Abstract—Preeclampsia is a life-threatening disorder characterized by maternal gestational hypertension and proteinuria that results from placental dysfunction. Placental abnormalities include abnormal syncytiotrophoblast and a 50% reduction in placental expression of the transcription factor Gcm1. In mice, homozygous deletion of Gcm1 prevents syncytiotrophoblast differentiation and is embryonic lethal. We used heterozygous Gcm1 mutants (Gcm1+/−) to test the hypothesis that hypomorphic expression of placental Gcm1 causes defective syncytiotrophoblast differentiation and maternal and placental phenotypes that resemble preeclampsia. We mated wild-type female mice with Gcm1+/− fathers to obtain wild-type mothers carrying ≈50% Gcm1+/− conceptuses. Gcm1+/− placentas had syncytiotrophoblast abnormalities including reduced gene expression of Gcm1-regulated SynB, elevated expression of sFlt1, a thickened interhemal membrane separating maternal and fetal circulations, and electron microscopic evidence in syncytiotrophoblast of necrosis and impaired maternal-fetal transfer. Fetoplacental vascularity was quantified by histomorphometry and microcomputed tomography imaging. In Gcm1+/−, it was ≈30% greater than wild-type littermates, whereas placental vascular endothelial growth factor A (Vegfa) expression and fetal and placental weights did not differ. Wild-type mothers carrying Gcm1+/− conceptuses developed late gestational hypertension (118±2 versus 109.6±0.7 mm Hg in controls; P<0.05). We next correlated fetoplacental vascularity with placental Gcm1 expression in human control and pathological pregnancies and found that, as in mice, fetoplacental vascularity increased when GCM1 protein expression decreased (R² = −0.45; P<0.05). These results support a role for reduced placental Gcm1 expression as a causative factor in defective syncytiotrophoblast differentiation and maternal and placental phenotypes in preeclampsia in humans. (Hypertension. 2012;59:732-739.) ● Online Data Supplement

Key Words: syncytin ■ placenta ■ preeclampsia ■ angiogenesis ■ VEGFA ■ placental growth factor ■ sFlt1

P}lacental dysfunction is believed to be the major cause of one of the most common and serious complications of human pregnancy, preeclampsia (PE). This potentially life-threatening hypertensive disorder adversely impacts ≈5% of all pregnancies and has no known cure. In PE, growing evidence implicates abnormalities in syncytiotrophoblast (SynT) in the villous exchange region of the placenta.1–6 SynTs are thin, fetal-derived cells that lie between the maternal blood flowing through the villous exchange region of the placenta and the fetal blood flowing through fetal vessels in the highly vascularized villi. This surface is essential for fetomaternal communication and exchange and is formed through the differentiation of SynT from the underlying villous cytotrophoblast cells under the control of the transcription factor GCM1 (glial cells missing 1). 1 Gcm1 in the conceptus is almost exclusively expressed in a subset of trophoblast in the placenta8,9 and is a critical regulator of the SynT cell type.10

In the PE placenta, Gcm1 expression is reduced by ≈50%,4 as expression of the immediate downstream target of Gcm1,11 Syn-1.5,6 That SynT is abnormal and potentially causative in PE is supported by abnormal levels of SynT-produced factors, such as pregnancy-associated plasma protein A, human chorionic gonadotropin, and inhibin in maternal blood during early pregnancy in women at high risk for the later development of PE.12,13 When PE develops, the abnormal syncytial surface is prothrombotic, leading to infarctions within the intervillus space,14 and is excessively
shed as aponecrotic fragments into the maternal circulation; both are believed to be factors contributing to clinical signs of disease.

The availability of Gcm1 deletion mutants provides an opportunity to explore the function of Gcm1 in vivo. Homozygous deletion mutants show the critical importance of Gcm1; embryonic lethality results from absent SynT differentiation and failure to form the exchange region known as the labyrinth in mice. Relative to placental differences between species, the placentas of humans and mice are strongly similar, although differences exist. In both species, maternal blood perfuses an intervillous space lined by fetal-derived trophoblasts that encase the fetoplacental vasculature within the exchange region. In both species, Gcm1 is expressed in trophoblast cells differentiating into SynT.

