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Trophoblasts regulate the placental hematopoietic niche through PDGF-B signaling

Akanksha Chhabra[1,2], **Andrew J. Lechner**[2], **Masaya Ueno**[2], **Asha Acharya**[6], **Ben Van Handel**[2], **Yanling Wang**[2], **M. Luisa Iruela-Arispe**[1,2,5], **Michelle D. Tallquist**[6], and **Hanna K.A. Mikkola**[1,2,3,4,5,*]

[1]Molecular Biology Institute, University of California, Los Angeles, Los Angeles, CA 90095, USA

[2]Department of Molecular Cell and Developmental Biology, University of California, Los Angeles, Los Angeles, CA 90095, USA

[3]Department of Microbiology, Immunology and Molecular Genetics, University of California, Los Angeles, Los Angeles, CA 90095, USA

[4]Eli and Edythe Broad Center for Regenerative Medicine and Stem Cell Research, University of California, Los Angeles, Los Angeles, CA 90095, USA

[5]Jonsson Comprehensive Cancer Center, University of California, Los Angeles, Los Angeles, CA 90095, USA

[6]Department of Molecular Biology, University of Texas Southwestern Medical Center at Dallas, Dallas, TX 75390, USA

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, PDGFRβ, 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 erythroid differentiation to specific anatomical niches during development.

*Correspondence: hmikkola@mcdb.ucla.edu.

Supplemental Information: Supplemental information includes four Figures, one Table, Supplemental Experimental Methods and References.

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 post-natal life. This is achieved by segregating fetal hematopoiesis into multiple waves and different microenvironmental niches that protect undifferentiated HSPCs (hematopoietic stem/progenitor cells) 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 chorioallantoic mesenchyme, while 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 (Barcena 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 PDGFRβ and PDGFRα, influencing cell differentiation, proliferation, migration and survival in various organs (Tallquist and Kazlauskas, 2004). *Pdgf-b*$^{-/-}$ and *Pdgfrβ*$^{-/-}$ embryos die perinatally with strikingly similar phenotypes (Leveen et al., 1994; Soriano, 1994). In the placenta, PDGF-B is expressed in the endothelium, some hematopoietic cells and trophoblasts, whereas PDGFRβ 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 midgestation (Ohlsson et al., 1999). Later in gestation, the embryos also develop anemia, thrombocytopenia, and hypocellular fetal liver, as well as kidney and heart defects (Leveen 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 staining 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 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 (Figure S1A), but not GP1b-β which implied 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 FcRγ) (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 pro-erythroblasts 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 pro-erythroblasts and basophilic erythroblasts (Figure 2A), while 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.

As the increase in erythroblasts was also noted in circulating blood, a 1h 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 or spleen, in *Pdgf-b*$^{-/-}$ embryos showed an increase in BrdU^{+}CD71^{+} erythroblasts (Figure 2C). 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 Pdgfrβ*$^{fl/fl}$ embryos to delete PDGFRβ in hematopoietic cells. FACS analysis at E14.5 revealed a complete loss of PDGFRβ protein on c-Kit+ HSPCs, as in *Pdgfrβ*$^{-/-}$ 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 (Figure 3A, B). Ectopic definitive erythropoiesis in *Pdgfrβ*$^{-/-}$ placentas was further confirmed by FACS analysis (accumulation of CD71^{+}Ter119^{+} erythroblasts, Figure S3D) and CFU-E assay (Figure 3C), while *Tie2Cre Pdgfrβ*$^{fl/fl}$ placentas showed no signs of erythropoiesis. Similar data were obtained when both PDGF receptors, *Pdgfrβ* and *Pdgfra,* were deleted in hematopoietic cells (data not shown). These data imply that the ectopic definitive erythropoiesis in *Pdgf-b*$^{-/-}$ and *Pdgfrβ*$^{-/-}$ 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

As *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); these abnormalities were not observed at E13.5-15.5 when ectopic erythropoiesis was already evident (Figure S4A) (Leveen et al., 1994). By E16.5 and E18.5 both *Pdgf-b*$^{-/-}$ and *Pdgfrβ*$^{-/-}$ embryos showed anemia (Table S1); however, the first sign of reduced blood cell counts were observed only after E13.5, when the erythroblast clusters were already evident in the placenta (Figure S4B and Figure 1B). Furthermore, reduction in fetal liver size in *Pdgf-b*$^{-/-}$ embryos was observed only in late gestation (Figure 1B) (Figure S4C). These data indicate that the ectopic definitive erythropoiesis in *Pdgf-b*$^{-/-}$ placentas precedes other macroscopic defects and anemia.

