Trophoblasts Regulate the Placental Hematopoietic Niche through PDGF-B Signaling

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SUMMARY

The placenta is a hematopoietic organ that supports hematopoietic stem/progenitor cell (HSPC) generation and expansion without promoting differentiation. We identified PDGF-B signaling in trophoblasts as a key component of the unique placental hematopoietic microenvironment that protects HSPCs from premature differentiation. Loss of PDGF-B or its receptor, PDGFRß, induced definitive erythropoiesis in placental labyrinth vasculature. This was evidenced by accumulation of CFU-Es and actively proliferating definitive erythroblasts that clustered around central macrophages, highly reminiscent of erythropoiesis in the fetal liver. Ectopic erythropoiesis was not due to a requirement of PDGF-B signaling in hematopoietic cells but rather in placental trophoblasts, which upregulated Epo in the absence of PDGF-B signaling. Furthermore, overexpression of hEPO specifically in the trophoblasts in vivo was sufficient to convert the placenta into an erythropoietic organ. These data provide genetic evidence of a signaling pathway that is required to restrict erythropoiesis to specific anatomical niches during development.

INTRODUCTION

The goals of developmental hematopoiesis are to generate differentiated blood cells for the fetus while establishing a pool of undifferentiated hematopoietic stem cells (HSCs) for postnatal life. This is achieved by segregating fetal hematopoiesis into multiple waves and different microenvironmental niches that protect undifferentiated hematopoietic stem/progenitor cells (HSPCs) or promote differentiation (Mikkola and Orkin, 2006). The first wave of hematopoiesis begins in the yolk sac with the formation of primitive erythroblasts that fulfill the immediate oxygen needs of the embryo, and macrophages that assist in tissue remodeling (Palis et al., 2001). In the second wave, the yolk sac generates a transient pool of definitive progenitors that seed the fetal liver to launch definitive erythropoiesis and myelopoiesis. Finally, in the third wave, the multipotent, self-renewing HSCs develop in the major arteries in the AGM (aorta-gonad mesonephros) region, the placenta, and the yolk sac, after which they expand in the placenta and the fetal liver before colonizing the bone marrow (Chen et al., 2009; Mikkola and Orkin, 2006; Rhodes et al., 2008; Zovein et al., 2008). Several niche cells, such as endothelial, endosteal, and mesenchymal cells, and macrophages regulate HSCs in the bone marrow (Chow et al., 2011; Kiel and Morrison, 2008). However, the cellular and molecular mechanisms promoting stemness versus differentiation in fetal hematopoietic niches remain undefined.

The function of the placenta as a hematopoietic site was recognized only recently (Alvarez-Silva et al., 2003). The placenta is a unique hematopoietic organ that can generate multipotent HSPCs de novo and support their expansion without promoting differentiation (Gekas et al., 2005; Ottersbach and Dzierzak, 2005; Rhodes et al., 2008; Zeigler et al., 2006). HSPCs are generated in the large vessels in the chorionic villi mesenchyme, whereas the placental vascular labyrinth provides a niche where HSPCs expand (Rhodes et al., 2008). The human placenta is also populated by HSPCs throughout most of gestation (Bárcena et al., 2009; Robin et al., 2009; Serikov et al., 2009). However, the niche cells and signals that compose the unique placental hematopoietic microenvironment are unknown.

The structure of the placental vascular labyrinth is compromised in mouse embryos that lack PDGF-B signaling (Ohlsson et al., 1999). PDGF-B signals through receptor tyrosine kinases PDGFRß and PDGFRα, influencing cell differentiation, proliferation, migration, and survival in various organs (Tallquist and Kazlauskas, 2004). Pdgfr−/− and Pdgfrα−/− embryos die perinatally with strikingly similar phenotypes (Levèen et al., 1994; Soriano, 1994). In the placenta, PDGF-B is expressed in the endothelium, some hematopoietic cells, and trophoblasts, whereas PDGFRß is expressed in pericytes and trophoblasts (Andrae et al., 2008; Holmgren et al., 1992). Loss of the ligand or the receptor leads to a decrease in placental trophoblasts and pericytes, and dilation of vasculature after mid-gestation (Ohlsson et al., 1999). Later in gestation, the embryos...
also develop anemia, thrombocytopenia, and hypocellular fetal liver, as well as kidney and heart defects (Levéen et al., 1994; Soriano, 1994).

