HUMAN DEVELOPMENT

The human chorion contains definitive hematopoietic stem cells from the fifteenth week of gestation

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ABSTRACT

We examined the contribution of the fetal membranes, amnion and chorion, to human embryonic and fetal hematopoiesis. A population of cells displaying a hematopoietic progenitor phenotype (CD34+ CD45low) of fetal origin was present in the chorion at all gestational ages, associated with stromal cells or near blood vessels, but was absent in the amnion. Prior to 15 weeks of gestation, these cells lacked hematopoietic in vivo engraftment potential. Differences in the chemokine receptor and β1 integrin expression profiles of progenitors between the first and second trimesters suggest that these cells had gestationally regulated responses to homing signals and/or adhesion mechanisms that influenced their ability to colonize the stem cell niche. Definitive hematopoietic stem cells, capable of multilineage and long-term reconstitution when transplanted in immunodeficient mice, were present in the chorion from 15-24 weeks gestation, but were absent at term. The second trimester cells also engrafted secondary recipients in serial transplantation experiments. Thus, the human chorion contains functionally mature hematopoietic stem cells at mid-gestation.

KEY WORDS: Human hematopoietic stem cells, Chorion, Extraembryonic development, Hematopoietic development

INTRODUCTION

Mammalian development entails asynchronous growth of the embryo/fetus and extraembryonic structures – principally, the placenta and membranes. The placenta, which develops in advance of the embryo/fetus to support its growth, was shown to be a hematopoietic organ in mice (Gekas et al., 2005; Ottersbach and Dzierzak, 2005) and humans (Barcena et al., 2009, 2011; Robin et al., 2009; Serikov et al., 2009). The placenta is composed of highly vascularized tree-like chorionic villi that sprout from the chorionic plate (CP) (Fig. 1A; Fig. S1A). The villous surface is covered by trophoblasts. During most of the first trimester, the chorion is also covered with villi, which regresses into a bilayer of cytотrophoblasts (CTBs) and stroma (Fig. 1B) to form the smooth chorion (SC) that surrounds the embryo/fetus, as the amniotic cavity enlarges (Benirschke et al., 2006). We reasoned that the common developmental origin of the placenta and the chorion (Hamilton and Boyd, 1960) might entail the simultaneous existence of cells with hematopoietic potential at both sites.

The anatomy of the fetal membranes is depicted in Fig. 1. The amnion, the inner layer, is the most proximal membrane to the embryo/fetus (Fig. 1A,B) and includes an epithelial monolayer. Beneath lies an avascular stromal layer (Luckett, 1975). The SC, the outer layer that lies adjacent to the decidual cells that line the uterus (Fig. 1A,B), comprises multiple layers of CTBs and a stroma that fuses with the amnion stroma in the mature fetal membrane (Bourne, 1962; Cross, 1998). The amniotic epithelium and CTBs are ectodermal derivatives; their stromata arise from the extraembryonic mesoderm. Initially, the chorion and amnion are physically separated and, as the pregnancy progresses, the amnion is positioned closer to the chorion and the adjacent stromal layers fully fuse at ~17-20 weeks of gestation (Ilancheran et al., 2009), forming the mature structure of the amnion-chorion (Fig. 1B; Fig. S1D).

The mouse chorion contains myeloid and definitive erythroid progenitors prior to chorio-allantoic fusion and before establishment of the fetal circulation within the placenta, suggesting the in situ generation of hematopoietic progenitors (Zeigler et al., 2006). Previously, we reported the presence of a population expressing high levels of CD34 and low levels of CD45 (CD34+CD45low cells) in whole human fetal membranes, but their niche and, more importantly, their functional status as hematopoietic precursors have not been established (Barcena et al., 2011). This population also resides in the chorionic villi of the placenta and contains hematopoietic stem cells (HSCs) (Barcena et al., 2011). Here, we asked whether the comparable region of the human chorion (Fig. 1B, dark blue) contains HSCs throughout gestation.

RESULTS

Hematopoietic progenitors in the extraembryonic compartment are restricted to the chorion and chorionic villi

To determine the exact location of phenotypically defined hematopoietic precursors observed in the amniochorion (Barcena et al., 2011) we isolated cells from the amnion, the chorion and, as a control, the chorionic villi from the same placentas across gestation and analyzed CD34 and CD45 (PTPRC) expression. The anatomical regions analyzed are depicted in Fig. S1. The 40 weeks of human pregnancy are often divided into trimesters: first (0-13 weeks), second (14-27 weeks) and third (28-40 weeks) trimester. The chorion samples contained both the SC and the CP, which was denuded of villi by manual dissection (Fig. S1A,B), and in those samples of amniochorion the amnion was separated from the chorion (Fig. S1C,E). The three tissues analyzed from first trimester samples were not subjected to any further processing after

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the enzymatic digestion of the tissues as described (Barcena et al., 2009), whereas second and third trimester tissues were processed further to obtain the light-density fraction. Fig. 1C shows the absence of cells co-expressing CD34 and CD45 in the amnion. By contrast, hematopoietic progenitors (CD34⁺⁺ CD45⁺⁻ cells) were readily detected in the chorion and the chorionic villi at all gestational ages. CD34⁻ CD45⁺ mature cells were observed in all samples and their frequency increased during gestation (Fig. 1C). Most of these cells are Hofbauer cells, i.e. CD14⁺ macrophages, which represent the most abundant mature hematopoietic cells in extraembryonic tissues (Barcena et al., 2009).

