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Research Article

Reproductive biology

Preeclampsia remains a clinical challenge due to its poorly understood pathogenesis. A prevailing notion is that increased placental production of soluble fms-like tyrosine kinase-1 (sFlt-1) causes the maternal syndrome by inhibiting proangiogenic placental growth factor (PlGF) and VEGF. However, the significance of PlGF suppression in preeclampsia is uncertain. To test whether preeclampsia results from the imbalance of angiogenic factors reflected by an abnormal sFlt-1/PlGF ratio, we studied PlGF KO (*Pgf*^{-/-}) mice and noted that the mice did not develop signs or sequelae of preeclampsia despite a marked elevation in circulating sFlt-1. Notably, PlGF KO mice had morphologically distinct placentas, showing an accumulation of junctional zone glycogen. We next considered the role of placental PlGF in an established model of preeclampsia (pregnant catechol-O-methyltransferase-deficient [COMT-deficient] mice) by generating mice with deletions in both the *Pgf* and *Comt* genes. Deletion of placental PlGF in the context of COMT loss resulted in a reduction in maternal blood pressure and increased placental glycogen, indicating that loss of PlGF might be protective against the development of preeclampsia. These results identify a role for PlGF in placental development and support a complex model for the pathogenesis of preeclampsia beyond an angiogenic factor imbalance.

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Loss of placental growth factor ameliorates maternal hypertension and preeclampsia in mice

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Preeclampsia remains a clinical challenge due to its poorly understood pathogenesis. A prevailing notion is that increased placental production of soluble fms-like tyrosine kinase-1 (sFlt-1) causes the maternal syndrome by inhibiting proangiogenic placental growth factor (PlGF) and VEGF. However, the significance of PlGF suppression in preeclampsia is uncertain. To test whether preeclampsia results from the imbalance of angiogenic factors reflected by an abnormal sFlt-1/PlGF ratio, we studied PlGF KO (*Pgf*^{-/-}) mice and noted that the mice did not develop signs or sequelae of preeclampsia despite a marked elevation in circulating sFLT-1. Notably, PlGF KO mice had morphologically distinct placentas, showing an accumulation of junctional zone glycogen. We next considered the role of placental PlGF in an established model of preeclampsia (pregnant catechol-O-methyltransferase-deficient [COMT-deficient] mice) by generating mice with deletions in both the *Pgf* and *Comt* genes. Deletion of placental PlGF in the context of COMT loss resulted in a reduction in maternal blood pressure and increased placental glycogen, indicating that loss of PlGF might be protective against the development of preeclampsia. These results identify a role for PlGF in placental development and support a complex model for the pathogenesis of preeclampsia beyond an angiogenic factor imbalance.

Introduction

Preeclampsia is a common and serious hypertensive disorder affecting approximately 5% of pregnancies. The underlying biology of the disease is complex and not fully understood (1). Poor invasion of the placenta, characterized by incomplete remodeling of uterine spiral arteries early in pregnancy, is thought to promote the altered production of placenta-derived factors in the latter half of pregnancy in response to hypoxia that results from the vascular remodeling defect (2–4). These factors induce maternal endothelial activation and inflammation, which manifest clinically as hypertension and varying degrees of end-organ injury. Significant attention has been given to the role of the circulating angiogenic factors soluble fms-like tyrosine kinase-1 (sFlt-1, also known as soluble VEGFR-1) and placental growth factor (PlGF) in this process as well as their potential utility as early biomarkers of the disease (5–8). In pre-

eclampsia, elevated sFlt-1 — a truncated, soluble form of Flt-1 lacking the tyrosine kinase domain — acts as a decoy receptor, functioning as a molecular sponge to limit signaling induced by elevated levels of circulating proangiogenic ligands and preventing their action on target cells (9–11). In rodents, adenovirus-mediated overexpression of sFlt-1 or treatment with anti-VEGF antibodies or recombinant sFlt-1 induces hypertension, proteinuria, and kidney glomerular injury in pregnancy (11–13). These observations provided the foundation for a widely accepted model for preeclampsia in which an angiogenic factor imbalance, characterized by an abnormally high ratio of sFlt-1 to PlGF and VEGF, is thought to be an essential component of disease pathogenesis (reviewed in refs. 1, 2, 14).

PlGF, a member of the VEGF family, was originally discovered in the placenta, where it was proposed to regulate vascular development and trophoblast growth and differentiation (15). Unlike VEGF, which is required for angiogenesis and endothelial cell maintenance and binds both Flt-1/sFlt-1 and Flk-1, PlGF is redundant for vascular development and selectively binds Flt-1/sFlt-1 (16–18). PlGF stimulates endothelial cell growth, migration, and survival and plays a central role in pathologic angiogenesis, including in cancer and tissue ischemia (19, 20). In addition to signaling through Flt-1, PlGF promotes angiogenesis by competing

Authorship note: VHG is deceased.