However, whereas humans have a single SynT layer, mice have 2 layers; SynT-II is adjacent to the fetal vasculature, and it is intimately connected to SynT-I via gap junctions and nutrient transporters and, unlike SynT-I, only SynT-II expresses Gcm1. In humans, the SynT surface is fully exposed to maternal blood, whereas in mice the SynT-I surface is partially covered by a discontinuous layer of sinusoidal trophoblast giant cells (sTGCs). Given the difficulty in establishing causal relationships in human studies, here we exploit similarities between humans and mice by using heterozygous Gcm1 deletion mutants to determine whether reduced Gcm1 expression in mice causes changes in trophoblast morphology, fetoplacental vascularity, and pregnancy outcomes that resemble PE in humans.

Materials and Methods

Mouse and human experiments were approved by institutional review committees. For complete methods please see the online-only Data Supplement.

Mouse Model

CD1 females mated to males with heterozygous deletion of Gcm1 (Gcm1+/−) carried 50% Gcm1+/− and 50% wild-type conceptuses. Control pregnancies were CD1 females mated with CD1 males. At embryonic day 13.5 (E13.5) and E17.5, placentas were microdissected to remove decidual tissue and frozen for protein extraction. For STAT1+/− and wild-type littermates (Figure S1 in the online-only Data Supplement).

Human Patients

Placental villous tissue and matched histological samples were obtained from a BioBank. Samples were from pregnancies with PE, intrauterine growth restriction (IUGR; and without PE), and from gestation-matched preterm controls (Table). PE was diagnosed as new-onset gestational hypertension, proteinuria, and reversal of hypertension and proteinuria by 12 weeks postpartum. IUGR was diagnosed as fetal sex and gestation-adjusted birth weights less than the third centile and absent end-diastolic blood flow velocity in umbilical artery Doppler waveforms.

Histology and Imaging

In mice, images from midline placental sections were quantified by histomorphometry to obtain areas of placental components or evaluated by transmission electron microscopy. Counts of Ki67 positive cells in the labyrinth excluded sinusoidal trophoblast giant cells. In humans, the proportion of fetoplacental vasculature in villi was quantified in hematoxylin-eosin-stained sections. Microcomputed tomography was used to quantify fetoplacental arteries and arterioles ≥50 μm in diameter, as published. Capillaries were examined in vascular corrosion casts as published.

Table. Human Clinical Outcomes and Histomorphometry Data

<table>
<thead>
<tr>
<th>Variables</th>
<th>Preterm Controls (N=5)</th>
<th>PE (N=5)</th>
<th>IUGR (N=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestational age at delivery, wk</td>
<td>30.9±0.8</td>
<td>30.5±1.2</td>
<td>30.7±1.2</td>
</tr>
<tr>
<td>Range, wk</td>
<td>28–33</td>
<td>28–35</td>
<td>28–34</td>
</tr>
<tr>
<td>Systolic BP, mm Hg</td>
<td>112±5</td>
<td>185±5†</td>
<td>138±7</td>
</tr>
<tr>
<td>Diastolic BP, mm Hg</td>
<td>61±6</td>
<td>109±4†</td>
<td>86±4*</td>
</tr>
<tr>
<td>Fetal weight, g</td>
<td>1772±127</td>
<td>1364±200</td>
<td>998±179*</td>
</tr>
<tr>
<td>Placental weight, g‡</td>
<td>271±23</td>
<td>189±31</td>
<td>163±23*</td>
</tr>
<tr>
<td>Fetal vasculature in villi, %</td>
<td>11.3±1.1</td>
<td>19.1±1.2</td>
<td>11.1±1.4</td>
</tr>
</tbody>
</table>

BP indicates blood pressure; PE, preeclampsia; IUGR, intrauterine growth restriction.

Maternal Phenotypes

Arterial pressure in awake mice was measured by tail-cuff plethysmography, urine protein by the Bradford method, urine creatinine by the Jaffe reaction, and sFLT1 protein in maternal plasma by ELISA.