Epo expression is increased in placentas deficient for PDGF-B signaling by hypoxia independent mechanism

Since 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., 1992; Holmgren et al., 1991). As previous reports had documented increased Epo (Erythropoietin) 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).

Since 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 *Pgk)* (Figure S4E) were 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, which can be distinguished by their location and large nucleus (Figure 4 A, B) (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.

To determine if PDGF-B signaling through PDGFRβ in trophoblasts regulates Epo expression, lentiviral shRNA was used to knockdown *Pdgfrβ* in human BeWo trophoblast cells. (Figure 4C). Transduced cells were cultured with PDGF-B for 48 hours, after which secreted 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 human *EPO* (hEPO); injection under zona space results in integration of the vector into trophoblasts, whereas the extraembryonic mesoderm and the embryo remain unaffected (Figure S4F and G). Similar to *Pdgf-b*$^{-/-}$ and *Pdgfrβ*$^{-/-}$ placentas, placentas overexpressing hEPO displayed clusters of Ki-67^{+} definitive erythroblasts and macrophages in placental vasculature (Figure 4E). FACS analysis also confirmed accumulation of CD71^{+}Ter119^{+} erythroblasts in placentas overexpressing hEPO and the blood of these embryos (Figure S4H). These results suggest that increased Epo expression in the trophoblasts is responsible for induction of ectopic definitive erythropoiesis in placental vasculature. These data highlight the requirement for tight control of Epo levels in placental trophoblasts (Figure 4F), and nominate PDGF-B signaling as a key mechanism for maintaining the unique placental hematopoietic microenvironment by regulating Epo levels.

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 key components of the unique placental hematopoietic niche.

Trophoblasts are tropho-ectoderm derived epithelial-like cells that facilitate implantation and maternal blood flow, and secrete growth factors and hormones (Simmons and Cross, 2005). We show that loss of PDGF-B signaling provokes upregulation of Epo in sinusoidal trophoblast giant cells (S-TGCs), which secrete factors in fetal circulation (Simmons et al., 2007). We discovered that S-TGCs express PDGFRβ (receptor), but not PDGF-B (ligand), which is mainly expressed in the endothelium during mid/late gestation, suggesting paracrine signaling between endothelium and trophoblasts. Moreover, mouse embryos that lack PDGF-B in the endothelium evidence similar placental structural defects as in *Pdgf-b*$^{-/-}$ and *Pdgfrβ*$^{-/-}$ embryos, including reduction in trophoblasts, further indicating that the endothelium is a critical source of PDGF-B in the placenta (Bjarnegard et al., 2004). Trophoblasts also express other growth factors and hormones that support hematopoiesis, such as VEGF, PLP-E and PLP-F (Breier et al., 1995; Lin et al., 1997; Simmons et al., 2008). However, unlike Epo, these factors are not expressed in labyrinth S-TGCs (Lin et al., 1997; Simmons et al., 2008 and data not shown), implying a different cellular origin and regulation. Future studies will be needed to elucidate how the different trophoblast subtypes contribute to the placental hematopoietic microenvironment.

Our study revealed that the ectopic erythropoiesis in placentas lacking PDGF-B signaling was induced by upregulation of Epo in trophoblasts. Epo promotes erythropoiesis by regulating proliferation and differentiation of erythroid progenitors (Wu et al., 1995). Althobe drastically upregulated during anemia and hypoxia (Ebert and Bunn, 1999), we excluded these cugh Epo levels can onditions as a major cause of Epo upregulation in PDGF-B deficient embryos. Furthermore, although co-existence of placental defects, hypocellular liver and anemia has been described also in other knockout mouse models such as p38α MAPK, SOCS3, or c-Met (Adams et al., 2000; Marine et al., 1999; Roberts et al., 2001; Sasaki et al., 2000; Tamura et al., 2000), Epo upregulation and ectopic definitive erythropoiesis in the placenta has been described so far only in embryos deficient for PDGF-B signaling.