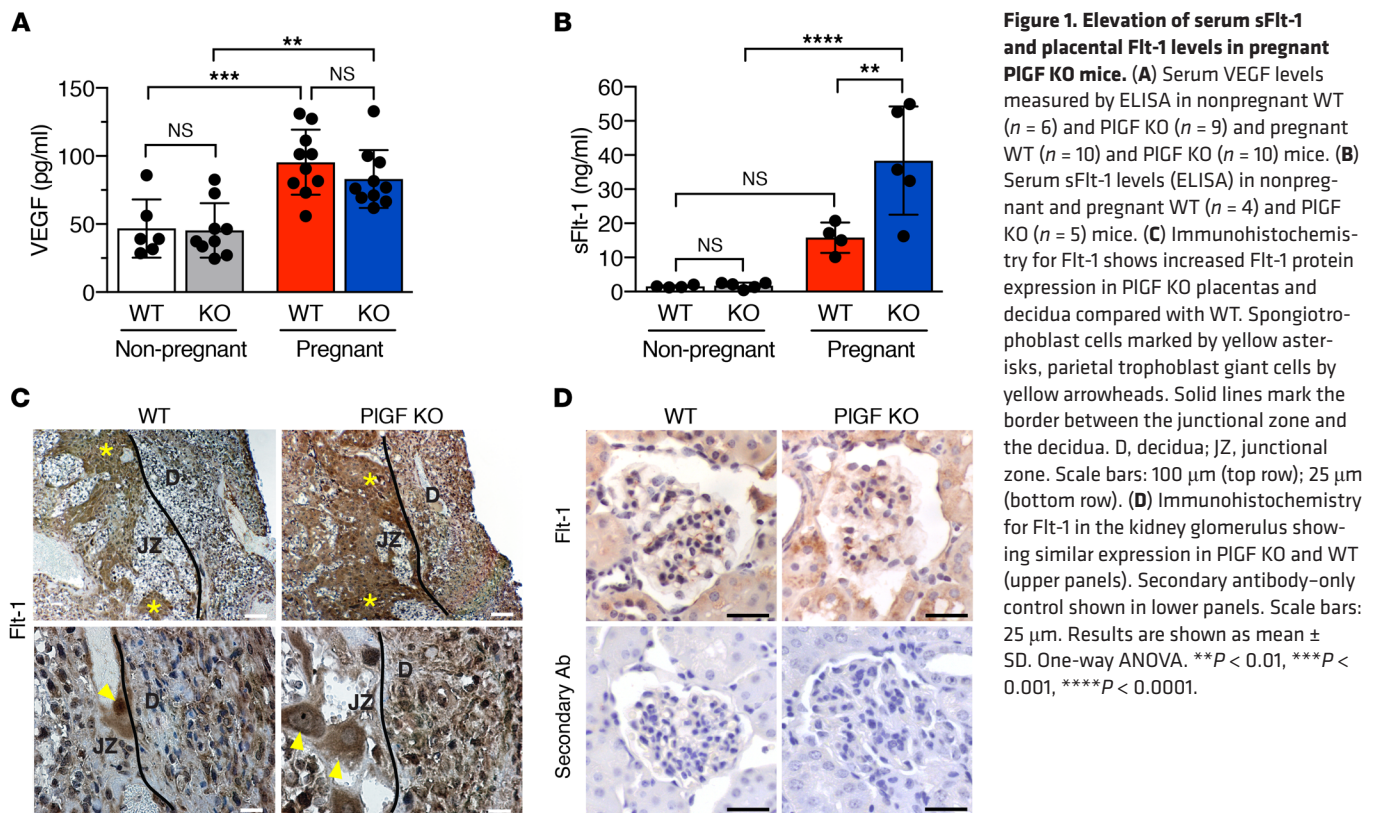
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with VEGFA for Flt-1, freeing VEGFA to signal through Flk-1 (17). PlGF is upregulated in pregnancy, with levels peaking in the serum of pregnant women in the early third trimester (10, 21, 22). In pregnant mice, PlGF has been shown to influence maternal cardiovascular physiology (23). In the placenta, it is expressed in trophoblast giant cells and decidual natural killer cells (21, 24). Although it is dispensable for embryogenesis (16), recent data support its function in fetal cerebrovascular development (25). Despite interest in the utility of PlGF for clinical diagnostics, the role of PlGF in preeclampsia and in placental development is not well understood.

To delineate the pathogenic role of sFlt-1 and PlGF in preeclampsia, we used PlGF KO mice to create an angiogenic factor imbalance in pregnancy. We also examined the role of PlGF in preeclampsia by generating mice with genetic deletion of both PlGF and the enzyme catechol-O-methyltransferase (COMT). Reduced COMT activity and low plasma levels of its metabolic product, 2-methoxyestradiol (2-ME), are observed in preeclampsia (26–32). Mechanistically, 2-ME has been shown to promote extravillous trophoblast invasion (33) and migration (29) and to dysregulate HIF-1 α function (34–36). Treatment of first trimester placental villous explants with 2-ME rescued altered levels of sFlt-1 and hypoxia-inducible factor 1- α (HIF-1 α) induced by hypoxia (36). We previously reported that pregnant COMT KO mice develop a preeclampsia-like phenotype characterized by hypertension, proteinuria, renal glomerular endotheliosis, increased placental hypoxia, an influx of decidual natural killer cells, and elevated circulating sFlt-1 (37). Treatment of pregnant COMT KO mice with 2-ME rescued the preeclampsia-like phenotype, while treatment of WT mice with an inhibitor of COMT (Ro41-0960) mimicked the COMT KO

phenotype (37). Additional findings consistent with preeclampsia have been reported in various studies of pregnant COMT-deficient mice, including fetal growth restriction (37, 38), abnormal uterine artery Doppler waveforms (38), an exaggerated response to angiotensin II (39), and an abnormal metabolome (40). We hypothesized that PlGF deficiency would induce a preeclampsia-like phenotype by promoting an antiangiogenic state defined by excess sFlt-1 and that loss of PlGF in the setting of COMT deficiency would exacerbate the phenotype. Our findings, however, suggest that PlGF is protective against the development of preeclampsia-like features in COMT KO mice and that placental PlGF plays a role in normal placental development and metabolism.

Results

Loss of PlGF in pregnancy results in elevated sFlt-1 levels. We used mice with genetic deletion of PlGF ($Pgfl^{-/-}$; PlGF KO) to evaluate the effect of PlGF deficiency on serum angiogenic factor levels in pregnancy. PlGF expression examined by immunofluorescence was confirmed in placentas of WT mice, but not PlGF KO mice, with expression primarily in parietal trophoblast giant cell and spongiotrophoblast populations, consistent with prior studies (data not shown; refs. 21, 24). Serum VEGF and sFlt-1 levels were measured in PlGF KO and WT mice by ELISA and were found to be elevated in pregnant compared with nonpregnant mice, as expected (Figure 1, A and B). Whereas serum VEGF levels in pregnant KO and WT mice were similar (Figure 1A), sFlt-1 levels were significantly elevated in pregnant PlGF KO mice compared with pregnant WT (Figure 1B). Immunostaining for Flt-1 revealed increased Flt-1 expression in the junctional zone spongiotrophoblasts, parietal trophoblast giant cells, and asso-