**Immunolocalization of chorionic CD34⁺⁺ CD45⁺⁻ cells throughout gestation**

To identify the hematopoietic niche in the chorion, we localized CD34⁺⁺ CD45⁺⁻ cells using immunofluorescence and confocal microscopy (Fig. 2). The sensitivity of immunofluorescence techniques does not allow high and low levels of CD34 expression to be distinguished, as is achieved by FACS. Therefore, we searched for cells coexpressing CD34 and CD45. Immunolocalization detected a similarly low frequency of chorionic hematopoietic progenitors as that observed by FACS analyses (Fig. 1C). These cells resided primarily within the mesenchymal compartment (Fig. 2A). During early gestation, when villi are forming, clusters of CD34⁺⁺ CD45⁺⁺ cells were observed next to vimentin⁺ cells (Fig. 2B). Regardless of age, these cells were frequently found in close contact with vimentin⁺ mesenchymal cells in the CP (Fig. 2C,D). CD34⁺⁺ CD45⁺⁺ cells were also found in a predominantly perivascular location in the SC (Fig. 2E,F), near vessels containing CD34⁻ CD45⁻ endothelial cells. In addition, the number of individual and clusters of CD34⁺⁺ CD45⁺⁺ cells significantly increased from first to second trimester. In conclusion, CD34⁺⁺ CD45⁺⁺ cells were frequently found associated with vimentin⁺ stromal cells in first trimester chorion, as well as in second trimester CP, and were primarily positioned near or within the vasculature in second trimester SC.

**The frequency and number of CD34⁺⁺ CD45⁺⁻ cells vary across gestation**

Hematopoietic progenitors were quantified from 51 chorion samples of different gestational ages. We defined a gate that contained the population of CD34⁺⁺ CD45⁺⁻ cells (Fig. 3A). Primitive hematopoietic progenitors and HSCs are known to display low levels of CD45 on their cell surface and to increase CD45 expression as they differentiate (Mayani et al., 1993). We found chorionic CD34⁺⁺ CD45⁺⁻ cells at low frequency throughout gestation (0.01-1.9%, n=51). The density of CD34⁺⁺ CD45⁺⁻ cells, measured as the mean number of cells/g tissue, was highest at
5-8 weeks of gestation (Fig. 3B). In the first trimester, they gradually diminished to \( \sim 10^4 \) cells/g and this density persisted for the rest of gestation. Although there was variability among samples, the total number of CD34++ CD45low cells in the chorion substantially increased towards term (Fig. 3C): first trimester \((n=21)\), \(12-58 \times 10^3\) cells/tissue; second trimester \((n=21)\), \(1.0-21 \times 10^4\) cells/tissue; and third trimester \((n=9)\), \(0.8-15 \times 10^5\) cells/tissue. These data show that the density of CD34++ CD45low cells was highest in the first trimester, whereas absolute numbers were highest at term. These findings are in line with our previous observations in chorionic villi of the human placenta (Barcena et al., 2009).

CD34++ CD45low cells in the chorion have a similar phenotypic profile to the equivalent intraembryonic population

The antigenic profiles of the light-density chorion cells are shown in Fig. 4 \((n=3)\). At mid-gestation, there are abundant CD34− CD45+ mature cells, mainly Hofbauer cells, and CD34− CD45− cells, mostly comprising stromal cells (data not shown). Analysis of CD34++ CD45low cells (gated in Fig. 4A) showed that they express little CD38 and CD133 (PROM1), low levels of CD117 (KIT) and CD4, and medium to high levels of HLA-DR, CD31 (PECAM1), CD90 (THY1), CD95 (FAS), TIE2 (TEK, CD202b, angiopoietin receptor 2) and CD71 (TFRC). These data suggest that the CD34++ CD45low population is heterogeneous, possibly including both hematopoietic and endothelial lineage (TIE2+) cells (Fig. 4B). In addition, this population displayed variable levels of CD13 (ANPEP), CD33 and EPOR (data not shown) and did not coexpress KDR (VEGFR2, CD309) or CD150 (SLAMF1) (Fig. 4B). Thus, the CD34++ CD45low cells in the chorion displayed a phenotypic profile similar to that of the most primitive subset (CD38−/low) of the intraembryonic counterpart population from fetal liver and bone marrow (BM), and was very similar to that of placental hematopoietic progenitors (Barcena et al., 2009; Bárcena et al., 1996; Muench et al., 1997).