The role of Epo in normal placental biology is largely unknown. Epo signaling acts also in endothelial cells, supporting their survival, proliferation and migration (Anagnostou et al., 1990; Carlini et al., 1995), and it has been suggested that Epo signaling can regulate PDGF-B expression in the endothelium (Janmaat et al., 2010). EpoR, the receptor for Epo, is expressed both in placental vasculature and trophoblasts (Fairchild Benyo and Conrad, 1999; Sawyer et al., 1989), suggesting that Epo may also have an autocrine function in trophoblasts. Although the physiological role of Epo signaling in the different placental cell types is still largely undefined, the embryos lacking Epo signaling possess smaller placentas (personal communication - Hong Wu, UCLA). Our data also indicates that the placental Epo levels have to be tightly regulated, as the overexpression of Epo in the trophoblasts is sufficient to induce ectopic definitive erythropoiesis in the placenta.

There are major differences in the regulation of Epo in the fetus and adult. While Epo production in the adult is restricted to the kidney, during development, the sites of Epo production change temporally. Epo is first expressed in the yolk sac and the placenta (Conrad et al., 1996; Ebert and Bunn, 1999; Yasuda et al., 2002), after which its expression transitions to the fetal liver (Lee et al., 2001) and at around birth to the kidney (Dame et al., 1998; Koury et al., 1988). Systemic Epo in the adult does not pose a risk for ectopic erythropoiesis as HSPCs reside in their niche in the bone marrow, whereas during fetal life, HSPCs traffic between the different hematopoietic niches, exposing HSPCs to signals they encounter during migration. Furthermore, it has been suggested that fetal progenitors are more sensitive to Epo than their adult counterparts (Linch et al., 1982). Hence, the niche restricted Epo production may be essential to facilitate HSPC migration between the fetal hematopoietic organs without exposing them to premature differentiation.

The discovery of PDGF-B signaling in the trophoblasts as an important regulator of local Epo levels in the placenta reveals a developmental stage and niche specific mechanism for regulating Epo expression, which is critical for governing the fate of HSPCs during their developmental journey. This work gives new insights into the goal of recreating the different types of hematopoietic niches *in vitro,* as well as furthers our understanding of the etiology of developmental defects originating from the placenta.

Experimental Procedures

Documentation of ectopic erythropoiesis in the placenta

Placenta and other hematopoietic tissues from *Pdgf-b*$^{-/-}$, *Pdgfrβ*$^{-/-}$, *Tie2Cre Pdgfrβ*$^{fl/fl}$ and control embryos were analyzed for evidence of proliferation and differentiation of erythroid cells. See Supplementary Methods for details for IHC, IF, FACS. Erythroid progenitors in placentas were quantified by CFU-E assay (Methocult M3334, Stem Cell Technologies) 3-4 days after plating. Colonies were imaged using a Canon Powershot G6 camera connected to a Zeiss Axiovert 40 CFL microscope via a 52 mm Hama adapter. Complete blood count (CBC) analysis was performed using Hemavet (Drew Scientific). Manual blood counts were conducted with a hemacytometer (Fisher Scientific) using Trypan Blue staining (Sigma-Aldrich). Cytospins from blood, placenta and fetal liver were generated using a Shandon Cytospin 4 (Thermo Electron Corporation). Air-dryed slides were stained with MGG stain (Sigma-Aldrich). Images were taken using an Olympus BX51 microscope with a DP72 camera. Pregnant mice were injected intraperitoneally with BrdU 1h before harvesting embryos (FITC BrdU Flow Kit, BD Pharmingen). Tissues were processed for FACS or fixed frozen in O.C.T. for immunostaining. See Supplemental Experimental Methods for details.

Verification of direct regulation of EPO expression in trophoblasts by PDGF-B signaling

IF was used to localize Epo, PDGFRβ and PDGF-B in the placenta. PDGF-B signaling mediated regulation of EPO in trophoblasts was assessed by lentiviral shRNA knockdown of PDGFRβ (Open Biosystems, clone B11) in human BeWo trophoblast cell line. Secreted EPO was measured 48 hours after PDGF-B (10ng/ml) (Peprotech) stimulation at 8% oxygen using human Epo ELISA Quantikine Kit (R&D Systems). See Supplemental Experimental Methods for details.