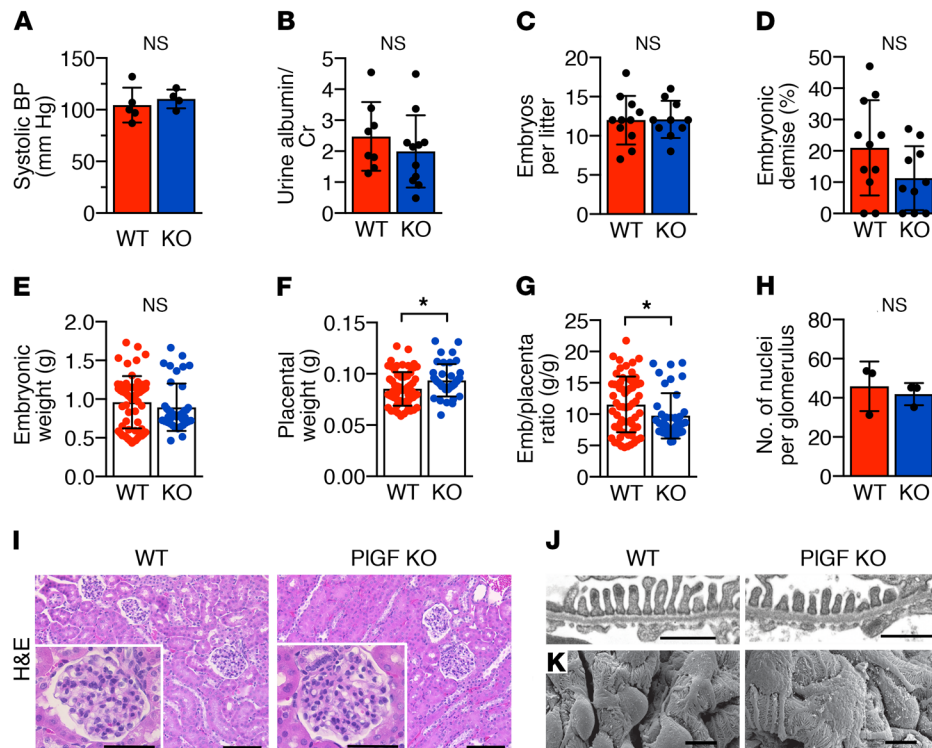


Figure 2. Pregnant PIGF KO mice do not develop signs or complications of preeclampsia. (A) Systolic blood pressure (BP) of pregnant WT ($n = 5$) and PIGF KO ($n = 4$) mice. (B) Proteinuria measured by spot urine albumin/creatinine (Cr) ratios for pregnant WT ($n = 8$) and KO mice ($n = 11$). Blood pressure and urine protein measured between E17 and E20. (C) Total number of embryos per litter, includes resorbing embryos ($n = 10$ litters). (D) Percentage of resorbed embryos per litter ($n = 10$ litters). (E–F) Weights of individual embryos (E) and placentas (F) from WT ($n = 63$ embryos/placentas from 7 litters) and KO ($n = 40$ embryos/placentas from 4 litters) mice collected between E17 and E20. (G) Embryo/placenta ratio decreased in PIGF KO ($n = 40$ embryos from 4 litters) compared with WT ($n = 62$ embryos from 7 litters). (H) Number of nuclei per glomerulus ($n = 3$ mice, 6–10 glomeruli per mouse). (I) Representative images of H&E-stained kidney sections from the indicated groups showing normal tubules and glomeruli. Scale bars: 100 μm ; 50 μm (inset). (J) Normal podocyte foot processes, glomerular basement membrane, and slit diaphragms by transmission electron microscopy and (K) normal podocyte foot processes by scanning electron microscopy for both groups. Scale bars: 1 μm (J); 5 μm (K). Results are shown as mean \pm SD. Two-tailed, unpaired t test. * $P < 0.05$.

ciated decidua of KO placentas (Figure 1C and Supplemental Figure 1, A and B; supplemental material available online with this article; <https://doi.org/10.1172/JCI99026DS1>), but not in the kidneys of PIGF KO mice (Figure 1D). These results suggest that the elevated circulating sFlt-1 levels in pregnant PIGF KO mice originated from increased placental Flt-1 expression.

Pregnant PIGF KO mice do not develop signs of preeclampsia. Hypertension and proteinuria, the classic features of preeclampsia, were evaluated in pregnant PIGF KO and WT mice. Tail-cuff blood pressures measured at E17–E20 were similar for PIGF KO and WT mice (Figure 2A), as were urine albumin/creatinine ratios (Figure 2B). Preeclampsia is also associated with increased rates of fetal growth restriction and fetal demise due to placental insufficiency. Therefore, we compared the litter sizes, resorption rates, and embryonic weights and found no significant differences between KO and WT mice (Figure 2, C–E). These findings are in concordance with the previously reported normal fertility and litter size of PIGF KO mice (16, 23). The embryo/placenta ratio was significantly decreased in PIGF KO compared with WT (Figure 2G) due to increased placental weight in PIGF KO mice (Figure 2F and Supplemental Table 1).

Glomerular capillary endotheliosis and podocyte injury are signature kidney lesions associated with preeclampsia. Histological

analyses of the maternal kidneys revealed normal kidney architecture in pregnant PIGF KO mice (Figure 2I). Detailed examination of glomerular structure also showed a similar number of cells per glomerulus (Figure 2H) and no differences in renal corpuscle, glomerular tuft, or Bowman's space areas (Supplemental Figure 2, A and B). Transmission electron microscopy (Figure 2J) and scanning electron microscopy (Figure 2K) analyses revealed intact podocyte foot processes and absence of glomerular endotheliosis and hyperproliferation, indicating normal glomerular ultrastructure in pregnant PIGF KO mice. Collectively, these results show that PIGF KO mice do not show manifestations of preeclampsia despite an abnormal elevation in circulating sFlt-1.

