential prior to vascularization. Following explant culture, they give rise to myeloid and definitive erythroid cells that express early-stage markers such as CD117 (c-kit) and CD41.

CD34^{++/+}CD45⁺ cells in term extraembryonic tissues

To investigate the tissue compartment in which the putative hematopoietic progenitors and stem cells reside, we performed immunolocalization experiments on frozen sections of the placenta. Sections of term (38 wk) placenta stained with anti-CD45 and anti-CD34 antibodies revealed few, yet clearly detectable double positive cells (Fig. 4). The frequency of these cells is expected to be low from our previous FACS data (between 0.07-0.3% of the total light density fraction of cells). The CD34⁺CD45⁺ population was found predominantly within the villous stroma and frequently associated with CD34⁺CD45⁻ endothelial cells. We have reported similar immunolocalization results earlier in gestation³⁶. Immunolocalization

experiments performed on fetal membranes showed that CD34⁺CD45⁺ cells were found almost exclusively, in close physical association with vimentin-positive putative stroma ³⁶.

The presence of hematopoietic progenitors/HSCs in term placenta has been reported by several groups. It has been calculated that the HSC content of term placenta is about 10% of the reported number of HSCs present in one unit of UCB ^{37,41}. This number will likely increase in the future as we continuously improve our methods of processing the placental tissue. Our data shows that the total number of CD34⁺⁺/CD45^{low} cells in villi at term was 100 times higher than in the first trimester and 10 times higher than in the second trimester, reflecting the extensive growth of the placenta during development. Table 1 shows the total number of CD34⁺⁺/CD45^{low} cells present in 5 sets of villus and chorion samples obtained at birth. On average, there were 10 times more CD34⁺⁺/CD45^{low} cells in the placenta than in the chorion and the number of these cells in the placenta was similar to those reported for UCB. In a study involving 300 UCB samples the mean number of total CD34⁺CD45⁺ cells, assessed by FACS, was 3.1×10^6 cells per UCB unit ⁴². Similar data have been reported by other groups, where the number of CD34⁺CD45⁺ cells ranged from 0.5 – 2.6×10^6 per UCB unit ^{43,44}. Our data shows that the number of CD34⁺⁺/CD45^{low} cells ranges from 1.0 – 12.2×10^6 in placentas and 0.1 – 2.5×10^6 in chorionic membranes, indicating that there are abundant hematopoietic progenitors in these extraembryonic tissues at term. In one specimen of term placenta (sample D, Table 1), we were able to obtain UCB, chorion, and the blood that washes out of the placenta prior the tissue processing. The numbers of CD34⁺CD45⁺ cells in these tissues obtained by FACS were: 1.5×10^6 from 72 ml of UCB, 5.8×10^4 from 150 ml of placental blood, 12.2×10^6 from the placenta weighting 590 g and 1×10^6 from a portion of the chorionic membrane weighting 55 g. These results indicated that the villi from this subject was a particularly rich source of CD34⁺CD45⁺ cells when compared with other placental samples, and our processing of UCB yielded equivalent numbers of CD34⁺CD45⁺ cells to those reported in the literature. In addition, the placental wash also contained a moderate number of CD34⁺CD45⁺ cells, likely derived from fetal blood present in the placenta. Future experiments will assess the efficiency of different methods to perfuse the placenta and obtain the maximum volume of fetal blood, to thus increase the number of CD34⁺CD45⁺ cells obtained from UCB.

CD34⁺⁺CD45^{low} cells from placenta have hematopoietic activity in vivo and contain HSCs