Statistical Analysis

Wild-type littermates from CD1×Gcm1+/− matings were used as controls for Gcm1+/− conceptuses. Statistical significance (P<0.05) was determined using Wilcoxon sign-ranked test for quantitative RT-PCR data, 2-tailed Student t test (genotype effects), or 2-way ANOVA (genotype and gestation effects), followed by the Tukey post hoc test. Results show mean±SE.

Results

Abnormal Trophoblast Differentiation

Gcm1 in mice is specifically expressed in the SynT-II layer of the labyrinth where it regulates expression of its downstream target, SynB. In the Gcm1+/− labyrinth, expression of Gcm1 mRNA and protein was decreased by ~50% relative to wild-type littermates (Figure S1 in the online-only Data Supplement). Gcm1 deficiency resulted in a dysregulation of trophoblast differentiation as shown by an ~35% decrease in SynB mRNA expression (Figure S1A), an ~2-fold increase in cell proliferation within the labyrinth (Figure S2), and prominent ultrastructural abnormalities of the 2 syncytiotrophoblastic layers (SynT-I and SynT-II) at E17.5 (Figure 1). There was evidence of necrosis in SynT-II including pale cytoplasm, numerous vacuoles, and swollen and degenerating mitochondria in transmission electron microscopy images (Figure 1A through 1C). The Gcm1+/− SynT-I layer appeared to differentiate normally in that expression of its marker gene, SynA, was not significantly affected (1.0±0.2- and 1.3±0.3-fold change relative to wild-type littermates, at E13.5 and E17.5, respectively; P>0.05). Nevertheless, the SynT-I layer exhibited ultrastructural abnormalities, including increased electron density and the presence of large fluid-filled vacuoles (Figure 1A and 1B). SynT-I and SynT-II appeared thicker in...
Gcm1+/− placenta compared with wild-type littermate controls at both E13.5 and E17.5 (Figure 1A and 1B). In addition, loss of apical membrane integrity in focal areas of the sTGCs (in direct contact with maternal blood) was observed in transmission electron microscopy images of the Gcm1+/− placenta at E17.5 (Figure 1D).

Abnormal Placental Histomorphology and Vascularity

At E13.5, the Gcm1+/− labyrinth was significantly decreased in cross-sectional area, and there were significant increases in the proportion of maternal blood spaces and in the thickness of the interhemal membrane relative to wild-type litters (Figure 2A through 2D). There was also an increase in the area of clustered cuboidal cells within the Gcm1+/− labyrinth (Figure 2C). In contrast, at E17.5, Gcm1+/− placentas had a significantly higher proportion of fetal blood spaces in the labyrinth compared with wild-type litters (Figure 2G).

The latter is consistent with increased fetoplacental vascularity at E15.5 in Gcm1+/− placentas, as shown by significant increases in the total number and length of arterial segments with diameters >50 μm by microcomputed tomography (Figure 3) and apparent increases in the density of capillaries in vascular casts and in histological sections (Figure S3A through S3C).

Intriguingly, the mRNA and protein expression profile of VEGFA, a potent proangiogenic molecule thought to play an important role in placental angiogenesis,31 demonstrated decreased expression across gestation within the Gcm1+/− placenta relative to that of wild-type litters (Figure 4A and 4B). A similar examination of the mRNA and protein expression of another proangiogenic molecule, placental growth factor (PGF) demonstrated a modest increase in expression at E13.5 in the Gcm1+/− placenta and no difference in expression by E17.5 (late gestation) compared with littermate controls (Figure 4A and 4C).
Fetal and Placental Growth

Despite pronounced abnormalities in trophoblast differentiation and placental morphology, Gcm1+/- conceptuses grew normally; at E17.5 there were no significant differences between fetal weights of Gcm1+/- (1.2±0.1 g) versus wild-type litters (1.1±0.1 g) or in their placental weights (92±15 versus 90±10 mg, respectively). There was no evidence for an effect of paternal genotype on litter size or number of resorptions.