PIGF KO placentas exhibit increased junctional zone glycogen and decreased labyrinth area. The mouse placenta is divided into 3 compartments: the decidua on the maternal side, the middle junctional zone, and the vessel-dense labyrinth layer on the fetal side. Alterations in the overall placental architecture were evident on histological analyses of H&E-stained cross-sections, which revealed a significantly thicker junctional zone and thinner labyrinth layer in PIGF KO placentas (Figure 3, A and D). Expansion of the junctional zone appeared to be due primarily to increased numbers of vacuolated glycogen trophoblast cells (Figure 3A). Glycogen accumulation in PIGF KO placentas was confirmed

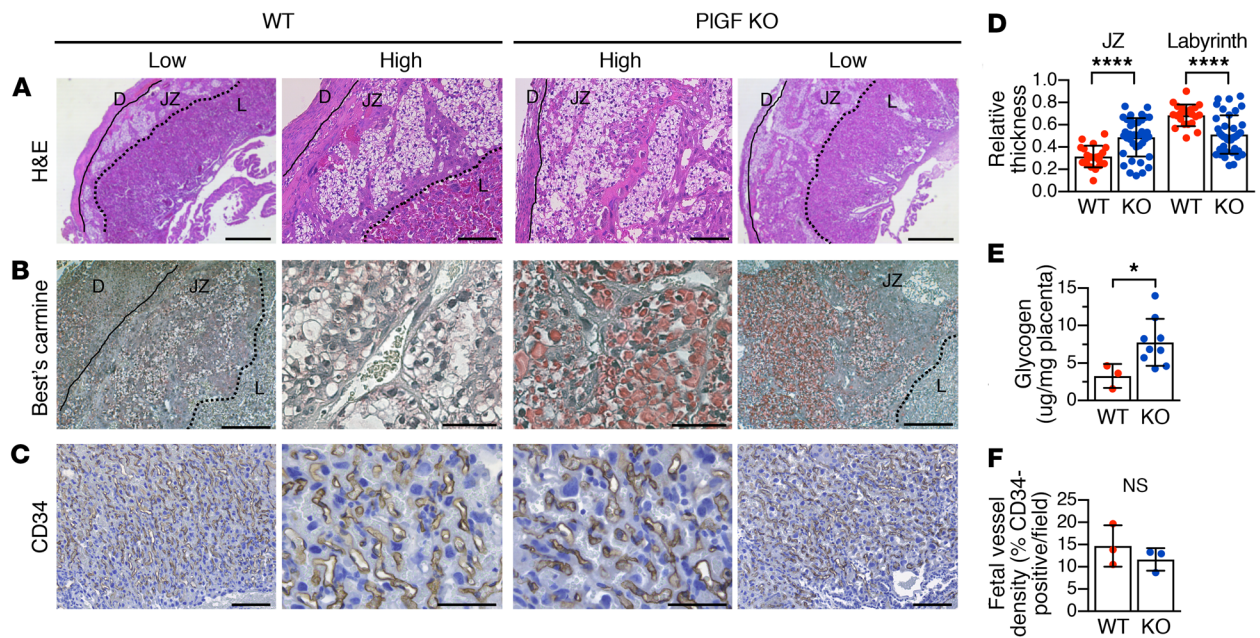


Figure 3. Changes in morphology and glycogen content in PlGF KO placentas. (A) Representative low- and high-magnification images of H&E-stained cross-sections of placentas showing increased numbers of junctional zone glycogen trophoblast cells, which appear as islets of cells with clear cytoplasm due to the high content of glycogen-containing vacuoles. Scale bars: 500 μ m (low); 100 μ m (high). (B) Specific staining of glycogen with Best's carmine (red) in the junctional zone (E19). Scale bars: 200 μ m (low); 50 μ m (high). Solid and dotted lines mark the borders between the junctional zone and the decidua and junctional zone and labyrinth, respectively. (C) Immunohistochemistry for CD34 showing similar staining of fetal vasculature in the labyrinth. Scale bars: 100 μ m (low); 50 μ m (high). (D) Quantification of placental layer measurements expressed as a proportion of total placental thickness. At least 2 midplacental cross sections were analyzed from each placenta from WT ($n = 21$ placentas from 7 litters) and KO ($n = 38$ placentas from 5 litters) mice. (E) Quantification of relative glycogen content (μ g glycogen/mg placental tissue) in WT ($n = 3$) and KO ($n = 9$) placentas. (F) Quantification of CD34 staining (% positive area) of the labyrinth in WT and KO placentas ($n = 3$ placentas from 3 litters, 3 representative high-power fields per placenta) L, labyrinth. Results are shown as mean \pm SD (D, E) and mean \pm SEM (F). Two-tailed, unpaired t test. * $P < 0.05$; **** $P < 0.0001$.

with the glycogen-specific stain Best's carmine (Figure 3B) and quantification of tissue glycogen content (Figure 3E). Parietal trophoblast giant cells (demarcating the junctional zone-decidual border) and spongiotrophoblasts in the junctional zone appeared normal in PlGF KO placentas (Supplemental Figure 3, A and B).

We next examined the labyrinth layer, which comprises a dense network of fetal and maternal blood vessels. Comparisons of PlGF KO with WT placentas revealed a similar density of endothelial cell-lined fetal vessels identified by CD34 immunohistochemistry (Figure 3, C and F). Mononuclear sinusoidal trophoblast giant cells lining the maternal vessels had normal morphology by H&E (Supplemental Figure 3C) and pan-cytokeratin staining (Supplemental Figure 3D). Overall, labyrinth vessel architecture was similar in PlGF KO and WT placentas, but labyrinth area in PlGF KO placentas was decreased compared with that in WT (Figure 3D). These placental changes were associated with increased placental weight in PlGF KO mice (Figure 2F). Collectively, these results show that PlGF loss leads to junctional zone glycogen accumulation, a reduction in labyrinth area, and a larger placenta, but does not significantly affect trophoblast morphology or labyrinth architecture.

Loss of PlGF ameliorates the preeclampsia-like phenotype in COMT-deficient mice. Since we observed an increase in sFlt-1 levels and a placental phenotype in PlGF KO mice, but an absence of preeclampsia manifestations, we hypothesized that loss of PlGF might be protective. We tested the effects of PlGF loss in preeclampsia using the COMT KO model, which develops a preeclampsia-like

phenotype (37). PlGF KO and COMT KO mice were bred to generate double KO (DKO) mice ($Pgf^{-/-}; Comt^{-/-}$). DKO females were mated to COMT KO males (DKO^{xPlGF+}) or DKO males (DKO^{xDKO}) to assess the role of placental PlGF on the maternal preeclampsia phenotype. This mating scheme produced pregnancies with placentas expressing ($Pgf^{+/-}; Comt^{-/-}$, placental PlGF^{+/-}) or lacking ($Pgf^{-/-}; Comt^{-/-}$, placental PlGF^{-/-}) PlGF, respectively (Figure 4A). Lack of placental PlGF in pregnant DKO^{xDKO} mice was associated with a lower systolic blood pressure (Figure 4B) and a trend toward decreased proteinuria (Figure 4C) when compared with DKO^{xPlGF+} pregnant females, indicating partial rescue of the preeclampsia phenotype caused by COMT deficiency. The relative reduction in blood pressure in pregnant DKO mice lacking placental PlGF was associated with a nonsignificant elevation of sFlt-1 compared with that in pregnant DKO^{xPlGF+} mice (Figure 4D), in which sFlt-1 levels approximate the levels observed in the COMT KO model of preeclampsia. Blood pressure, proteinuria, and sFlt-1 data for PlGF, COMT, and DKO mice are summarized in Supplemental Table 2. Similarly to PlGF KO litters, which show a decreased embryo/placenta ratio, DKO^{xDKO} litters also showed a decreased embryo/placenta ratio (Figure 4G and Supplemental Table 1), which appeared to be driven by a significant reduction in embryonic weight (Figure 4E), as placental weight was not dependent on the PlGF genotype (Figure 4F).