Here, we demonstrate that loss of PDGF-B signaling alters the placental hematopoietic niche by upregulating Epo (Erythropoietin) levels in placental trophoblasts, which triggers ectopic erythropoiesis in placental vasculature. These data establish trophoblasts as important niche cells and PDGF-B signaling as a critical molecular cue that prevent premature differentiation of HSPCs in the placenta.

RESULTS

**Pdgf-b^−/−** Embryos Display Ectopic Erythropoiesis in Placental Labyrinth during Midgestation

To identify the cellular and molecular components of the placental hematopoietic microenvironment, we asked whether the compromise of the labyrinth structure in placentas that lack PDGF-B signaling affects hematopoiesis. Consistent with previous reports, the placentas in Pdgf-b^−/− embryos exhibited dilation of labyrinth blood vessels and a reduction in trophoblasts by E13.5 (Figure 1A). Interestingly, H&E revealed the presence of clusters of blast-like cells with high nucleus to cytoplasmic ratio within the Pdgf-b^−/− labyrinth vasculature (Figure 1B). These blast clusters were first identified at E13.5, and by E18.5 they contained cells that resembled maturing erythroblasts.

To define the identity of the blast cells in Pdgf-b^−/− placental vasculature, immunohistochemistry (IHC) and immunofluorescence (IF) were performed. The blast cells did not express embryonic β-H1 globin, arguing against them being primitive erythroid cells (Figure 1C). Most of the blast cells expressed the HSPC marker c-Kit and erythroid marker Ter119, suggesting that they were definitive erythroid precursors (Figure 1C). Ki67 staining indicated that the blast cells were actively cycling (Figure 1D). Methylcellulose colony assay confirmed a drastic increase in erythroid-committed progenitors, CFU-Es, in Pdgf-b^−/− placentas (Figure 1E). These results suggested that the placenta in Pdgf-b^−/− embryos had started to support definitive erythropoiesis, a process that should be restricted to the fetal liver at this stage.

We next investigated whether the blast cells in the placenta differentiated into other hematopoietic lineages. Some blast cells expressed CD41, a marker of nascent HSPCs and megakaryocytes/platelets (see Figure S1A available online), but not GP1b-β, which implied that they were not megakaryocytes. The blast cells neither expressed HSC markers Sca1 or CD150, nor the B-lymphoid marker B220; however, some expressed the pan-hematopoietic marker CD45. Strong CD45 expression (Figure S1A) was also observed in large cells that were identified as macrophages based on expression of F4/80, but not other myeloid markers (Mac1, Gr1, or FcγR) (Figure S1A). These macrophages clustered together with the blast cells in the vasculature of Pdgf-b^−/− placentas (Figure 1F) in structures that were highly reminiscent of the erythroblast-macrophage islands that are characteristic of tissues supporting erythroid differentiation.

Ectopic Erythropoiesis in the Absence of PDGF-B Signaling Is Specific to the Placenta

FACS analysis for erythroid maturation markers (Zhang et al., 2003) confirmed a significant increase in proerythroblasts in placentas and circulating blood of Pdgf-b^−/− embryos (Figure S2A). Morphological analysis of hematopoietic cells in the placenta and blood in Pdgf-b^−/− embryos verified the presence of proerythroblasts and basophilic erythroblasts (Figure 2A), whereas electron microscopy of the erythroblasts in Pdgf-b^−/− placentas confirmed the characteristic heterochromatin pattern as observed in fetal liver erythroblasts (Figure 2B). Thus, the ectopic erythropoiesis in Pdgf-b^−/− placentas had all the hallmarks of definitive erythropoiesis.

Because the increase in erythroblasts was also noted in circulating blood, a 1 hr BrdU incorporation assay was performed to determine if the ectopic erythropoiesis was unique to the placenta. FACS analysis revealed that only the placenta, and not blood, fetal liver, yolk sac, or spleen, in Pdgf-b^−/− embryos showed an increase in BrdU+/CD71+ erythroblasts (Figure 2C and data not shown). IF also evidenced prominent clusters of BrdU cells in the vasculature of Pdgf-b^−/− placentas (Figure 2C). Thus, in the absence of PDGF-B, the placental labyrinth vasculature specifically becomes a permissive environment for the proliferation and differentiation of definitive erythroid precursors.