Clinical Signs of PE in Mothers

CD1 females mated with Gcm1+/- males carried litters in which ≈50% of conceptuses were Gcm1+/-.. These mothers had significantly higher mean arterial pressure at E17.5 compared with CD1 mated with CD1 males (118±2 versus 109.6±0.7 mm Hg, respectively; Figure 5A). The increase in maternal pressure in late gestation was positively correlated with the number of Gcm1+/- pups within the litter ($R^2=0.54; P<0.05; \text{Figure } 5B$). CD1 mothers carrying mixed litters did not have proteinuria in late gestation; they excreted 0.7 mg of protein per milligram of creatinine in controls. As in human PE placentas,\textsuperscript{32} the Gcm1+/- labyrinth demonstrated a 2-fold increase in sFlt1 mRNA expression relative to the wild-type littermate controls (Figure 5C). However, in contrast to human PE,\textsuperscript{32} there was no significant increase in maternal plasma sFlt1 protein (Figure 5D).

Correlation Between GCM1 and Fetoplacental Vascularity in Humans

Human clinical outcomes for patients with PE, with IUGR, and for gestation-matched controls are shown in the Table. The proportion of fetal vascular space within the villous tissue of patients with PE was significantly elevated relative to gestation-matched controls and IUGR placentas (Table). Across all of the groups, the proportion of fetal vascular space within villous tissue was negatively correlated with GCM1 protein expression ($R^2=-0.45; P<0.05; \text{Figure } 6$).

Discussion

In the current study, hypomorphic expression of placental Gcm1 caused defective SynT differentiation and maternal and placental phenotypes resembling PE in humans. As anticipated, heterozygous Gcm1 gene deletion reduced placental Gcm1 expression by ≈50%, a decrement similar to that in human PE placentas.\textsuperscript{4} SynT-II cells exhibited prominent abnormalities, including reduced expression of SynB and ultrastructural evidence of necrosis.\textsuperscript{30} Both SynT-I and SynT-II appeared thickened and ultrastructurally abnormal. Nevertheless Gcm1+/- fetuses were normally grown. Similarly, fetuses are normally grown in the majority of PE pregnancies,\textsuperscript{33} despite evidence of hypomorphic Gcm1 expression and SynT dysfunction.\textsuperscript{1-6} An unexpected and striking finding of the current study was increased fetoplacental...
vascularity in the Gcm1+/− mouse placentas. We show a similar increase in fetoplacental vascularity correlates with decreased GCM1 protein expression in human placental villi. Finally, results show that conceptuses with hypomorphic Gcm1 expression caused maternal hypertension in late gestation in otherwise healthy, wild-type mothers. Hypomorphic Gcm1 expression caused abnormal differentiation of SynT-II cells, as shown by a reduction in expression of

Figure 3. Arterial vascularity in the labyrinth at embryonic day (E) 15.5 by microcomputed tomography (CT). A, Images from a Gcm1+/− and wild-type littermate control (Wt). B, Color-coded arterial tree to show the anatomic distribution of vessel diameters. C and D, Quantitative analysis showing significant overall increases in (C) the total number of vessel segments and (D) the total length of vessel segments in arterial trees of Gcm1+/− placentas (●) vs Wt (□). Increased vascularity was most prominent in the smallest diameter range (ie, 50–100 μm). *P<0.05. N=11 per genotype (2–3 conceptuses per genotype from 5 pregnancies).

Figure 4. Expression of proangiogenic factors at embryonic day (E) 13.5 and E17.5. A, mRNA expression of Vegfa and Pgf in labyrinth-enriched Gcm1+/− samples normalized using 3 housekeeping genes and expressed as a fold change relative to wild-type littermate controls (Wt; hashed line at 1.0). Vegfa mRNA was significantly reduced at E13.5 and E17.5 in Gcm1+/−, whereas Pgf mRNA was significantly increased at E13.5 only. B, Placental VEGFA protein by ELISA was significantly decreased at both ages. C, Placental PGF protein by ELISA was not significantly changed. Number of pregnancies for each gestational age and genotype was N=6. *P<0.05.
the Gcm1-regulated fusogenic protein SynB and by prominent ultrastructural abnormalities that directly paralleled the described focal necrosis abnormalities observed in the SynT layer in human PE.30,34 SynT-II is tightly integrated with the adjacent SynT-I layer. SynT-I uniquely expresses Syncytin A (SynA).10 We found that SynA expression was not altered in Gcm1/H11001/H11002 placentas suggesting normal differentiation of SynT-I, a non-Gcm1–expressing cell type.10 Similarly, no change in SynA expression was observed in homozygous Gcm1 knockout placentas. Nevertheless, morphology of the SynT-I layer was markedly abnormal in Gcm1/H11001/H11002 placentas; transmission electron microscopy imaging showed that it appeared thicker, contained more electron dense material, and contained many large, fluid-filled vacuoles. SynT-I and SynT-II are intimately connected by gap junctions and ultimately function as a single syncytial layer across which maternal-fetal transfer occurs.21,22 Thus, it is likely expression was observed in homozygous Gcm1 knockout placentas.10 Nevertheless, morphology of the SynT-I layer was markedly abnormal in Gcm1+/− placentas; transmission electron microscopy imaging showed that it appeared thicker, contained more electron dense material, and contained many large, fluid-filled vacuoles. SynT-I and SynT-II are intimately connected by gap junctions and ultimately function as a single syncytial layer across which maternal-fetal transfer occurs.21,22 Thus, it is likely