Based on the findings in PlGF KO placentas, we anticipated that PlGF loss would lead to an increase in glycogen content

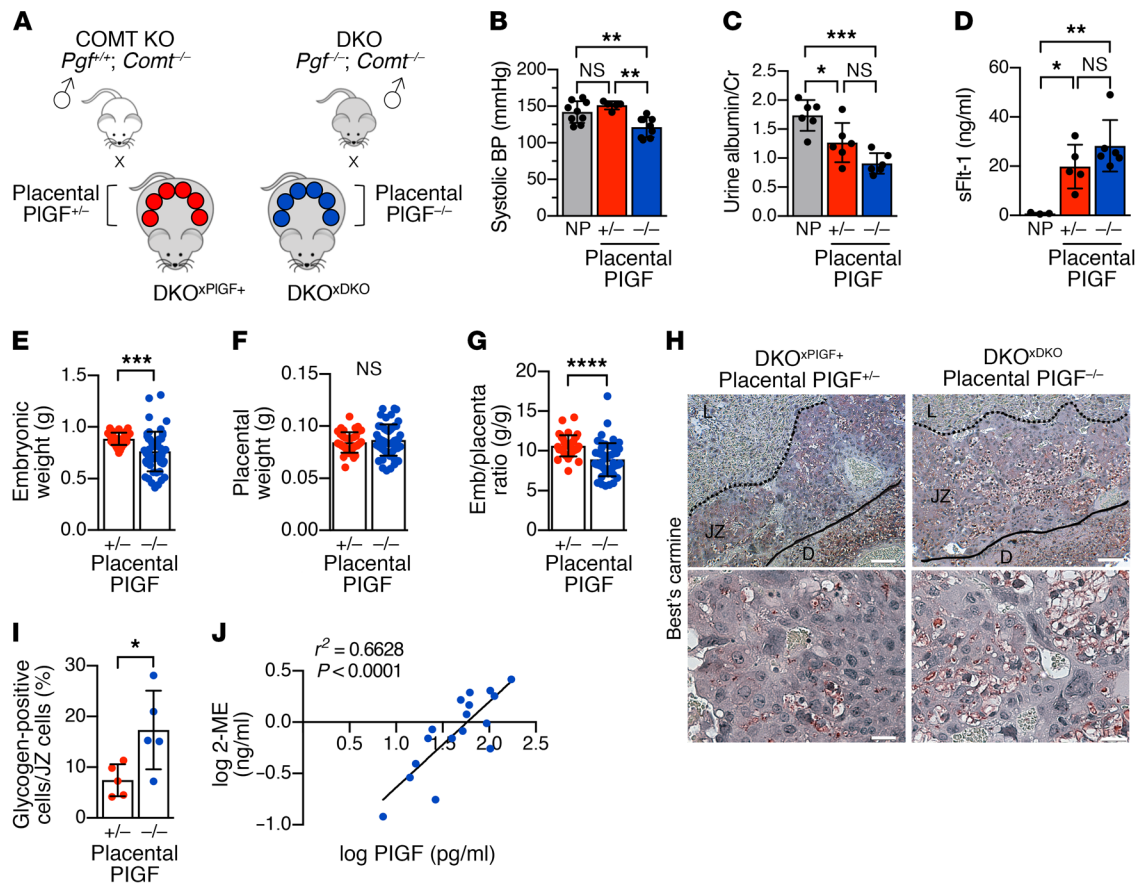


Figure 4. Loss of PlGF ameliorates preeclampsia-like phenotype in COMT KO mice. (A) Schematic of the breeding strategy and embryo/placenta genotypes. (B) Systolic blood pressure in nonpregnant DKO ($Pgf^{-/-}; Comt^{-/-}$; $n = 9$) and pregnant DKO^{xPIGF+} ($n = 5$) and DKO^{xDKO} ($n = 9$) mice at E17. (C) Proteinuria measured by spot urine albumin/creatinine ratio ($n = 6$). (D) Plasma sFlt-1 measured by ELISA in nonpregnant DKO ($n = 3$), DKO^{xPIGF+} ($n = 5$), and DKO^{xDKO} ($n = 6$) at E17. (E–F) Weights of embryos (E) and placentas (F) collected at E17 from DKO^{xPIGF+} ($n = 38$ embryos from 5 litters) and DKO^{xDKO} ($n = 52$ embryos from 7 litters) cohorts. (G) Embryo/placenta ratio decreased in DKO^{xDKO} (placental PIGF^{-/-}). (H) Representative images of the placenta and of the junctional zone showing Best's carmine glycogen staining of E17 placentas. Solid and dotted lines mark the borders between the junctional zone and the decidua and the junctional zone and labyrinth, respectively. Scale bars: 100 μ m (top row); 25 μ m (bottom row). (I) Ratio (%) of glycogen-positive cells to total junctional zone cells per visual field ($n = 5$ placentas). (J) Linear regression analysis of the relationship between plasma PIGF and 2-ME levels in preeclampsia (human cases; $n = 17$). PIGF samples here are also represented in Supplemental Figure 5C. NP, nonpregnant. Results are shown as mean \pm SD. One-way ANOVA (B–D), 2-tailed, unpaired *t* test (E–H), or Pearson's correlation test (J). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

in DKO^{xDKO} placentas. At baseline, COMT KO mice exhibited decreased placental glycogen that was rescued with 2-ME treatment (Supplemental Figure 4, A and B). In the DKO context, this loss of glycogen in COMT-deficient placentas was represented in the DKO^{xPIGF+} mice, which showed a comparable percentage of glycogen-positive cells in the junctional zone compared with COMT KO mice (Figure 4H and Supplemental Figure 4B). The glycogen content in DKO^{xDKO} placentas lacking PIGF was indeed increased compared with that in DKO^{xPIGF+} placentas, confirmed by Best's carmine stain, indicating partial rescue of the reduced glycogen phenotype seen in COMT KO placentas (Figure 4, H and I). In preeclamptic women, alterations in placental glycogen content have previously been reported (41–43). This was confirmed in our study by measuring tissue glycogen content in human placentas from normotensive control versus preeclamptic pregnancies (Supplemental Figure 5A).