**Erythropoiesis in Pdgf-b^−/− Placentas Is Not Due to an Intrinsic Requirement for PDGF-B Signaling in Hematopoietic Cells**

To determine if the ectopic definitive erythropoiesis observed in Pdgf-b^−/− placentas could be due to a direct requirement of PDGF-B signaling in hematopoietic cells, we examined the expression of the receptor, PDGFRβ, by FACS. Although no surface expression of PDGFRβ was detected in circulating primitive or fetal liver definitive erythroid cells (Figure S3A), PDGFRβ was expressed on a subset of c-Kit+ HSPCs in both the fetal liver and the placenta (Figure S3B).

Thus, we utilized conditionally targeted Tie2Cre Pdgfrα^fl/fl embryos to delete PDGFRα in hematopoietic cells. FACS analysis at E14.5 revealed a complete loss of PDGFRα protein on c-Kit+ HSPCs, as in Pdgfrα^−/− embryos (Figure S3C). However, in contrast to Pdgfrβ^−/− embryos that showed anemia in late gestation, no obvious hematological abnormalities were observed in Tie2Cre Pdgfrβ^fl/fl embryos (Table S1). Furthermore, Ki-67 and BrdU staining evidenced clusters of proliferating blast cells in Pdgfrβ^−/− but not Tie2Cre Pdgfrβ^fl/fl placentas (Figures 3A and 3B). Ectopic definitive erythropoiesis in Pdgfrβ^−/− placentas was further confirmed by FACS analysis (accumulation of CD71<sup>+</sup>Ter119<sup>+</sup> erythroblasts, Figure S3D) and CFU-E assay (Figure 3C), whereas Tie2Cre Pdgfrβ^fl/fl placentas showed no signs of erythropoiesis. Similar data were obtained when both PDGFαRs, Pdgfrβ and Pdgfrα, were deleted in hematopoietic cells (data not shown). These data imply that the ectopic definitive erythropoiesis in Pdgf-b^−/− and Pdgfβ^−/− placentas is not due to a requirement of PDGFRβ signaling in hematopoietic cells.

**Ectopic Definitive Erythropoiesis in PDGF-B-Deficient Placentas Occurs prior to Other Hematological Defects**

Because Pdgf-b^−/− embryos display abnormalities in multiple organs, we investigated whether hemorrhaging, anemia, and other organ defects could underlie the ectopic erythropoiesis in the placenta. Consistent with previous studies, Pdgf-b^−/− embryos displayed bleeding and edema perinatally (E18.5); however, these abnormalities were not observed at E13.5–E15.5.
when ectopic erythropoiesis was already evident (Figure S4A) (Levéen et al., 1994). By E16.5 and E18.5 both Pdgf-b−/− and Pdgfrα−/− embryos showed anemia (Table S1); however, the first sign of reduced blood cell counts was observed only after E13.5, when the erythroblast clusters were already evident in the placenta (Figures S4B and 1B). Furthermore, reduction in fetal liver size in Pdgf-b−/− embryos was observed only in late gestation (Figure S4C). These data indicate that the ectopic definitive erythropoiesis in Pdgf-b−/− placentas precedes other macroscopic defects and anemia.

Figure 1. Pdgf-b−/− Embryos Support Ectopic Erythropoiesis in Placental Labyrinth Vasculature during Midgestation
(A) IHC for CD31 (endothelium) and cytokeratin (trophoblasts) documents progressive dilation of the labyrinth vasculature (arrows) in Pdgf-b−/− placentas at E13.5–E18.5. Scale bar, 100 μm.
(B) H&E reveals clusters of blast-like cells in Pdgf-b−/− placental vasculature at E13.5–E18.5 (arrows, insets). Scale bar, 100 μm.
(C) IHC shows that majority of the blast cells (arrow, inset) in Pdgf-b−/− placentas do not express β-H1 globin (primitive RBCs, arrowhead), but express c-Kit (HSPCs). Dlk marks placental vasculature. IF shows coexpression of c-Kit and Ter119 (erythroid cells) in the blasts (arrow). Scale bars, 50 μm.
(D) IHC for Ki-67 (proliferation) and cytokeratin implies that the blast cells (arrow, inset) in Pdgf-b−/− placentas are actively cycling. Scale bar, 50 μm.
(E) Bright-field image of CFU-Es. Increase in CFU-Es is observed in Pdgf-b−/− placentas compared to controls. Error bars represent SEM (n = 6 for each genotype; **p < 0.005).
(F) IF for F4/80 identifies macrophages within the blast cell clusters (DAPI) in Pdgf-b−/− placental vasculature (CD31) (arrow). Scale bar, 50 μm.
See also Figure S1.
Epo Expression Is Increased in Placentas Deficient for PDGF-B Signaling by Hypoxia-Independent Mechanism