Transplantation of chorion cells into immunodeficient mice shows that they contain HSCs

First, we identified the dissociation method that yielded the maximum number of repopulating HSCs. Collagenase digestion of the chorion failed to release cells that engrafted mice (Fig. 5A). Transplanted CD34++ CD45low cells and their mature progeny (CD34− CD45+ cells) were detected only after the chorion samples were subjected to serial enzymatic digestion by collagenase IA.
followed by trypsin (Fig. 5A). The level of human cell engraftment in the BM obtained with cells isolated using collagenase IA and trypsin was similar in both of the transplanted mice (~23%). The gating strategy employed to determine the frequency of human cells is shown in Fig. S2A. Multilineage engraftment was observed in these mice, with 24-30% B cells (CD19^+), 18-19% erythrocytes [CD235a (GYP A)^+], 7-8% myeloid cells [CD14^+ and CD66b (CEACAM8)^+], as well as 35-37% CD34^+ progenitor cells.

Fig. 3. The density and total number of CD34^{++} CD45^{low} cells changes during development. Light-density (LD) suspensions of human chorionic cells (SC+CP) were isolated and stained with monoclonal antibodies (mAbs) against CD34, CD45, as well as isotype-matched control mAbs. (A) Representative analysis of an 18-week chorion sample, showing the gate employed to determine the frequency of CD34^{++} CD45^{low} cells. (B) The density (mean number/g tissue) of CD34^{++} CD45^{low} cells at the indicated gestational windows (n=51); error bars indicate s.e.m. (C) The total number of CD34^{++} CD45^{low} cells contained in chorion samples (n=51) at different gestational ages was calculated by multiplying the percentage of cells in the gate (shown in A) by the total number of cells (5-12 weeks) or the number of LD cells (13-41 weeks). The number of samples analyzed of each gestational age is indicated in red.

Fig. 4. Phenotypic profile of chorionic CD34^{++} CD45^{low} cells. (A) LD suspensions of human chorionic cells (19 weeks of gestation) were stained with the indicated mAbs and PI. 1×10^6 viable cells were acquired for each analysis to ensure that at least 4×10^3 events were included in the gate containing CD34^{++} CD45^{low} cells. (B) Expression of the indicated markers labeled with PE (blue); data from isotype-matched controls are outlined in black. This experiment is representative of three experiments that analyzed the full marker set and 17 that analyzed a subset of the panel.
We investigated the hematopoietic engraftment potential of chorionic cells of different gestational ages. The vast difference in the number of cells that can be obtained from first versus second trimester chorion samples prevented us from cell sorting specific populations (i.e. CD34++ CD45low) across all gestational ages. In addition, the low frequency of CD34++ CD45low cells (0.01-1.9%) leads to very long cell sorts, which might affect cell purity and viability. Therefore, we transplanted unfractionated cell suspensions of enzymatically digested first trimester chorion samples and the light-density fraction of samples older than 12 weeks, which are enriched in CD34+ cells. In addition, for second trimester chorion samples (≥14 weeks), lineage depletion (lin−) was performed (as described in the supplementary Materials and Methods). The transplanted cells were obtained by processing the entire CP and the SC together (second trimester) or 30-50 g of third trimester tissues. Five to ten mice were transplanted with each specimen of 1-27×105 cells/mouse (first trimester), 0.1-22×10^5 cells/mouse (second trimester) or 2-6×10^5 cells/mouse (third trimester). Mice were analyzed from 9-30 weeks post-transplant, with the majority of analyses between 100 and 150 days. It has been reported that 7-10 weeks after transplant provides sufficient time to observe reconstitution derived from HSCs (Goyama et al., 2015; McKenzie et al., 2007), while more mature progenitors (CD38+) disappear by 4 weeks (Kerre et al., 2001). Mice transplanted with chorion cells obtained prior to 15 weeks of gestation showed no multilineage engraftment (Fig. 5B). In two cases, limited reconstitution by mostly myeloid- and/or T cells was observed in some or all of the mice (Fig. S3). With some variability, multilineage long-term hematopoietic reconstitution was detected from the fifteenth week of gestation onward (Fig. 5B). A general absence of repopulating HSCs was observed from late pre-term (34 weeks, 5 days) and term (38-39 weeks) chorionic cells: only 1 out of 26 mice transplanted displayed full multilineage reconstitution, while 7 out of 26 mice had partial human engraftment (Fig. 5B). Our data showed that definitive HSCs are present in the chorion from 10 weeks, whereas prior to 12 weeks no HSC activity was detected, and during the third trimester HSCs were extremely rare. In Fig. S3 we provide more detail on the transplantation experiments summarized in Fig. 5B.

Chorionic HSCs generate multilineage progeny in immunodeficient mice and can be serially transplanted