To determine whether placental cells have HSC properties, we tested their long-term and multilineage reconstituting capability when injected into NOD/SCID mice. For this purpose, 10^3 sorted CD34⁺⁺CD45^{low} and CD34⁺CD45^{low} cells were injected intravenously in conjunction with carrier cells (2×10^7 30Gy irradiated human fetal BM). The positive controls for these experiments were mice injected with 10^3 CD34⁺⁺CD45^{low} fetal liver cells from the same specimen used to obtain the placental cells. Negative controls were mice injected with carrier cells alone. After 10–12 weeks, the mice were sacrificed and the presence of human cells was investigated by flow cytometry performed on the mouse BM cell preparations. Results from a representative mouse are shown in Figure 5. The mice injected with both placental and fetal liver CD34⁺⁺CD45^{low} cells contained between 0.5 and 11% human cells representing multiple hematopoietic lineages (erythroid, myeloid, megakaryocytic and lymphoid). In contrast, the mice transplanted with CD34⁺CD45^{low} cells showed no evidence of human engraftment, further reinforcing the notion that earlier in gestation this population represents a more mature type of hematopoietic progenitors than CD34⁺⁺CD45^{low} cells. Furthermore, the NOD/SCID BM contained an abundant population of primitive hematopoietic progenitors expressing the HSC-markers CD133 and high levels of CD34. These data indicate that CD34⁺⁺CD45^{low} placental cells contain HSCs. Other groups have also reported similar findings using placental cells obtained at birth ^{37,38}.

Concluding remarks

One relevant question that arises from the data reviewed here is why the placenta and chorion are hematopoietic sites. From the developmental point of view we could propose that, before the placenta is fully connected to the embryonic circulatory system and has continuous access to circulating HSCs generated in the embryo, it might provide an intrinsic site of primitive hematopoiesis, and is therefore capable to support *in situ* the need for mature hematopoietic cells. Hofbauer cells, a macrophage-like and abundant cell type in the placenta, and erythrocytes, necessary to properly oxygenate the developing embryo and the placenta itself, are detectable in pre-circulation placenta. A recent report showed that the early first trimester placenta provides a site for terminal maturation/enucleation of primitive erythroid cells and that Hofbauer cells may play a role in this enucleation process⁴⁵. This data resonates with our findings that the placental chorionic villi has the highest density of hematopoietic progenitors/HSCs prior the 9th week of gestation, diminishing more than 7-fold for the remainder of the gestation³⁶. This may be a reflection of a developmentally regulated switch, as the embryo/fetus becomes able to produce abundant immature and mature hematopoietic cells accessible to the placenta in the blood stream. The presence of primitive hematopoietic progenitors/HSCs in the chorionic membrane is a new finding and it might be due to the common developmental origin of this membrane and the placenta, thus permissive/supportive niches would develop in both extraembryonic tissues. The timing of appearance of hematopoietic progenitors/HSCs in these two tissues, the localization of the hematopoietic niches at different gestational ages and the nature of the cellular and molecular signals provided by the chorion and the placental villi niches are not fully characterized. Ongoing research in our laboratory will likely shed light to these aspects of the hematopoietic function of extraembryonic tissues/organs.

The concept of harvesting HSCs from chorion and placenta for the purpose of banking and improving UCB transplantation is novel and it could contribute an important advancement for expanding the field of UCB transplantation in pediatric and, particularly, in adult patients in whom increasing the number of CD34⁺ donor cells present in the graft is a critical issue. Related to the limited cell dose of UCB-HSCs, the slow engraftment results in low numbers of neutrophils and platelets during the first 4-5 months post-transplant in patients transplanted with UCB, compared with BM, increases their chance of infection and mortality. In addition, in order to transplant UCB not only into pediatric patients, but also into adults presenting with hematological malignancies -i.e. acute leukemia or chronic leukemia-, it is necessary to pool at least 2 or more units of UCB to thus reach the target number of donor CD34⁺ cells⁴⁶. The strategy of using multiple UCB units per patient greatly diminishes the availability of HLA-matched UCB and poses a problem for transplantation into adult patients with non-malignant hematological disease, since in this case, HLA disparity has a strong negative effect on engraftment, GVHD and overall survival. In addition, despite continuous efforts to recruit new donors, and a large number of donors already recruited in the USA and international registries (13 million volunteer BM donors and 7 million UCB donors at the end of 2008), approximately 50-70% of the patients in need of life-saving HSC transplants are still unable to find a suitable adult donor in a timely manner. In Table 2, we summarized diseases that have been and can be treated with UCB transplantation and could thus benefit from expanding current banks. Note the abundance of non-malignant hematological and non-hematological diseases that would not benefit from double UCB transplant, but could be treated with an increased pool of HSCs obtained by a single donor.