Because the ectopic erythropoiesis in Pdgf-b−/− and Pdgfrβ−/− placentas was neither explained by lack of signaling in hematopoietic cells nor anemia, we hypothesized that this phenotype may be due to the absence of PDGF-B signaling in the cells of the microenvironment, many of which express the receptor (Figure 4B) (Andrae et al., 2008; Holmgren et al., 1991, 1992). Because previous reports had documented increased Epo levels in Pdgf-b−/− embryos (Kaminski et al., 2001), we asked whether Epo was upregulated in Pdgf-b−/− placentas. Interestingly, a profound increase in Epo expression was noted specifically in placental trophoblasts in both Pdgf-b−/− (Figure 4A) (8.22 ± 6.64-fold increase at E15.5) and Pdgfrβ−/− embryos (3.32 ± 1.19-fold increase).

Because Epo expression can be upregulated in the placenta during hypoxia (Trollmann et al., 2008), we asked if the structural defects in Pdgf-b−/− placentas could lead to insufficient fetal-maternal exchange and increased hypoxia. However, no significant increase in pimonidazole incorporation (Figure S4D) or mRNA transcripts for known hypoxia indicators (Glut1, Ldh-A, and Pgkl) (Figure S4E) was observed, suggesting that lack of PDGF-B signaling does not lead to severe hypoxia in the placenta.

PDGF-B Signaling Is Required in Trophoblasts to Suppress Epo Secretion

To investigate if Epo upregulation in the placenta could be caused by a direct requirement of PDGF-B signaling in trophoblasts, we localized the expression of PDGFRβ and Epo. Both
PDGFRβ and Epo were expressed in cytokeratin+ sinusoidal trophoblast giant cells (S-TGCs), which can be distinguished by their location and large nucleus (Figures 4A and 4B) (Simmons et al., 2007). In comparison, the main source of the ligand, PDGF-B, in the placenta during mid-late gestation is the endothelium (Figure 4B). This suggested that PDGF-B signaling between endothelium and trophoblasts directly regulates Epo levels in the placenta.
Figure 4. Loss of PDGF-B Signaling in Trophoblasts Induces Epo Overexpression, which Promotes Ectopic Erythropoiesis

(A) IF for Epo, cytokeratin (trophoblasts), and DAPI (nuclei) (insets) documents upregulation of Epo in S-TGCs (arrows) in Pdgf-b⁻/⁻ placenta. Scale bar, 50 μm.
(B) IF for PDGFRβ, PDGF-B, cytokeratin, and CD34 (endothelium), and DAPI (insets) in E15.5 wild-type (WT) placentas reveals PDGFRβ expression in S-TGCs (arrows), whereas PDGF-B is expressed in the endothelium (asterisks). Scale bar, 50 μm.
(C) RT-PCR verifies knockdown of PDGFRβ in BeWo trophoblast cells. Error bars represent SEM. Representative example from three experiments is shown (n = 9 for each vector; **p < 0.005).
(D) ELISA for hEPO documents significant increase in hEPO secretion per cell in shPDGFRβ BeWo cells after 48 hr in culture. Error bars represent SEM. Representative example from two experiments is shown (n = 6 for each vector; **p < 0.005).
(E) H&E for placenta overexpressing hEPO in the trophoblasts (EPO OE) shows clusters of blast cells (green arrows, insets). IHC shows the absence of β-H1 globin (primitive RBCs, arrowheads), but presence of Ter119 (yellow arrows, insets) expression in the blast cells (arrows, insets). IHC for Ki67 indicates proliferation of the blasts (red arrows, insets) in placental vasculature (Dlk). F4/80 identifies macrophages (asterisks, insets) in the blast cell clusters. Scale bar, 100 μm.
To determine if PDGF-B signaling through PDGFRβ in trophoblasts regulates Epo expression, lentiviral shRNA was used to knock down Pdgfrβ in human BeWo trophoblast cells (Figure 4C). Transduced cells were cultured with PDGF-B for 48 hr, after which secreted human EPO (hEPO) levels were measured by ELISA. Strikingly, BeWo cells transduced with shPDGFRβ showed a significant increase in hEPO secretion (Figure 4D) as compared to empty vector, implying a direct function of PDGF-B signaling in regulating EPO secretion in trophoblasts.