Thickening of the basement membrane has also been associated with preeclampsia and other conditions of placental insufficiency, such as hypertension and fetal growth restriction (44, 45).

Thus, we examined placental expression of basement membrane proteins laminin and entactin (also known as nidogen-1) in DKO and COMT KO placentas. Both proteins were more abundant in PIGF-expressing placentas from DKO^{xPIGF+} compared with DKO^{xDKO} mice by immunofluorescence (Supplemental Figure 6A). Consistent with this finding, increased staining of these basement membrane proteins was observed in COMT KO placentas compared with WT or COMT KO treated with 2-ME (Supplemental Figure 6B). In summary, we have shown that PIGF loss in the COMT KO preeclampsia model leads to increased glycogen content and reduced basement membrane protein deposition in the placenta, which were associated with a reduction in maternal blood pressure and alterations in embryonic growth.

Correlation between PIGF and 2-ME in plasma from preeclamptic women. In the COMT KO mouse, low levels of 2-ME (metabolite of COMT enzyme activity) are associated with placental hypoxia and increased HIF-1 α expression (37). Because low PIGF levels are also strongly associated with placental hypoxia and preeclampsia, we evaluated the relationship between PIGF and 2-ME

levels in human plasma. First, we validated previous findings by our group and others showing reduced 2-ME (26–29, 31, 32) and PlGF levels (6, 10, 11) in preeclamptic compared with control women (Supplemental Figure 5, B and C). Then we assessed their relationship in patient plasma and found a significant positive, linear correlation between PlGF and 2-ME among women with preeclampsia (Figure 4).

Discussion

Numerous studies have established the association between an elevated sFlt-1/PlGF ratio and preeclampsia (5, 10, 11, 46–49). However, whether this biochemical imbalance is directly related to disease etiology remains largely unknown. We studied mice with genetic deletion of PlGF to address whether the resultant angiogenic factor imbalance would be sufficient to induce preeclampsia. We found that pregnant PlGF KO mice displayed an abnormally elevated level of circulating sFlt-1, but lacked manifestations of preeclampsia, in contrast with mice lacking COMT, despite a mean serum concentration of sFlt-1 that was greater than peak concentrations observed in human preeclampsia and in other rodent models of preeclampsia (10, 11). Upregulation of placental Flt-1 and sFlt-1 receptors was presumed to occur in response to ligand depletion. Notably, loss of placental PlGF in the COMT KO preeclampsia model ameliorated the maternal hypertension phenotype of those mice. Furthermore, deletion of PlGF altered placental morphology, supporting a role for PlGF in placental development.

Our study demonstrated that PlGF KO placentas have a distinct morphology characterized by enlargement of the junctional zone due to glycogen accumulation and a reduction in the area of the labyrinth compartment. Glycogen trophoblasts, a population of specialized trophoblast cells found in the junctional zone and decidua, are rich in glycogen-containing vacuoles and have been compared with invasive extravillous cytotrophoblasts of the human placenta (50, 51). Although the primary purpose of placental glycogen is a matter of debate, it is thought to serve as an important energy source for fetal growth and can be utilized to maintain placental function under hypoxic conditions (51–55). Many studies have reported alterations in placental metabolism and glycogen content in preeclampsia and fetal growth abnormalities in human and rodent pregnancy, although it is challenging to make definitive conclusions from these studies due to sample and methodological differences (41–43, 51, 56). What is evident is that the significance of increased placental glycogen alterations is context dependent, for instance, indicating excess nutrient availability in diabetes as opposed to impaired utilization or compensation for placental dysfunction in preeclampsia. There is evidence that increased glycogen may serve a protective function. In cancer cells, for example, increases in cellular glycogen storage have been shown to maintain cellular ATP levels as a protective mechanism against bioenergetic stress (57). In genetic mouse models, placental glycogen has been shown to play a role in preserving fetal growth (53). Furthermore, increased placental glycogen in human preeclampsia cases was reported in conjunction with a significant increase in placental glycogen synthase activity, suggesting a metabolic state favoring glycogen synthesis in preeclampsia (41). Collectively, the literature suggests that placental glycogen effectively

acts as a barometer for disturbances in fetoplacental metabolism and that its availability may be an important etiologic component of some pregnancy conditions and a consequence of others.

We found that loss of PlGF in both WT and COMT KO backgrounds resulted in a significant increase in placental glycogen and a reduction in the embryo/placenta ratio, a parameter used as a rough proxy for placental function (58, 59). We speculate that this phenotype represented a compensatory response to disturbances in placental function that have been documented in the COMT-deficient background (37), and are inferred in the PlGF-single KO model based on increased sFlt-1 levels and the reduction in labyrinth area, which directly affects placental nutrient exchange capacity. The placental phenotype resulting from PlGF loss had a net beneficial effect for the mother, demonstrated by the absence of preeclampsia in pregnant PlGF KO mice and a reduction in blood pressure in pregnant DKO^{xDKO}. The change in labyrinth area was not entirely surprising, given the established role of PlGF in angiogenesis. Based on our histologic findings and the localization of Flt-1 receptor to trophoblasts residing in the junctional zone and at the decidual border (i.e., glycogen trophoblast cells, spongiotrophoblast cells, and endovascular trophoblast cells), we postulate that the location of PlGF action in the placenta is primarily the junctional zone (60). Whether PlGF directly regulates the activity of key enzymes involved in glycogen metabolism or whether additional mechanisms of compensation, such as local upregulation of VEGF, occurred is not known; these are areas of interest for future studies. Our findings suggest that PlGF loss had a protective effect potentially mediated through alterations in placental metabolism, which strongly correlates with placental glycogen content and preeclampsia (41, 42, 51, 56, 61, 62).