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**Figure 5.** Effect of Gcm1+/− conceptuses in CD1 mothers on maternal arterial pressure and placental and plasma sFlt1 levels. **A**, Arterial pressure in CD1 mothers carrying mixed litters of wild-type (Wt) and Gcm1+/− conceptuses (■) was significantly higher at E17.5 than in CD1 mothers with Wt conceptuses only (□) when expressed as a change (△) from the nonpregnant state (NP). **B**, Maternal arterial pressure was positively correlated with the number of Gcm1+/− conceptuses at E17.5. R² value P<0.05. **C**, mRNA expression of the antiangiogenic factor sFlt1 was higher in Gcm1+/− placentas expressed relative to Wt littermate controls at E17.5 (hashed line at 1.0). **D**, Maternal plasma sFLT1 protein was not significantly different whether CD1 mothers carried only Wt fetuses (□) or mixed litters (■) at E17.5. Number of pregnancies for each gestational age and paternal genotype was N=6 in **A**, **C**, and **D**, and N=10 in **B**. *P<0.05.

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**Figure 6.** GCM1 protein expression and its correlation with villous fetoplacental vascularity in human placentas. **A**, Western blot showing GCM1 protein expression in placentas from preterm control (C), pre-eclamptic (PE), and intrauterine growth-restricted (IUGR) pregnancies at delivery. β-Actin (BAct) was used as the loading control. **B**, There was a significant negative correlation between placental GCM1 expression and the proportion of villous tissue composed of fetal vasculature in C, PE, and IUGR placentas (N=5 per group). R² value P<0.05.
that a primary limitation in transport across the abnormal SynT-II layer contributed to abnormal SynT-I morphology by impeding the egress of protein and vacuoles from SynT-I.

Abnormal SynT-II in Gcm1<sup>+/−</sup> placentas additionally influenced the discontinuous sTGCs<sup>3</sup> that overlie SynT-I in the maternal blood spaces of the labyrinth. Apical membrane integrity of sTGC was lost in localized areas, and cytoplasmic cellular contents were visible within the adjacent maternal blood space. This was unexpected given that the sTGCs are only loosely connected to the underlying SynT-I layer through desmosomal attachments<sup>23</sup> and are not in direct contact with SynT-II cells. Nevertheless, results suggest that hypomorphic Gcm1 expression in the PE placenta in human pregnancy may play a similar causative role in promoting the shedding of placental debris into the maternal circulation. This debris is thought to be responsible, at least in part, for the widespread maternal endothelial dysfunction and hypertension in PE.<sup>12</sup> In mice, sTGCs release hormones such as placental lactogen, into the maternal circulation,<sup>20</sup> so abnormalities observed in this cell type in the current study may also alter maternal responses to pregnancy by an endocrine mechanism.