Documentation of ectopic erythropoiesis in the placenta upon trophoblast specific EPO overexpression

The effect of hEPO overexpression in placental trophoblasts *in vivo* was assessed using trophoblast specific lentiviral gene manipulation. Lentiviral vector particles overexpressing human EPO from the Ubiquitin promoter were injected under the zona pellucida of blastocysts, which were transferred to pseudo-pregnant females. Ectopic erythropoiesis in transduced placentas (EPO OE) was assessed using IF, IHC and FACS. See Supplemental Experimental Methods for details.

Graphical and Statistical Analysis

Mathematical analysis and statistics were performed using Graphpad Prism Software. Unpaired student t tests were used to calculate p-values, and data is reported as mean±SEM.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Trophoblasts are major signaling centers in the placental hematopoietic niche
- PDGF-B signaling in placental trophoblasts regulates Epo levels
- Tight regulation of placental Epo is essential to prevent ectopic erythropoiesis

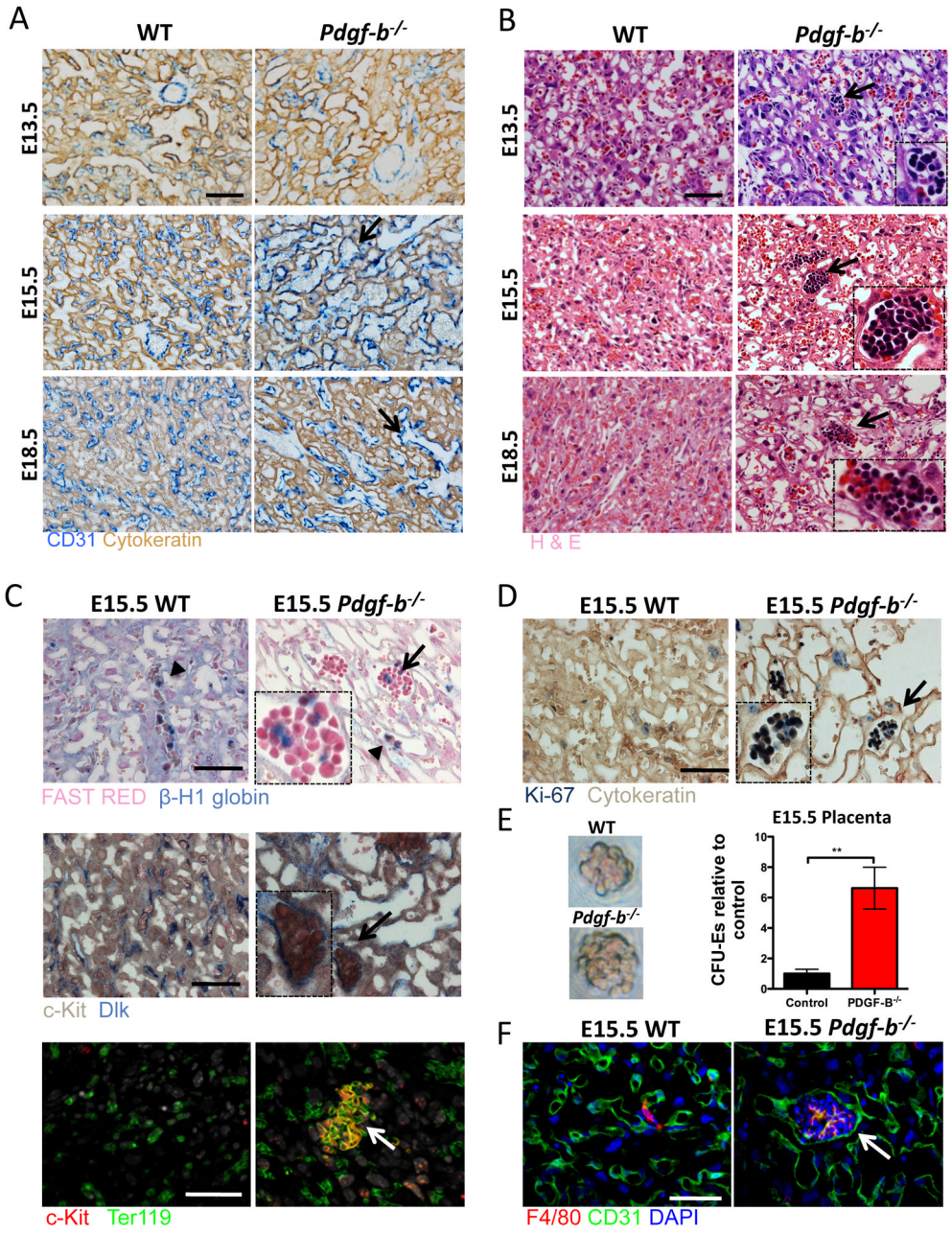

Figure 1. *Pdgf-b*$^{-/-}$ embryos support ectopic erythropoiesis in placental labyrinth vasculature during midgestation

(A) IHC for CD31 (endothelium) and cytokeratin (trophoblasts) documents progressive dilation of the labyrinth vasculature (arrow, inset) in *Pdgf-b*$^{-/-}$ placentas at E13.5-E18.5. Scale bar = 100μm.

(B) H&E staining reveals clusters of blast-like cells in *Pdgf-b*$^{-/-}$ placental vasculature at E13.5-E18.5 (arrow, inset). Scale bar = 100μm.

(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 co-expression of c-Kit and Ter119 (erythroid cells) in the blasts (arrow) Scale bar = 50μm.

(D) IHC for Ki-67 (proliferation) and cytokeratin imply that the blasts cells (arrow, inset) in *Pdgf-b*$^{-/-}$ placentas are actively cycling. Scale bar = 50μm.
(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-value < 0.05).
(F) IF for F4/80 identifies macrophages within the blast cell clusters (DAPI) in *Pdgf-b*$^{-/-}$ placental vasculature (CD31) (arrow). Scale bar = 50μm.
(See also Supplementary Figure 1)