In conclusion, we think that our observations that human placenta and chorionic membrane contain sizable numbers of HSCs suggest an important additional and novel source of HSCs that could be easily obtained at birth. These findings might translate into improved cellular

therapies in the clinical setting of HSC transplantation. Funding from the National Blood Foundation has played a critical role in our effort to study and characterize these sources of HSC.

Acknowledgments

We thank the staff and faculty at San Francisco General Hospital Women's Options Center for assistance in the collection of human fetal tissues. We gratefully acknowledge Ms. Tara Rimbaldo (Laboratory of Cell Analysis, UCSF) for the cell sorting of placental, bone marrow, chorionic membrane and UCB samples and Ms. Jean Perry (RN MS NP, UCSF Department of Obstetrics, Gynecology and Reproductive Sciences) for her assistance in obtaining full-term placental, chorionic membrane and UCB samples. We also wish to thank Ms. Melissa Schirmer for laboratory managerial work and Mr. Keith Jones for administrative assistance.

Sources of Support: This work was supported by a grant from the National Blood Foundation (A.B.), as well as support from NIH grant R21HD055328, (A.B.) and from Blood Systems (M.O.M.).

References

1. Weissman IL. The road ended up at stem cells. *Immunol Rev.* 2002; 185:159–74. [PubMed: 12190929]
2. Prindull G, Prindull B, Meulen N. Haematopoietic stem cells (CFUc) in human cord blood. *Acta Paediatr Scand.* 1978; 67(4):413–6. [PubMed: 676726]
3. Wang JC, Doedens M, Dick JE. Primitive human hematopoietic cells are enriched in cord blood compared with adult bone marrow or mobilized peripheral blood as measured by the quantitative in vivo SCID-repopulating cell assay. *Blood.* 1997; 89(11):3919–24. [PubMed: 9166828]
4. Broxmeyer HE, Kurtzberg J, Gluckman E, et al. Umbilical cord blood hematopoietic stem and repopulating cells in human clinical transplantation. *Blood Cells.* 1991; 17(2):313–29. [PubMed: 1680502]
5. Gluckman E, Thierry D, Lesage S, et al. Cord blood banking for human hematopoietic cell transplantation. *Prog Clin Biol Res.* 1992; 377:591–8. discussion 9-602. [PubMed: 1359566]
6. Wagner JE, Kernan NA, Steinbuch M, et al. Allogeneic sibling umbilical-cord-blood transplantation in children with malignant and non-malignant disease. *Lancet.* 1995; 346(8969):214–9. [PubMed: 7616801]
7. Kurtzberg J. Update on umbilical cord blood transplantation. *Curr Opin Pediatr.* 2009; 21(1):22–9. [PubMed: 19253461]
8. Fisher SJ, Cui TY, Zhang L, et al. Adhesive and degradative properties of human placental cytotrophoblast cells in vitro. *J Cell Biol.* 1989; 109(2):891–902. [PubMed: 2474556]
9. Barcena A, Muench MO, Kapidzic M, Fisher SJ. A new role for the human placenta as a hematopoietic site throughout gestation. *Reprod Sci.* 2009; 16(2):178–87. [PubMed: 19208786]
10. Bárcena A, Muench MO, Galy AHM, et al. Phenotypic and functional analysis of T-cell precursors in the human fetal liver and thymus. CD7 expression in the early stages of T- and myeloid-cell development. *Blood.* 1993; 82:3401–14. [PubMed: 7694684]
11. Prakobphol A, Genbacev O, Gormley M, et al. A role for the L-selectin adhesion system in mediating cytotrophoblast emigration from the placenta. *Dev Biol.* 2006; 298(1):107–17. [PubMed: 16930583]
12. Golfier F, Bárcena A, Harrison MR, Muench MO. Fetal bone marrow as a source of stem cells for in utero or postnatal transplantation. *Br J Haematol.* 2000; 109(1):173–81. [PubMed: 10848797]
13. Moore MA, Metcalf D. Ontogeny of the haemopoietic system: yolk sac origin of in vivo and in vitro colony forming cells in the developing mouse embryo. *Br J Haematol.* 1970; 18(3):279–96. [PubMed: 5491581]
14. Keller G, Lacaud G, Robertson S. Development of the hematopoietic system in the mouse. *Exp Hematol.* 1999; 27(5):777–87. [PubMed: 10340392]
15. Cumano A, Ferraz JC, Klaine M, et al. Intraembryonic, but not yolk sac hematopoietic precursors, isolated before circulation, provide long-term multilineage reconstitution. *Immunity.* 2001; 15(3):477–85. [PubMed: 11567637]