A remarkable finding in the current study was that fetal growth was not compromised despite marked abnormalities in SynT-I, SynT-II, and sTGC cell morphology, as well as augmented interhemal membrane thickness. It is possible that normal fetal growth was protected by increased fetoplacental vascular density in the Gcm1<sup>+/−</sup> labyrinth. Increased fetoplacental vascularity may have been caused by feedback mechanisms invoked by impaired transfer caused by the thicker, malfunctioning fetal/maternal exchange barrier. However, abnormal SynT-I in SynA knockout placentas also causes abnormal interhemal membrane morphology, but in that case results in fetoplacental hypovascularity, fetal growth restriction, and fetal lethality by E13.5.<sup>35</sup> Furthermore, Gcm1 overexpression results in fetoplacental hypovascularity, leading to fetal growth restriction in late-gestation in mice.<sup>36</sup> Thus, it is more likely that dysregulated SynT-II differentiation directly influences fetoplacental vascularization through altered signaling to the adjacent fetal endothelial and mesenchymal cells. How angiogenesis is augmented in the face of decreased mRNA and protein expression of the potent proangiogenic factor VEGFA, increased mRNA expression of the antiangiogenic sFlt1, and only modest increases in PGF at E13.5 is not known. It is possible that alternate unidentified angiogenic mechanisms are activated within the Gcm1<sup>+/−</sup> placenta. The same paradox among villous trophoblast pathology<sup>2,30,36</sup> high placental expression of the antiangiogenic sFlt1<sup>32</sup> and augmented villous angiogenesis (current study) occurs in PE where the majority of women give birth to infants of normal weight.<sup>33</sup> Indeed, the current results in Gcm1<sup>+/−</sup> mice prompted the examination and discovery of a similar negative correlation between placental vascularity and GCM1 protein expression in placental biopsies from normal and pathological pregnancies (PE and IUGR). Thus, this mouse model provides an excellent opportunity to explore this apparent incongruity and to advance our understanding of the influence of SynT on fetoplacental vascularization.

Hypomorphic Gcm1 expression resulted in significantly increased maternal arterial pressure in late gestation, although how hypomorphic Gcm1 in SynT-II caused this change is not known. Mechanisms other than increased circulating sFLT1 must be driving maternal hypertension, because, despite increased placental mRNA expression of sFlt1 in the Gcm1<sup>+/−</sup> placenta, sFLT1 protein in the maternal circulation was not significantly elevated. Maternal proteinuria was also absent, suggesting that renal function was unimpaired. It is possible that placental debris from sTGC entered the maternal circulation and caused endothelial cell inflammation, increased peripheral vascular resistance, and hypertension (as in human PE<sup>1,3</sup>), or that abnormal endocrine signals from the dysfunctional sTGC are responsible. The increase in arterial pressure in this mouse model was modest, and this could mean that, alone, the hypomorphic expression of Gcm1 in the placenta of women with PE is insufficient to completely explain their hypertension. This is consistent with evidence suggesting that a combination of placental dysfunction and underlying maternal predispositions to endothelial dysfunction may be required for PE in women.<sup>37,38</sup> Alternatively, it is also possible that relatively mild maternal effects were observed, because not all of the conceptuses were Gcm1<sup>+/−</sup>; this cannot be achieved in a wild-type mother by natural breeding, because Gcm1 deletion is homozygous lethal. Wild-type conceptuses may diminish maternal effects by effectively diluting circulating factors released by Gcm1<sup>+/−</sup> placentas and/or by releasing protective factors into the maternal circulation. Indeed, a positive correlation was observed when maternal arterial pressure was plotted against the number of Gcm1<sup>+/−</sup> conceptuses within the pregnancy. Interestingly, evidence supports a similar mechanism in human twin pregnancies complicated by IUGR where a healthy co-twin appears to be protective; PE affects 6.8% of singleton pregnancies complicated with IUGR versus 1.5% in twin pregnancies when only 1 twin has IUGR.<sup>39</sup>

Thus, results of the current study show that hypomorphic Gcm1 expression in SynT can elevate maternal arterial pressure in late-gestation in mice. This means that hypomorphic placental Gcm1 expression may not just correlate with human PE but could also play a causative role in maternal hypertension, one of the hallmark signs of PE. This conclusion is supported by the recent identification of a fetal variant in the Gcm1 gene that is associated with gestational hypertension in human pregnancy.<sup>40</sup>

**Perspectives**

Hypomorphic placental Gcm1 expression caused dysregulated SynT-II differentiation, abnormal morphology and dysfunction of the interhemal membrane, and augmented fetoplacental vascularity, and caused maternal hypertension in late gestation. In addition, we showed in humans that there is a similar inverse correlation between fetoplacental vascularity of placental villi and Gcm1 expression. These results, therefore, support a role for reduced placental Gcm1 expression as a causative factor in defective SynT differentiation and maternal and placental phenotypes in PE in humans.