In plasma samples from preeclamptic women, we found a significant positive correlation between levels of 2-ME and PlGF. Reduced COMT activity and 2-ME have consistently been associated with preeclampsia and placental hypoxia and metabolism (29, 37, 40, 62–64). We speculate that suppression of PlGF in the context of low 2-ME levels in preeclampsia might promote placental glycogen accumulation as an adaptive response to mitigate oxidative stress. The COMT KO mouse has decreased placental glycogen content (Supplemental Figure 4, A and B), which at first seemed to contradict this proposed relationship between suppressed 2-ME/PlGF and increased placental glycogen. However, measurements of PlGF levels revealed that PlGF is not suppressed in COMT KO mice compared with WT (Supplemental Figure 4C). Therefore, differences in PlGF levels may partially explain the relative lack of placental glycogen in COMT KO mice compared with PlGF KO mice and human preeclampsia. Deletion of PlGF in COMT mice (DKO^{xDKO}) did indeed result in increased placental glycogen, lending further support to potential coordinated or overlapping roles for PlGF and 2-ME in regulating placental glycogen and metabolism in preeclampsia.

Despite significant interest in PlGF as a predictor and diagnostic biomarker of preeclampsia (5, 6, 65), the precise role of PlGF in the placenta and preeclampsia is not well understood. A recent study by Aasa and colleagues examined the effect of PlGF loss on maternal cardiovascular physiology (23). Pregnant PlGF KO mice were found to have changes in systolic blood pressure, cardiac output, ventricular mass, and expression of cardiac

stress-related genes in addition to a mild renal phenotype at E16. Alterations in maternal cardiac parameters occurred at E14 and E16 and normalized by E18, leading the authors to propose a limited window for PlGF activity during pregnancy with respect to maternal cardiovascular adaptations. Early transient increased uterine artery resistance was reported in PlGF KO mice; this did not have a significant impact on fetal blood flow (by umbilical artery Doppler), fetal/neonatal growth, or litter size, consistent with our litter data. Any discrepancies between our studies can likely be explained by differences in study design and methodology. We focused our evaluation late in gestation, when preeclampsia occurs most commonly; Aasa et al. highlighted a maternal phenotype that was evident at earlier time points than the ones we examined (23). Although they reported a difference in blood pressure in PlGF KO mice compared with WT C57BL/6, the absolute difference in blood pressure at the late time points of gestation was minimal and not inconsistent with our results. Variations in blood pressure may also be due to differences in genetic background of the mice. Importantly, our studies had different objectives and therefore report outcomes that are not mutually exclusive.

We acknowledge that the PlGF KO model does not faithfully recapitulate the pattern seen in preeclampsia in which PlGF is reduced but still present. The existence of 4 PlGF splice variants in human compared with only 1 variant in mouse also suggests more complex PlGF biology and function in humans that remains to be explored in the context of preeclampsia (66). Furthermore, PlGF is a pleiotropic factor implicated in blood pressure regulation via the renin-angiotensin-aldosterone system (67, 68). This interaction was beyond the scope of our study, but may be relevant to the pathogenesis of preeclampsia (39, 69–72). Finally, the impact of local angiogenic factors, particularly VEGF, is an area of interest for future studies, as there is evidence that local regulation of angiogenic factors at the maternal-fetal interface is important for the maintenance of vascular integrity in the placenta (73).

Importantly, an elevated sFlt-1/PlGF ratio is not a prerequisite for the development of preeclampsia, nor is it unique to preeclampsia. As proposed in a recent review, elevated sFlt-1 and reduced PlGF levels may represent placental cellular stress or failure of homeostatic mechanisms in general, rather than preeclampsia per se (3). A similar profile of angiogenic factors is observed in a host of other conditions associated with compromised placental function (74), including fetal growth restriction (75, 76), fetal death (77, 78), multiple gestation (79, 80), systemic lupus erythematosus (81), and pregnancies resulting from in vitro fertilization (82). Our findings support a complex and multifaceted model for the pathogenesis of preeclampsia, consistent with the current consensus that preeclampsia is a syndrome with multiple etiologies. Therefore, designating a single pathophysiological model is not accurate (61). Therapeutic approaches have been proposed based on the idea that attenuating the antiangiogenic environment in preeclampsia will improve clinical status and delay disease progression (13, 83, 84). However, studies revealing disease-promoting functions of PlGF (85–89) support the need for further mechanism-focused research, and therefore, interventions aiming to alter angiogenic factors in preeclampsia should be approached with caution. Here, we provide evidence

for the broader impact of PlGF on placental development, potentially via regulation of fetal-placental metabolism.

Methods

Mice. PlGF KO mice (*Pgfl^{-/-}*; mixed background, Swiss/SV129) were previously generated by deletion of exons 3–6 (16) and provided by P. Carmeliet. Homozygous adult PlGF KO and WT matings produced pregnant females carrying litters of PlGF KO and WT embryos, respectively. *Pgfl^{-/-}; Comt^{-/-}* DKO mice were generated by breeding COMT KO (mixed, C57BL/6/SV129) and PlGF KO (mixed, Swiss/SV129) mice. DKO females were bred to either DKO males (DKO^{xDKO}) or COMT KO males (DKO^{xPlGF+}) to produce pregnancies with DKO (*Pgfl^{-/-}; Comt^{-/-}*) embryos and PlGF-positive (*Pgfl^{+/-}; Comt^{-/-}*) embryos, respectively. For COMT KO experiments, pregnant COMT KO and WT mice were injected subcutaneously daily with 10 ng 2-ME (Sigma-Aldrich) or placebo (olive oil), respectively, starting from E10, as previously described (37). The day the vaginal plug was observed was taken to be 12 hours after fertilization and set as E0.5.

ELISA, urinary protein, and blood pressure measurements. Blood and urine samples were collected from nonpregnant and pregnant PlGF KO and WT mice between E17 and E20 and from DKO^{xDKO} and DKO^{xPlGF+} mice at E17. Serum or plasma sFlt-1, VEGF, and PlGF levels were measured using Quantikine ELISA kits (R&D Systems). Urine protein levels were estimated using a colorimetric assay (Sigma-Aldrich), as previously described (12). Blood pressure was monitored using a programmable tail-cuff sphygmomanometer (SC-1000, Hat-teras Instruments), as previously described (37).