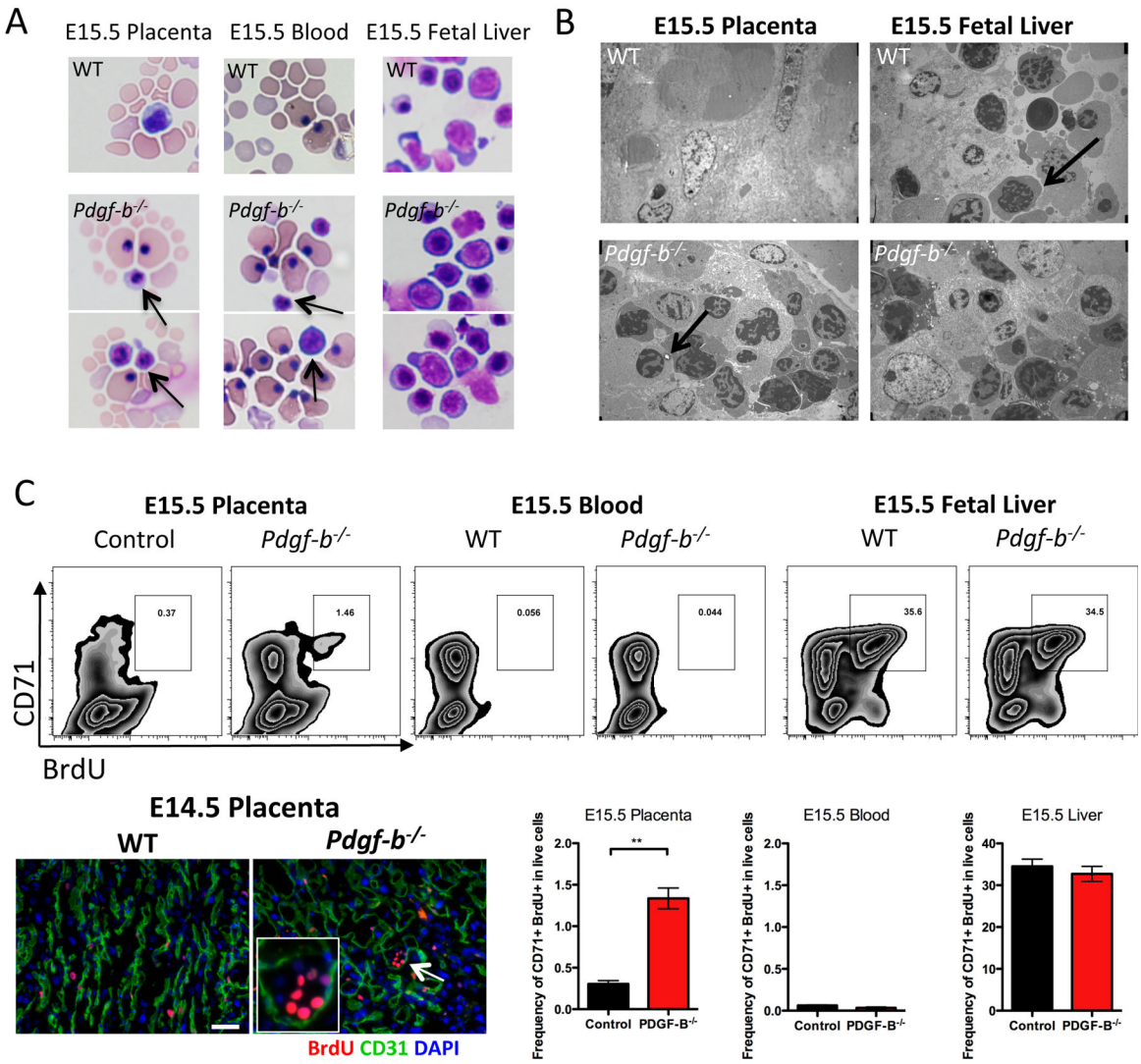

Figure 2. Loss of PDGF-B promotes proliferation of erythroid precursors specifically in the placenta

(A) MGG staining shows proerythroblasts and basophilic erythroblasts (arrows) in *Pdgf-b*$^{-/-}$ placenta and blood.

(B) Electron microscopy (mag 2900X) documents similar morphology of the erythroblasts (arrow) in *Pdgf-b*$^{-/-}$ placentas as in normal fetal liver (arrows).

(C) FACS after 1h BrdU incorporation shows increase in BrdU^{+}CD71^{+} erythroblasts only in placentas of E14.5 *Pdgfb*$^{-/-}$ embryos. Representative examples from three experiments are shown, n=6 for each genotype. IF for BrdU, CD31 (endothelium) and DAPI (nuclei) verify proliferation of the blast cells in placental vasculature (arrow, inset). Scale bar = 50μm.

(See also Supplementary Figure 2)