16. Peault B, Tavian M. Hematopoietic stem cell emergence in the human embryo and fetus. *Ann N Y Acad Sci.* 2003; 996:132–40. [PubMed: 12799291]
17. Till JE, McCulloch EA. A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. *Radiat Res.* 1961; 14:213–22. [PubMed: 13776896]
18. Dancis J, Jansen V, Gorstein F, Douglas GW. Hematopoietic cells in mouse placenta. *Am J Obstet Gynecol.* 1968; 100(8):1110–21. [PubMed: 5643190]
19. Dancis J, Jansen V, Brown GF, et al. Treatment of hypoplastic anemia in mice with placental transplants. *Blood.* 1977; 50(4):663–70. [PubMed: 901940]
20. Melchers F. Murine embryonic B lymphocyte development in the placenta. *Nature.* 1979; 277(5693):219–21. [PubMed: 317835]
21. Caprioli A, Jaffredo T, Gautier R, et al. Blood-borne seeding by hematopoietic and endothelial precursors from the allantois. *Proc Natl Acad Sci U S A.* 1998; 95(4):1641–6. [PubMed: 9465069]
22. Alvarez-Silva M, Belo-Diabangouaya P, Salaun J, Dieterlen-Lievre F. Mouse placenta is a major hematopoietic organ. *Development.* 2003; 130(22):5437–44. [PubMed: 14507780]
23. Gekas C, Dieterlen-Lievre F, Orkin SH, Mikkola HK. The placenta is a niche for hematopoietic stem cells. *Dev Cell.* 2005; 8(3):365–75. [PubMed: 15737932]
24. Ottersbach K, Dzierzak E. The murine placenta contains hematopoietic stem cells within the vascular labyrinth region. *Dev Cell.* 2005; 8(3):377–87. [PubMed: 15737933]
25. Terstappen LW, Huang S, Safford M, et al. Sequential generation of hematopoietic colonies derived from single nonlineage-committed CD34+CD38- progenitor cells. *Blood.* 1991; 77:1218–27. [PubMed: 1705833]
26. Issaad C, Croisille L, Katz A, et al. A murine stromal cell line allows the proliferation of very primitive human CD34+/CD38- progenitor cells in long-term cultures and semisolid assays. *Blood.* 1993; 81:2916–24. [PubMed: 7684620]
27. Muench MO, Cupp J, Polakoff J, Roncarolo MG. Expression of CD33, CD38, and HLA-DR on CD34+ human fetal liver progenitors with a high proliferative potential. *Blood.* 1994; 83(11):3170–81. [PubMed: 7514903]
28. Steen R, Tjonnfjord GE, Egeland T. Comparison of the phenotype and clonogenicity of normal CD34+ cells from umbilical cord blood, granulocyte colony-stimulating factor-mobilized peripheral blood, and adult human bone marrow. *J Hematother.* 1994; 3(4):253–62. [PubMed: 7537606]
29. Hogan CJ, Shpall EJ, Keller G. Differential long-term and multilineage engraftment potential from subfractions of human CD34+ cord blood cells transplanted into NOD/SCID mice. *Proc Natl Acad Sci U S A.* 2002; 99(1):413–8. [PubMed: 11782553]
30. Baum CM, Weissman IL, Tsukamoto AS, et al. Isolation of a candidate human hematopoietic stem-cell population. 1992; 89:2804–8.
31. Lansdorp PM, Sutherland HJ, Eaves CJ. Selective expression of CD45 isoforms on functional subpopulations of CD34+ hemopoietic cells from human bone marrow. *J Exp Med.* 1990; 172(1):363–6. [PubMed: 1694223]
32. Papayannopoulou T, Bricce M, Broudy VC, Zsebo KM. Isolation of *c-kit* receptor expressing cells from bone marrow, peripheral blood, and fetal liver: Functional properties and composite antigenic profile. *Blood.* 1991; 78:1403. [PubMed: 1715789]
33. de Wynter EA, Buck D, Hart C, et al. CD34+AC133+ cells isolated from cord blood are highly enriched in long-term culture-initiating cells, NOD/SCID-repopulating cells and dendritic cell progenitors. *Stem Cells.* 1998; 16(6):387–96. [PubMed: 9831864]
34. Osawa M, Hanada K, Hamada H, Nakauchi H. Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. *Science.* 1996; 273:242–5. [PubMed: 8662508]
35. Zanjani ED, Almeida-Porada G, Livingston AG, et al. Engraftment and multilineage expression of human bone marrow CD34- cells in vivo. *Ann N Y Acad Sci.* 1999; 872:220–31. discussion 31-2. [PubMed: 10372125]
36. Bárcena A, Kapidzic M, Muench MO, et al. The human placenta is a hematopoietic organ during the embryonic and fetal periods of development. *Dev Biol.* 2009; 327:24–33. [PubMed: 19073167]