Multiparametric FACS analyses were performed on the BM, spleen and liver of animals engrafted with CD59+ second trimester lin− chorion cells (22 weeks) using previously detailed methods (Varga et al., 2010). Total BM samples were analyzed to ensure the detection...
of CD235a+ erythrocytes, which were otherwise depleted during the isolation of light-density cells from spleen and liver samples. At 148 days post-transplant, the median level of human cell engraftment in the BM was 21.6% and ranged between 4.5 and 58% \((n=10)\). Human chimerism was higher among splenocytes, which contained a median of 82% human cells (range 30.4-93.7%). Fig. 6 shows multilineage engraftment in a mouse with a high level of human chimerism. We used CD59 to identify all human cells, including mature CD235a+ erythrocytes (Fig. S2B). CD45+ leukocytes contained a substantial CD34+ hematopoietic progenitor population. The human cells were at different stages of maturation, from HSCs (CD34++ CD133+) and early progenitors (CD34+++) to intermediate progenitors of the B-cell (CD34+ CD19+) cells, myeloid (CD34+ CD33+) cells and erythroid (CD34+ CD235a+) lineages. In addition, the BM of transplanted mice contained mature B cells (CD34− CD19+), mature myeloid cells (CD34− CD45+ CD33+), erythrocytes (CD34− CD235a+), T cells (CD3+) and natural killer (NK) cells [CD56 (NCAM1)+ CD3− CD7+]. In the spleen, we detected erythrocytes, monocytes (CD14+ cells), platelets [CD41 (ITGA2B)+ CD42b (GP1BA)+] and CD3+ CD4+ and CD3+ CD8+ T cells. CD34++ CD133+ CD38−/low cells were also observed in the liver, consistent with previous observations that the adult mouse liver supports this primitive population of hematopoietic precursors (Muench et al., 2014).

To further verify long-term reconstitution by HSC engraftment, serial transplantation was performed in which the BM of primary recipients was transplanted into secondary recipients. Light-density lin− chorion cells from a 24-week sample were transplanted into two primary recipients and their BM was harvested 68 days later. Multilineage engraftment was observed in the BM of both mice, with frequencies of 43% or 53% human cells (data not shown). BM was pooled for transplantation into secondary recipients, which each received the equivalent of 36% of the BM content of a single femur. The BM of secondary recipients was analyzed after 92 days and contained 19-21% human cells (Fig. 7). The primitive progenitor population (CD34++ CD133+ CD38−/low) was readily detected, as well as B-cell progenitors, myeloid cells and erythrocytes. The myeloid population contained granulocytes (CD33+ CD66b+) and monocytes (CD33+ CD14+). In the spleen, platelets and T cells (single positive for CD4 or CD8) were present.
These data demonstrate that the mid-gestation chorion contains HSCs capable of long-term, multilineage hematopoiesis with secondary transplant potential in adult hosts.

**Extreme limiting-dilution analysis (ELDA) of chorion HSCs in immunodeficient mice**

ELDA (Albelda et al., 1990) was performed to determine the frequency of HSCs among chorionic cells from two mid-gestation lin− samples (20 weeks) (Hu and Smyth, 2009). Phenotypic analyses of the chorion cells prior to transplant showed that the frequency of potential HSCs (CD34++ CD45low CD133+) was low (1-4 cells in 1×10³ cells of the light-density, lin− fraction; data not shown). The estimated frequency of engrafted HSCs was 1 in 3.3×10⁴ light-density lin− cells in one experiment (Fig. 8A) and 1 in 8.2×10⁴ light-density lin− cells in the other (Fig. 8B). Using these values, we calculated that the transplantation of 33-132 (experiment 1) or 82-328 (experiment 2) CD34++ CD45low CD133+ cells produced multilineage, long-term engraftment in mice. These frequencies are similar to those reported for human BM HSCs (Guezguez et al., 2013).

There is no universally agreed upon definition of engraftment as used as an indicator of HSC activity in immunodeficient mice. We used a strict definition to define full engraftment (presence of three lineages) that included detection of CD34+ cells as an indication of ongoing hematopoiesis, although failure to detect human red cells was often the cause of partial engraftment. Partial engraftment was detected in many mice in the limiting-dilution analyses in a pattern consistent with the dose of cells transplanted (Fig. 8C,D). As some of these mice may have been engrafted with HSCs but failed to achieve sufficient multilineage engraftment for detection, our estimate of HSC numbers might be low. In addition, the R-values for the graphs shown in Fig. 8A,B were higher than 1, which implies that engraftment was hyper-responsive to dose. This behavior suggests multi-hit alternatives (Hu and Smyth, 2009), i.e. the presence of more than one cell population cooperating to produce full human cell reconstitution in mice or the cooperative effects of human hematopoietic cells in supporting their own growth and differentiation.

**Sorted CD34++ CD45low chorion cells are of fetal origin and reconstitute immunodeficient mice**

To determine the origin of HSCs residing in the chorion, we sorted CD34++ CD45low cells from a CP plus SC whole-chorion sample obtained from a male at 18.3 weeks of gestation. Since the number of cells obtained was very low, we expanded them for 3.5 days in vitro in the presence of cytokines, after which we performed fluorescent in situ hybridization (FISH) with probes specific for the X or Y chromosomes. The results indicated that this population was of fetal origin, as 98% of the cells were male (Fig. 9A). Next, we sorted CD34++ CD45low cells from a male SC sample at 23.6 weeks of gestation, and transplanted them into NSG-3GS mice (3×10³ cells/mouse). After 61 days, multilineage human engraftment was observed in the BM, including erythroid, lymphoid (B, T and NK cells) and myeloid cells (Fig. 9B). The spleen contained human platelets and T cells (Fig. 9C), while the liver contained a CD34++ CD133+ HSC population (Fig. 9D). The BM from all four of the transplanted mice showed full human reconstitution and they were pooled to sort CD34− CD45− cells, demonstrating that the mature hematopoietic cells generated in the transplanted mice are also of fetal origin (data not shown). From these results we concluded that the HSC potential of the chorion is a source of fetal hematopoietic cells with engraftment potential in adult recipients.
chorion samples resides within the CD34++ CD45low population and is not maternally derived.