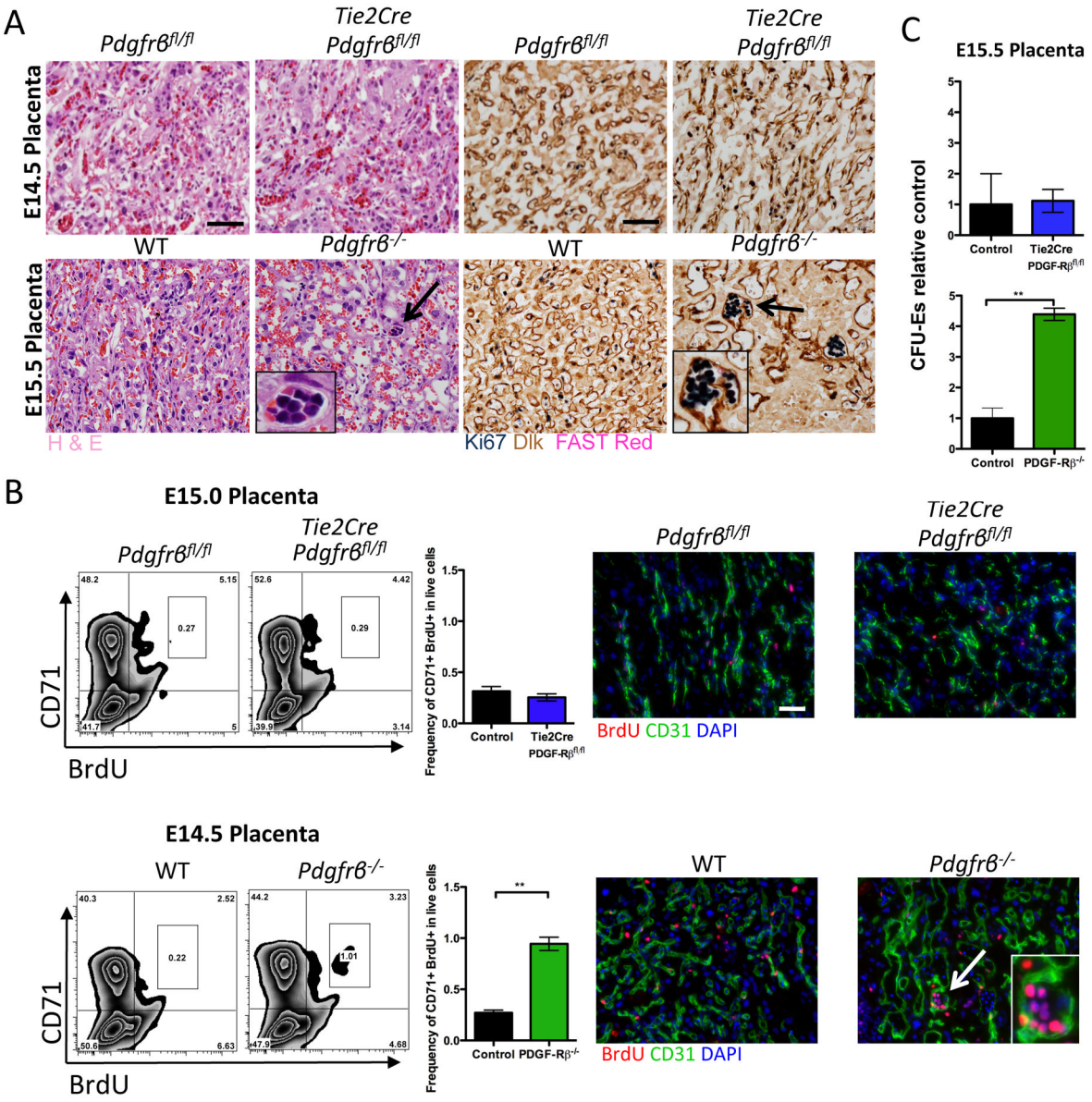

Figure 3. Erythropoiesis in *Pdgf-b*$^{-/-}$ placentas is not due to intrinsic requirement for PDGF-B signaling in hematopoietic cells

(A) H&E staining shows the presence of clusters of blast cells (arrow, inset) in E14.5 *Pdgfrβ*$^{-/-}$ placentas, but not in *Tie2Cre Pdgfrβ*$^{fl/fl}$ placentas. IHC for Ki-67 indicates active proliferation of blast cells (arrow, inset) in *Pdgfrβ*$^{-/-}$ placental vasculature (Dlk). Scale bar = 100μm.

(B) FACS analysis shows an increase in BrdU^{+}CD71^{+} erythroblasts in *Pdgfrβ*$^{-/-}$ placentas, but not in *Tie2Cre Pdgfrβ*$^{fl/fl}$ placentas. Representative examples from three experiments are shown (n=5 for each genotype) (** P-value < 0.005). IF for BrdU, DAPI (nuclei) and CD31 (endothelium) verifies the presence of clusters of proliferative blast cells in placental vasculature in *Pdgfrβ*$^{-/-}$ (arrow, inset) but not *Tie2Cre Pdgfrβ*$^{fl/fl}$ embryos. Scale bar = 50μm.

(C) Significant increase in CFU-Es is seen in *Pdgfrβ*$^{-/-}$ but not *Tie2Cre Pdgfrβ*$^{fl/fl}$ placentas (n=3 for each genotype). Error bars represent SEM (** P-value < 0.005).

(See also Supplementary Figure 3)