37. Robin C, Bollerot K, Mendes S, et al. Human placenta is a potent hematopoietic niche containing hematopoietic stem and progenitor cells throughout development. *Cell Stem Cell*. 2009; 5(4):385–95. [PubMed: 19796619]
38. Serikov V, Hounshell C, Larkin S, et al. Human term placenta as a source of hematopoietic cells. *Exp Biol Med (Maywood)*. 2009; 234(7):813–23. [PubMed: 19429852]
39. Corbel C, Salaun J, Belo-Diabangouaya P, Dieterlen-Lievre F. Hematopoietic potential of the pre-fusion allantois. *Dev Biol*. 2007; 301(2):478–88. [PubMed: 17010964]
40. Zeigler BM, Sugiyama D, Chen M, et al. The allantois and chorion, when isolated before circulation or chorio-allantoic fusion, have hematopoietic potential. *Development*. 2006; 133(21):4183–92. [PubMed: 17038514]
41. Bhatia M, Wang JC, Kapp U, et al. Purification of primitive human hematopoietic cells capable of repopulating immune-deficient mice. *Proc Natl Acad Sci U S A*. 1997; 94(10):5320–5. [PubMed: 9144235]
42. Solves P, Perales A, Moraga R, et al. Maternal, neonatal and collection factors influencing the haematopoietic content of cord blood units. *Acta Haematol*. 2005; 113(4):241–6. [PubMed: 15983430]
43. M-Reboredo N, Diaz A, Castro A, Villaescusa RG. Collection, processing and cryopreservation of umbilical cord blood for unrelated transplantation. *Bone Marrow Transplant*. 2000; 26(12):1263–70. [PubMed: 11223965]
44. Wu JY, Liao C, Xu ZP, et al. Banking and transplantation of umbilical cord blood in Guangzhou, China. *Cytotherapy*. 2006; 8(5):488–97. [PubMed: 17050254]
45. Van Handel B, Prashad SL, Hassanzadeh-Kiabi N, et al. The first trimester human placenta is a site for terminal maturation of primitive erythroid cells. *Blood*. 2010; 116(17):3321–30. [PubMed: 20628147]
46. Barker JN, Weisdorf DJ, DeFor TE, et al. Transplantation of 2 partially HLA-matched umbilical cord blood units to enhance engraftment in adults with hematologic malignancy. *Blood*. 2005; 105(3):1343–7. [PubMed: 15466923]

Figure 1. Anatomy of placenta and fetal membranes at term

The location of membranes (amnion and chorion) surrounding the fetus as well as the umbilical cord and placenta are shown. This image was obtained from the US National Library, NIH (<http://www.nlm.nih.gov/medlineplus>).

Figure 2. Comparative flow cytometric analyses of extraembryonic and intraembryonic hematopoietic progenitors

Freshly isolated light density cells from placenta, chorionic membrane and fetal liver (22 weeks of gestation) were stained with monoclonal antibodies against CD34-APC, CD45-FITC and CD38-PE. Cells were stained with propidium iodide (PI) for live cells gating. Quadrants were set based on the staining obtained using isotype-matched control antibodies.