Chemokine receptor and adhesion molecule expression of first and second trimester CD34++ CD45low chorion cells

We asked whether functional differences among chorion HSCs before and after 15 weeks of gestation could be due to differential expression of chemokine receptors and adhesion molecules involved in homing to, and retention in, the mouse BM. We focused on chemokine receptors previously reported to be expressed by HSCs from human umbilical cord blood and BM (Aiuti et al., 1997; Basu and Broxmeyer, 2009; Broxmeyer et al., 2005; de Wynter et al., 1998; Lévesque et al., 2003; Peled et al., 2000; Rosu-Myles et al., 2000; Su et al., 1997; Tarnowski et al., 2010; Torossian et al., 2014). First trimester CD34++ CD45low chorion cells expressed low levels of CXCR7 (ACKR3) and no detectable CD193 (CCR3) and CD195 (CCR5), but expression of the receptors increased in second trimester samples (Fig. 10A). Conversely, we detected CD191 (CCR1), which is expressed by CD34+ cells in the human BM (de Wynter et al., 1998; Su et al., 1997; Tarnowski et al., 2010; Torossian et al., 2014). First trimester CD34++ CD45low chorion cells expressed low levels of CXCR7 (ACKR3) and no detectable CD193 (CCR3) and CD195 (CCR5), but expression of the receptors increased in second trimester samples (Fig. 10A). Conversely, we detected CD191 (CCR1), which is expressed by CD34+ cells in the human BM (de Wynter et al., 1998; Su et al., 1997), only on first trimester CD34++ CD45low cells. These data suggest that the developmentally regulated expression of chemokine receptors could be responsible for the functional deficits of early gestation chorionic HSCs. In this context, the expression of CD191 alone failed to render functionally mature chorionic CD34++ CD45low cells capable of reconstitution. Instead, the cooperation among several chemokine pathways might be required for functional maturity in terms of homing.

Adhesion molecules are involved in HSC homeostasis, as they also regulate homing and retention of these cells to the BM niche. Thus, we asked whether chorionic CD34++ CD45low cells expressed adhesion molecules that play crucial roles in these processes, specifically β1 integrins – α4β1 (VLA-4, CD49d, ITGA4), α5β1 (VLA-5, CD49e, ITGA5) and α6β1 (VLA-6, CD49f, ITGA6) – as HSCs with repopulating capacity express these receptors (Imai et al., 2010; Notta et al., 2011; van der Loo et al., 1998). Fig. 10B shows high expression of CD49d and CD49e and medium levels of CD49f during the first trimester. In comparison, second trimester chorionic CD34++ CD45low cells lacked CD49d expression, displayed similar levels of CD49e and increased levels of CD49f. Other β1 integrins [α1β1 (CD49a, ITGA1), α2β1 (CD49b, ITGA2) and α3β1 (CD49c, ITGA3)] also implicated in BM adhesion were found to be expressed at similar levels regardless of gestational age (data not shown). Thus, the CD34++ CD45low cells isolated from the first and second trimester chorion had different integrin expression profiles. Correlating these results with the in vivo transplantation data suggested that the downregulation of CD49d and the upregulation of CD49f might be important signals for the functional maturation of chorionic HSCs.
DISCUSSION

Here we demonstrate the hematopoietic potential of the human chorion. It was previously reported that the mouse chorion possesses hematopoietic progenitors prior to the establishment of fetal circulation (Zeigler et al., 2006) or the chorio-allantoic fusion (Corbel et al., 2007), thus pointing to the likely in situ generation of hematopoietic progenitors expressing Runx1 and CD41. The notion that hematopoietic progenitors are engendered intrinsically in extraembryonic tissues was further supported by the finding of multilineage hematopoietic progenitors in mouse placenta of

Fig. 9. CD34++ CD45low human chorionic cells are of fetal origin and contain HSCs. (A) 1×10⁴ CD34++ CD45low cells were isolated by FACS from the CP+SC of a male fetus at 18.3 weeks of gestation and cultured for 3.5 days. FISH analysis showed that 98% of the interphase cells had a male (XY) signal. A photomicrograph of the cells that were analyzed is shown (green, X chromosome; orange, Y chromosome). CD34++ CD45low cells isolated from a male SC at 23.6 weeks of gestation were transplanted into NSG-3GS mice. Engraftment was analyzed 61 days later (n=4). Representative results showing hematopoietic engraftment of the BM (B), spleen (C) and liver (D) are shown. Open arrows indicate splenic platelets (C) and a likely population of HSCs in the liver (D). (E) The BM of four engrafted mice was pooled and CD34++ CD45+ cells isolated by FACS, all of which were male as assessed by FISH analysis. A photomicrograph of CD34++ CD45+ cells sorted from the mouse BM cells showed a male (XY) signal.
NCX1−/− (Slc8a1−/−) embryos, which lack heartbeat and, therefore, have no fetal blood circulation (Rhodes et al., 2008). These multipotent progenitors were able to produce erythroid, myeloid and lymphoid progeny in vitro. Although the self-renewing ability of mouse placental HSCs has been extensively documented (Gekas et al., 2005; Ottersbach and Dzierzak, 2005), the long-term repopulating activity of placental NCX1−/− hematopoietic progenitors has not been addressed and whether the murine chorion contains HSCs remains unknown.