Embryo and placental measurements. Pregnant mice were sacrificed at the time points indicated above. Weights of placentas and embryos were recorded. Embryonic demise was calculated by the number of resorptions divided by the total number of embryos for each pregnancy.

Histology. Animals were perfusion fixed with 4% paraformaldehyde in 0.1 M phosphate buffer. Paraffin-embedded organs were sectioned (5 μ m) and used for H&E staining, immunohistochemistry, and immunofluorescence. H&E-stained cross-sections from the mid-portion of the placenta were analyzed for trophoblast morphology and junctional zone/labyrinth architecture. Placental layers were measured to determine the relative thickness of the junctional zone and labyrinth, which was expressed as a proportion of total placental thickness. For histological analysis of kidney, randomly selected glomeruli were identified and quantified by counting the number of nuclei per 6 to 10 glomeruli per kidney. Areas of the renal corpuscle and glomerular tuft were quantified with ImageJ (NIH). The area of Bowman's space was calculated by subtracting glomerular tuft area from renal corpuscle area.

Immunohistochemistry and immunofluorescence. For immunohistochemistry, sections were deparaffinized and rehydrated, and antigen was retrieved at 98°C for 30 minutes in 10 mM citrate buffer, pH 6. Sections were incubated with blocking buffer, followed by primary antibody. The following primary antibodies were used: Flt-1 (Santa Cruz Biotechnology Inc., catalog sc-9029, 1:100; Abcam, catalog ab2350, 1:100); CD34 (Abcam, catalog ab81289, 1:500). Placental Flt-1 staining was processed using a VECTASTAIN ABC Kit (Vector Laboratories). For all other stains, sections were incubated with 4plus Biotinylated Goat Anti-Rabbit Secondary Antibody and streptavidin-HRP (BioCare Medical), followed by DAB peroxidase

substrate reagent. Sections were counterstained with hematoxylin, and DAB positivity was analyzed. Immunofluorescence analysis of frozen sections was performed as previously described (37). The following primary antibodies were used: Flt-1 (Abcam, catalog ab2350, 1:100); pan-cytokeratin (Sigma-Aldrich, catalog C1801, 1:200); laminin (Sigma-Aldrich, catalog L9393, 1:200); and entactin (also known as nidogen-1, Chemicon International, catalog MAB1946, 1:100). For quantification of CD34 and Flt-1, 3 visual fields were analyzed per placenta using ImageJ.

Electron microscopy of the kidney. Kidney tissues were fixed with 2.5% glutaraldehyde, 2% paraformaldehyde in 0.1 M cacodylate buffer. Kidney segments were processed for electron microscopy and viewed with an FEI Tecnai G2 Biotwin transmission electron microscope or a JEOL 6390 scanning electron microscope, as previously described (90).

Placental glycogen measurements. Best's carmine was used to stain placental glycogen. Deparaffinized sections were incubated for 20 minutes in Best's carmine staining solution, followed by differentiation solution and dehydration. Amylase treatment was used to evaluate the specificity of Best's carmine staining of glycogen.

Glycogen content was quantified by either digestion and precipitation from placental tissue or imaging of Best's carmine-stained sections. For tissue digestion, portions of placental tissue were weighed and boiled in 30% potassium hydroxide, 5% sodium sulfate bath for 30 minutes to release the glycogen. After cooling on ice, glycogen was precipitated with 1.1 volumes of 95% ethanol. Glycogen precipitates were dissolved in water and analyzed by a phenol sulfuric acid colorimetric method (91). Calculated glycogen content was normalized to the weight of the starting tissue and expressed as $\mu\text{g}/\text{glycogen}$ per mg placental tissue. For quantification of glycogen cells by imaging, the number of glycogen-positive cells (by Best's carmine staining) per visual field was counted and expressed as a percentage of total cells in the junctional zone.

Human sample collection and measurement of PlGF and 2-ME levels. Blood samples from preeclampsia cases and normotensive controls were collected from patients at the University of Pennsylvania (provided by SP and JFS), Mayo Clinic (provided by VDG), and Brigham and Women's (provided by TFM) Hospitals. Preeclampsia was diagnosed according to American College of Obstetricians and Gynecologists criteria (92). PlGF levels were measured using a Quantikine ELISA Kit (R&D Systems). Measurement of plasma 2-ME was performed by PPD Development as previously described (37). Outliers were identified using the ROUT method (robust regression and outlier removal method in GraphPad Prism 6, ref. 93); 7 outliers in the control group and 6 outliers in the PE group were identified, subsequently excluded from the data presented, and statistically analyzed, as shown in Supplemental Figure 5B. Human placenta biopsies were obtained from patients who were delivered at Brigham and Women's Hospital (patients of TFM). Placental glycogen content was quantified by the tissue digestion method described above.

Statistics. Data are presented as mean \pm SD or SEM, as indicated in the figure legends. Unpaired 2-tailed *t* test with Welch's correction, Mann-Whitney *U* test, or 1-way ANOVA with Tukey's multiple comparisons test was used as appropriate and specified in the

figure legends to determine significance. Statistical significance was defined as $P < 0.05$. GraphPad Prism 6 software was used for statistical analyses.

Study approval. All animal experiments were approved by the Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee. Studies involving human specimens were approved by the University of Pennsylvania, Mayo Clinic, and Brigham and Women's Hospital Institutional Review Boards. Written, informed consent was obtained from subjects prior to inclusion in the study.

Author contributions

JGP analyzed data, generated figures, and wrote the manuscript. KK and MK designed and performed experiments with DKO and COMT KO mice and contributed to Figures 1, 3, and 4 and Supplemental Figures 4–6. HS contributed to Figures 1–4 and Supplemental Figures 1–3. LX performed experiments with PlGF KO mice and contributed to Figures 1–3. YH contributed to Supplemental Figure 5. SBL contributed to Figure 4 and Supplemental Figure 5. VHG performed electron microscopy in Figure 2. SP, JFS, VDG, and TFM provided human specimens. PC provided PlGF KO mice. JFS, KHL, BMS, and PC provided intellectual input. VSL provided intellectual input and edited figures and manuscript. RK conceptually designed the strategy for this study, provided intellectual input, and helped write the manuscript.

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