**Overexpression of EPO in Placental Trophoblasts Is Sufficient to Induce Ectopic Erythropoiesis in Labyrinth Vasculature**

To investigate whether upregulation of Epo in placental trophoblasts was sufficient to induce erythropoiesis in the placenta, we utilized a lentiviral gene transfer strategy that targets placental trophoblasts (Okada et al., 2007). Blastocysts were injected with a lentiviral vector overexpressing hEPO; injection under zona space results in integration of the vector into trophoblasts (Okada et al., 2007). Blastocysts were sufficient to induce erythropoiesis in the placenta, whereas the extraembryonic mesoderm and the embryo remain unaffected (Figures S4F and S4G). Similar to Pdgf-b−/− and Pdgfrβ−/− placenta, placenta overexpressing hEPO displayed clusters of Ki-67+ definitive erythroblasts and macrophages in placental vasculature (Figure 4E). FACS analysis showed a significant increase in hEPO secretion (Figure 4D) as compared to empty vector, implying a direct function of PDGF-B signaling in regulating EPO secretion in trophoblasts.

**DISCUSSION**

Understanding the mechanisms by which the different fetal hematopoietic niches support the development of undifferentiated HSPCs versus promote lineage differentiation is a major goal for the field. We document that loss of PDGF-B or its receptor, PDGFRβ, induces premature differentiation of hematopoietic progenitors into definitive erythroid cells in the placenta. Ectopic erythropoiesis was caused by upregulation of Epo in placental trophoblasts due to a direct requirement of PDGF-B signaling in regulating Epo levels. These studies identify the trophoblasts and PDGF-B signaling as a key mechanism for maintaining the unique placental hematopoietic microenvironment by regulating Epo levels.

(F) Model for function of PDGF-B signaling in regulating Epo secretion from placental trophoblasts. PDGF-B from the endothelium (bright pink) activates PDGFRβ on trophoblasts (brown) and maintains Epo at basal levels. In the absence of PDGF-B signaling, placental trophoblasts upregulate Epo, which alters the placental hematopoietic niche and induces ectopic definitive erythropoiesis (dark purple/light pink) in association with macrophages (light blue). X indicates absence of PDGF-B signaling.

See also Figure S4.

Developmental Cell

PDGF-B Regulates the Placental Hematopoietic Niche
The effect of hEPO overexpression in placental trophoblasts was assessed using trophoblast-specific lentiviral gene manipulation. Lentiviral shRNA knockdown of PDGFRα, β, or Pdgfrb in BeWo trophoblast cell line. Secreted EPO was measured 48 hr after PDGF-B (10 ng/ml) stimulation at 8% oxygen using hEpo ELISA Quantikine (R&D Systems). 

Verification of Direct Regulation of Epo Expression in Trophoblasts by PDGF-B Signaling

IF was used to localize Epo, PDGFRα, and PDGFB in the placenta. PDGFB signaling-mediated regulation of EPO in trophoblasts was assessed by lentiviral shRNA knockdown of PDGFRα (Open Biosystems; clone B11) in human BeWo trophoblast cell line. Secreted EPO was measured 48 hr after PDGF-B (10 ng/ml) (PeproTech) stimulation at 8% oxygen using hEpo ELISA Quantikine Kit (R&D Systems). 

Graphical and Statistical Analysis

Mathematical analysis and statistics were performed using GraphPad Prism Software. Unpaired Student’s t tests were used to calculate p values, and data are reported as mean ± SEM.
Sokolowski, M., and Placental Lactogen-related genes is not associated with their position in the locus. BMC Genomics 9, 352.