To our knowledge, this is the first demonstration of transplantable, definitive HSCs in the human chorion, which are absent in the amnion. Specifically, the chorion contains CD34++ CD45low cells capable of long-term, multilineage reconstitution in mice and these cells could be serially transplanted into secondary recipients resulting in engraftment. By performing limiting-dilution transplantation studies, we estimated the frequency of HSCs to be 1 in 3.3-8.2×10⁴ light-density lin− cells.

Taken together, our data demonstrated that the chorionic membrane provides an extraembryonic site of hematopoiesis during human gestation that harbors a resident population of HSCs, albeit at low frequency.

The primary goal of our study was to map the emergence and presence of hematopoietic precursors in the chorion during gestation. In line with our previously reported observations in placenta (Barcena et al., 2009), the early gestation chorion (5-8 weeks) contained higher numbers of CD34++ CD45low cells/g tissue than observed later in development, although it is important to stress that this population of cells is always present at a low frequency (less than 1% of light-density chorion cells). Their density significantly drops from the ninth week onward, and is maintained at a constant level for the remainder of gestation. However, the transplantation of early gestational age chorion samples occasionally produced one or two hematopoietic lineages (e.g. unilineage T-cell reconstitution), but not multilineage engraftment in mice. By contrast, from the fifteenth week of gestation onward, the chorion consistently contained definitive HSCs. This observation resonates with a report by Ivanovs et al. (2011), in which the authors reported transient unilineage T-cell reconstitution when transplanting early placentas (26-41 days of gestation) into immunodeficient mice; multilineage, long-term engraftment

Fig. 10. CD34++ CD45low chorionic cells express several chemokine receptors and β1 integrins. (A) Freshly isolated LD suspensions of human chorionic cells from 9-week and 19-week samples were stained with the indicated mAbs and PI. Similar to the analysis performed in Fig. 4, a gate was set to include CD34++ CD45low cells. Staining with mAbs against chemokine receptors (CD184, CD193, CD195, CXCR7 and CD191) and isotype-matched controls is shown. The expression of each marker in the PE and Alexa Fluor 647 (AF647) channels is shown (blue); data from isotype-matched control mAbs are outlined in black. This experiment is representative of the analysis of n=3 for first trimester and n=4 for second trimester samples. (B) LD suspensions of chorionic cells from the same 9-week and 19-week specimens were stained with the indicated mAbs and PI. A gate was set to include CD34++ CD45low chorionic cells (not shown), and their staining with mAbs against CD49d (α4β1), CD49e (α5β1) and CD49f (α6β1) is shown in red, with the data from isotype-matched control mAbs outlined in black. n=6, three from the first and three from the second trimester.
was only accomplished when placentas of 15 weeks of gestation or older were transplanted.

The mechanisms that orchestrate changes in the functional status of extraembryonic HSCs as gestation advances are not known. Cells with the phenotype of hematopoietic progenitors that were isolated from early gestation tissues might possess limited potential and represent a primitive wave of hematopoietic progenitors unable to reconstitute mice. A potentially important player in the regulation of hematopoietic potential could be the hypoxic environment of extraembryonic tissues during early pregnancy. Since the placenta is not steadily perfused with maternal blood prior to 10-12 weeks of pregnancy (Rodesch et al., 1992), physiological hypoxia could play a role in regulating hematopoiesis. It has been suggested that HSCs in the BM reside in a hypoxic niche that maintains their repopulating potential and their quiescent state (Kubota et al., 2008), and hematopoietic tissues in the mouse embryo, such as the aorta-gonad-mesonephros (AGM) region, liver and placenta, are also hypoxic (Imanirad and Dzierzak, 2013). Likewise, the human chorion is poorly vascularized and possibly provides a hypoxic environment. We addressed the question of whether the oxygen level has a functional impact on early hematopoietic progenitors. Our data showed no consistent differences between CFU-C production at hypoxic versus normoxic conditions. However, we could not rule out the possibility that hypoxia may work indirectly via the niche, which is dismantled when we isolate the cells.