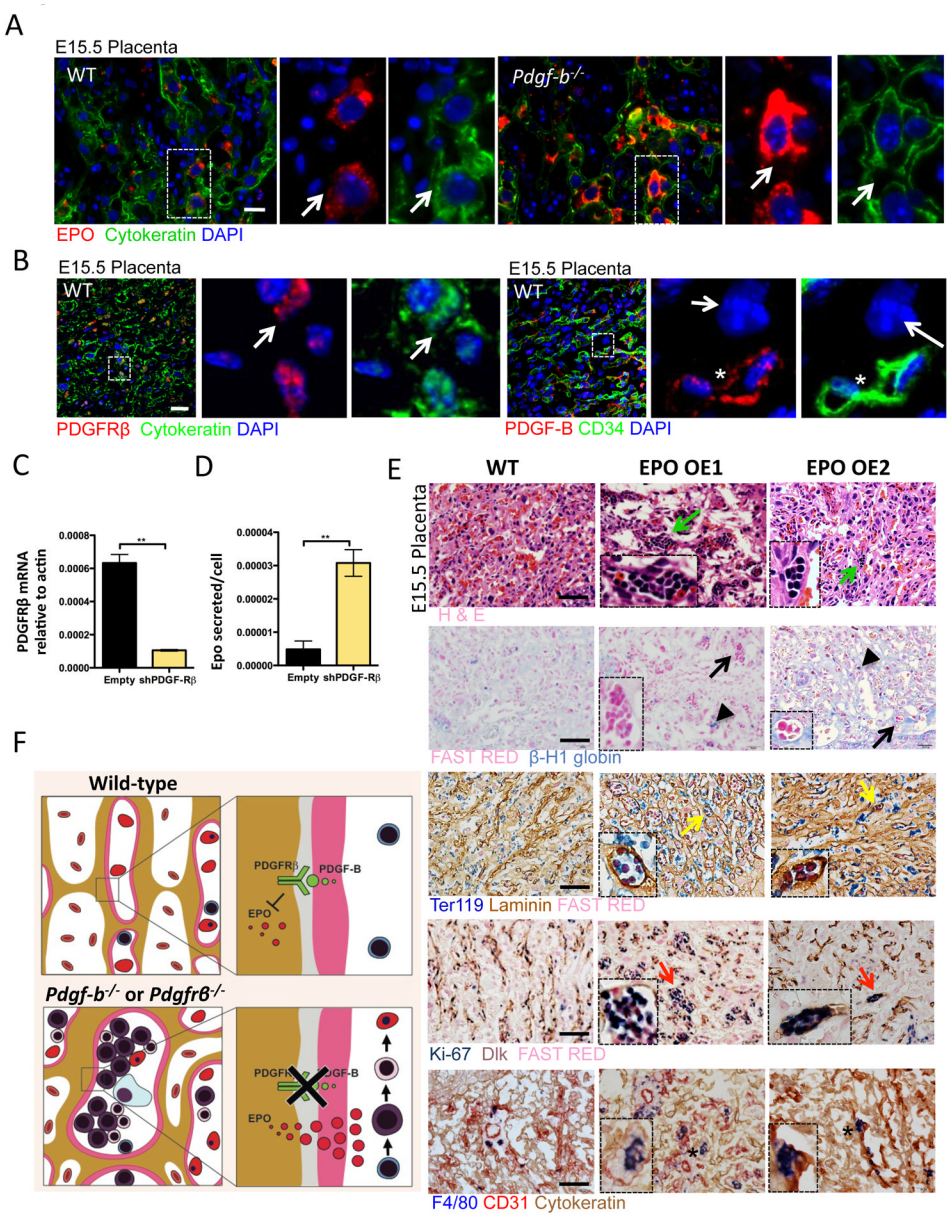

Figure 4. Loss of PDGF-B signaling in trophoblasts induces Epo overexpression, which promotes ectopic erythropoiesis

(A) IF for Epo, cytokeratin (trophoblasts), and DAPI (nuclei) (insets) documents upregulation of Epo in sinusoidal trophoblast giant cells (arrows) in *Pdgf-b*$^{-/-}$ placentas. Scale bar= 50μm.

(B) IF for PDGFRβ, PDGF-B, cytokeratin, CD34 (endothelium) and DAPI (insets) in E15.5 WT placentas reveals PDGFRβ expression in S-TGCs (arrows) while PDGF-B is expressed in the endothelium (asterisks). Scale bar= 50μm.

(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-value < 0.005).

(D) ELISA for hEPO documents significant increase in hEPO secretion per cell in shPDGFRβ BeWo cells after 48 hours in culture. Error bars represent SEM. Representative example from two experiments is shown (n=6 for each vector) (** P-value < 0.005).

(D) H&E staining for placentas over-expressing hEPO in the trophoblasts (EPO OE) show 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 indicate proliferation of the blasts (red arrows, insets) in placental vasculature (Dlk). F4/80 identifies macrophages (asterisks, insets) in the blast cell clusters. Scale bar = 100μm.

(E) 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).

(See also Supplementary Figure 4)