Figure 3. Hematopoietic progenitors are present in chorionic villi and the fetal chorionic membrane

Freshly isolated light-density cells from placenta and chorionic membrane (5, 15, 22 and 36 weeks of gestation) were stained with monoclonal antibodies against CD34-APC, CD45-PE, and PI for live cells gating. Quadrants were set based on the staining obtained using isotype-matched control antibodies.

Figure 4. Immunolocalization of CD34⁺CD45⁺ cells in term placenta

Tissue sections of a 38 wk full-term placental villus (delivered by C-section) were subjected to double indirect immunolocalization to evaluate expression of CD34 (FITC) and CD45 (Rhodamine). Nuclei were stained with DAPI (blue). Double positive cells appear yellow. The photographs were taken at 40x magnification. Inset on the top right corner shows a magnified CD34⁺CD45⁺ cell anchored to a CD34⁺CD45⁻ endothelial cell.

Figure 5. Long-term and multilineage hematopoietic reconstitution of NOD-SCID mice by human placental cells

CD34⁺⁺CD45⁺lin⁻ cells were isolated from 19 weeks of gestation placental villi by cell sorting and transplanted into 3 NOD-SCID mice. Hematopoietic reconstitution was observed 11 weeks after transplant in 2 of 3 mice. Human cells were identified in the mouse BM among PI⁻ live cells, followed by gating on human CD45⁺ cells lacking murine CD45 and TER119 antigen expression (not shown). Quadrants were drawn based on background staining of unreconstituted mice transplanted with carrier cells. Flow cytometric data demonstrating multilineage reconstitution is shown for one of the engrafted mice (11% of human reconstitution). From the upper right plot to lower left plot: 1) Erythroid reconstitution with CD71⁺CD235a⁺ precursors and CD235a⁺CD71⁻ mature red cells. 2) Myeloid reconstitution based on the expression of CD14 and CD15. 3) Active B-lymphopoiesis was observed based on the co-expression of CD19 and CD34. 4 & 5) The HSC populations CD34⁺⁺CD133⁺ and CD34⁺⁺CD38⁻ were also present in the reconstituted animal. 6) Megakaryocytes were identified by their expression of CD41. These data were previously reported in a note added in proof of our publication on the hematopoietic potential of human placenta³⁶.

Table 1

Abundant CD34^{++/+}CD45^{low} cells are present in chorionic villi and chorion at birth

Tissue	Gestational age (wks)	C-section (C) Labor (L)	# CD34 ^{++/+} CD45 ^{low} cells/g	Total # of CD34 ^{++/+} CD45 ^{low} cells
Chorion A	36	C	7×10^3	7.2×10^5
Placenta A	36	C	1.1×10^4	7.2×10^6
Chorion B	37	C	1.2×10^4	2.5×10^6
Placenta B	37	C	1.4×10^3	9.8×10^5
Chorion C	39	C	2.1×10^3	2.4×10^5
Placenta C	39	C	3.0×10^3	1.6×10^6
Chorion D	39	C	1.0×10^3	1.0×10^5
Placenta D	39	C	2.1×10^4	12.2×10^6
Chorion E	41	L	8.4×10^3	7.6×10^5
Placenta E	41	L	1.5×10^3	1.0×10^6

Table 2

Hematological diseases and some non-hematological diseases that have been successfully treated by UCB transplantation and will benefit from expansion of the number of HSCs present in UCB banks.

Malignant hematological diseases	Acute leukemias (ALL, AML, undifferentiated, bi-phenotypic), chronic leukemias (CLL, CML, juvenile CML, juvenile myelomonocytic leukemia), myelodysplastic syndromes (refractory anemias, chronic myelomonocytic leukemia), myeloproliferative disorders (acute myelofibrosis, polycythemia vera, essential thrombocytopenia).
Non-malignant hematological diseases	Non-malignant diseases: aplastic anemia, Fanconi anemia, paroxysmal nocturnal hemoglobinuria, pure red cell aplasia, thalassemia, sickle cell anemia.
Non-hematological diseases	Inherited metabolic diseases, such as Hurler's disease, Krabbe disease, mucopolysaccharidoses - MPS-, metachromatic leukodystrophy, and adrenoleukodystrophy.