An alternative explanation for the age-related functional disparities among chorionic hematopoietic precursors could be differences in their homing abilities and/or adhesion properties. We focused on receptors that are expressed by perinatal and postnatal HSCs. Our study shows that there are important differences among first and second trimester chorionic CD34++ CD54low cells with regards to their chemokine receptor expression profile. CD184 (CXCR4), the CXCL12 receptor, plays a crucial role in regulating HSCs homing to the BM (Aiuti et al., 1997; Broxmeyer et al., 2005; Lévesque et al., 2003; Peled et al., 2000). CD195 ligands (CCL3, CCL4 and CCL5) modulate the chemotaxis of CD34+ cells in cord blood via the CD184-CXCL12 pathway (Basu and Broxmeyer, 2009). CD195 is expressed at higher levels on cord blood HSCs than on those obtained from BM or mobilized to peripheral blood (Rosu-Myles et al., 2000), CXCR7, another CXCL12 receptor (Tarnowski et al., 2010), cooperates with CD184 in enhancing the effects of CXCL12 on cell cycling, survival and proliferation of CD34+ cells from peripheral blood (Torossian et al., 2014). In addition, CD34+ cells from umbilical cord blood express CD193 (Lamkihoued et al., 2003), and CD191 expression by CD34+ cells in cord blood confers superior engraftment of immunodeficient mice (de Wynter et al., 2001). Our data showed that first trimester chorionic progenitor cells express CD191 and low levels of CXCR7, features that do not render chorionic CD34++ CD54low cells transplantable. Second trimester CD34++ CD45low cells, although largely CD191+, consistently expressed CD184, CD193, CD195 and CXCR7. Therefore, transplantable second trimester chorionic HSCs share the chemokine receptor profile with HSCs from perinatal and postnatal origins.

Similarly, second trimester chorionic CD34++ CD45low cells share many characteristics with fetal liver and fetal BM HSCs/progenitors [expression of CD133, CD117, CD4, HLA-DR, CD95 (Fig. 4) and CD13, CD33 (data not shown)]. Despite the commonalities, definitive chorionic CD34++ CD45low cells display unique phenotypic properties, such as low-level CD38 expression and their β1 integrin expression profile (CD49d- CD49e+ CD49f+).

Second trimester fetal liver CD34++ CD45low cells coexpress CD49d (data not shown) and contain transplantable HSCs (Muench et al., 2014). Therefore, CD49d expression does not correlate with functional HSCs in the chorion. We conclude that the adhesion and homing properties of the extraembryonic niche might differ from those of the intraembryonic counterpart.

The origin of human extraembryonic HSCs is uncertain. Here we employed a FISH approach on second trimester male chorion samples to establish the fetal origin of sorted CD34++ CD45low cells, as well as to identify their progeny produced in vivo. Despite their fetal origin, CD34++ CD45low cells could arise in situ to later populate the embryo/organism. Alternatively, they might have an intraembryonic origin, colonizing extraembryonic structures once blood circulates. We cannot obtain chorion prior to 5 weeks of gestation and therefore, in all of our samples, the fetal-placental circulation was already established (Hamilton and Boyd, 1970). The AGM of 32-day-old human embryos (Carnegie stage 14) contains transplantable HSCs, preceding yolk sac, liver and placenta production of these cells (Ivanovs et al., 2011). Although suggestive, definitive proof that AGM-derived or liver-derived HSCs migrate to the placenta/chorion is lacking. Regardless, these extraembryonic structures could be sites of HSC generation. Our immunolocalization studies showed that the majority of the chorionic CD34++ CD45low cells were embedded within vimentin stromal cells rather than being associated with blood vessels, suggesting their possible origin from mesodermal progenitors in the stroma, which is derived from the extraembryonic mesoderm. This finding is in line with data from the murine system (Tanaka et al., 2012). We conclude that two separate hematopoietic niches coexist in the chorion: a predominant mesenchymal niche and a less common perivascular niche. The functional relationship between these two hematopoietic niches or whether cells traffic between these locations remains to be determined. It would be very interesting to define, at a molecular level, the similarities and differences between the niches, which could provide functional insights into the regulatory networks of these two locations.

**MATERIALS AND METHODS**

**Isolation of hematopoietic progenitors from the amnion, chorion and placenta**

Human tissues were obtained anonymously with the approval of the University of California, San Francisco Committee on Human Research. All donors gave written informed consent. Samples from 5-24 weeks of gestation were from elective pregnancy terminations at San Francisco General Hospital. Full-term specimens collected at the time of delivery were from Moffitt-Long Hospital, University of California, San Francisco, CA, USA. The gestational ages of first trimester specimens were estimated based on crown-rump lengths, measured by ultrasonography. The gestational ages of second trimester specimens were estimated by foot length. The ages are expressed in weeks and days (e.g. 20.5 weeks means 20 weeks, 5 days).

Hematopoietic cells from chorion and amnion (separated from one another by manual dissection) were isolated as previously described (Barcena et al., 2009). Age group-specific tissue processing is described in detail in the supplemental Materials and Methods.

**Flow cytometry**

Freshly isolated single-cell suspensions were stained with monoclonal antibodies (see Table S1) and analyzed using a two-laser FACScan or three-laser LSR II cytometer (BD Biosciences). CellQuest (BD Biosciences) or FlowJo version 9.2 software was employed for analyses.
which focused on live cells using an electronic gate to identify propidium iodide (PI)− events.

Human hematopoietic engraftment of mice
Animal studies were performed with the approval of the Institutional Animal Care and Use Committee at PMI Preclinical, San Carlos, CA, USA. Mice received humane care according to the criteria outlined by the National Academy of Sciences in the Guide for the Care and Use of Laboratory Animals as detailed in Varga et al. (2010). Founder NOD.Cg-Prkdcsclid I2rgtm1Wjl/Tg (CMV-IL3,CSF2,KITLG)1Eav/MloySzJ (NSG-3GS) mice were obtained from Jackson Laboratories and maintained, bred and transplanted in a restricted-access, specific pathogen-free vivarium at Blood Systems Research Institute, San Francisco, CA, USA. For additional details on the transplantation of human cells into mice, as well as the harvest of mouse tissues for engraftment analyses, see the supplementary Materials and Methods.

Human chimerism in mice was studied between 63 and 214 days post-transplant; the majority of analyses were performed between 100 and 150 days. Mice were sacrificed by carbon dioxide asphyxiation followed by cervical dislocation. BM was harvested using a syringe with a 27-gauge needle to flush the femurs with ~3 ml culture medium. Spleen and liver specimens were held in culture medium on ice until cell isolation. At the conclusion of the experiment, mice were examined for signs of poor health or pathologies such as tumors of the liver, spleen and/or thymus. Any moribund animals were removed from the study. Mouse BM, spleen and liver were processed as previously described (Varga et al., 2010).

The frequency of live human cells in the transplanted mice was determined based on the percentage of CD59+ cells that lacked the expression of mouse markers (Fig. S2). The presence of transplanted HSCs was determined by measuring engraftment of myeloid [CD33+, CD14+, CD66b+ and/or CD15 (FUT4)+], erythroid (CD235a+), lymphoid (CD19+ B cells, CD3+ T cells and CD56+ CD3− NK cells) and hematopoietic precursor (CD34+ CD133+ CD38−) cells by FACS. Engraftment of experimental mice was defined as a higher frequency of lineage marker-positive events in recipients as compared with control cells from an untransplanted mouse that were processed in parallel. Engraftment was defined as a minimum of five positive events above untransplanted controls. Full engraftment was defined as detection of all four cell types: myeloid, erythroid, lymphoid and precursors. Partial engraftment was defined as any engraftment less than full engraftment.

Hematopoietic progenitor assays
To enumerate CFU-Cs, methylcellulose clonal cultures of chorion cell suspensions were plated at a density of 2×103/35 mm plate (BD Falcon, BD Biosciences) in Methocult GF H4435 (StemCell Technologies). Colonies containing myeloid cells or CFU-GM (CFU-granulocyte-macrophage progenitors) and CFU-mix (CFU-granulocyte-macrophage and erythroid cells) were scored after growth for 3.5 weeks at 37°C in normoxic (20% O2) or physiologically hypoxic (1% O2) conditions.

Statistical analysis
For the estimation of HSC frequency in the mid-gestation chorion, ELDA (Albelda et al., 1990) was performed according to the method of Hu and Smyth (2009). We employed ELDA software (http://bioinf.wehi.edu.au/software/elda/) to graph the results, which are also shown as box and whisker plots; median chimerism levels are indicated by a notch in the box.

Immunolocalization and fluorescence microscopy
Chorion tissue sections (20 μm) were fresh-fixed in 4% paraformaldehyde for 90 min and processed as previously described (Prakobphol et al., 2006). The tissue sections were stained with monoclonal antibodies against CD34 (directly conjugated to FITC; clone 581, IgG1, BD Biosciences/PharMingen, 555821; 1:25), CD45 (clones 2B11+P7D7/26, IgG1, Dako/Agilent, GA75161-2; 1:50) and vimentin (clone SP20, Abcam, Ab92545; 1:100). For controls and secondary antibodies, see the supplementary Materials and Methods. Staining with DAPI in Vectashield mounting medium (Vector Laboratories) was used to visualize nuclei. Slides were imaged on a Leica DM 6000-CS microscope integrated with the Leica TCS SP5 confocal platform.

Cell sorting of CD34+ CD45low cells from the chorion
Once the light-density lin− single-cell suspensions were obtained from the chorion, the cells were stained with anti-CD34-APC (BD Biosciences, 555824) and anti-CD45-PE (Biolegend, 304008) monoclonal antibodies following manufacturer’s recommendations and sorted in a FACSAria cell sorter (BD Biosciences) as previously described (Barcena et al., 2009).

In vitro expansion of CD34+ CD45low cells for FISH
1×106 sorted CD34+ CD45low cells from an 18.3-week chorion (CP+SC) specimen were cultured in 48-well plates (BD Falcon, BD Biosciences) in 0.5 ml Stem Span II medium (StemCell Technologies) supplemented with 50 ng/ml Kit ligand, 100 ng/ml FLT3 ligand, 20 ng/ml thrombopoietin and 20 ng/ml interleukin 3; all the cytokines were from Peprotech, except FLT3 ligand, which was purchased from ORF Genetics (Kopavogur, Iceland). After 3.5 days in culture at 37°C, 5% CO2, the cells were harvested, fixed and deposited on slides for FISH.

FISH
2×105 sorted cells were processed immediately after isolation and FISH was performed using X chromosome-specific [Vysis CEP X (DXZ1) SpectrumGreen] and Y chromosome-specific [CEP Y (DYZ3) SpectrumOrange] probes from Abbott Molecular following the manufacturer’s protocol and as previously reported (Qi et al., 2015). For additional details, see the supplementary Materials and Methods. The cells were stained with DAPI II (Abbott Molecular) to visualize nuclei. FISH results were analyzed and documented using the CytoVision system (Leica).

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Competing interests
The authors declare no competing or financial interests.

Author contributions

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