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Disclosures
None.

References
15. Bainbridge et al Gcm1 and Pregnancy Outcomes in Mice

Effects of Reduced Gcm1 Expression on Trophoblast Morphology, Fetoplacental Vascularity, and Pregnancy Outcomes in Mice
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EFFECTS OF REDUCED Gcm1 EXPRESSION ON TROPHOBLAST MORPHOLOGY, FETO-PLACENTAL VASCULARITY AND PREGNANCY OUTCOMES IN MICE.

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MATERIALS AND METHODS

**Mouse Model**

Experiments were approved by the Animal Care Committee of the Toronto Centre for Phenogenomics (Toronto, ON, Canada) and were conducted in accord with guidelines established by the Canadian Council on Animal Care. Virgin female CD1 mice (an outbred wild type strain; Charles River, Canada), between 6-10 weeks of age, were mated with males with heterozygous deletion of the Gcm1 gene (Gcm1+/-) which were maintained on a CD1 background (a gift from Dr. J. Cross, University of Calgary). This breeding scheme yields pregnancies with ~50% offspring with heterozygous deletion of Gcm1 (Gcm1+/-) and ~50% wildtype (Wt) offspring. Wildtype littermates were used as intra-pregnancy controls. Additional controls were obtained by mating virgin CD1 females (6-10 weeks) with CD1 males. Noon of the day a vaginal plug was found was designated E0.5. Maternal weight, blood pressure, urine and maternal blood samples were collected prior to breeding and longitudinally across pregnancy. At E13.5 and E17.5, a sub-set of mothers were euthanized by cervical dislocation and the uterus was removed into RNAse-free, ice-cold PBS. Each implantation site was individually opened and the corresponding fetus removed and weighed, and a sample of fetus or yolk sac collected for genotyping. Placentas were removed from the uterine wall and microdissected to separate them from their decidua. Placentas were either flash frozen in liquid nitrogen for protein analysis, immersion fixed in 4% PFA for light microscopy, or fixed in 2% glutaraldehyde (in 0.1M sodium cacodylate buffer) for transmission electron microscopy. Some placentas were further microdissected to obtain labyrinth-enriched tissue. These samples were processed in RNAlater (Ambion, Applied Biosystems Canada) and stored at -80°C for mRNA analysis. After genotyping fetuses, samples for protein or mRNA analysis were pooled per pregnancy by genotype.

**Human patients**

Placental villous tissue (for GCM1 protein quantification) and matched histological samples (for histomorphometry) were obtained from the specimen archive of the Research Centre for Women’s and Infants’ Health BioBank at Mount Sinai Hospital ([http://biobank.lunenfeld.ca](http://biobank.lunenfeld.ca)). The study was conducted in accordance and with approval from the Mount Sinai Hospital Research Ethics Board (Toronto, ON) and patients had provided their informed consent. Samples were collected from placentas from PE pregnancies (N=5), pregnancies with intra-uterine growth restriction without PE (IUGR, N=5), and gestation matched idiopathic preterm rols (N=5). PE was diagnosed with new onset gestational hypertension, proteinuria, and reversal of hypertension and proteinuria by 12 weeks postpartum. Hypertension was defined as an increase of 30 mmHg systolic or 15 mmHg diastolic blood pressure compared to values obtained before 20 weeks gestation and an absolute blood pressure >140/90 mmHg. Proteinuria was defined as >300 mg/24 h collection or >2+ on voided or 1+ on catheterized random urine sample or a protein creatinine ratio >0.3. IUGR was diagnosed by fetal sex and gestational age-adjusted birth-weight <3rd centile accompanied by absent end-diastolic blood flow velocity in umbilical artery Doppler waveforms.

**Gene Expression**

RNA was extracted from the labyrinth-enriched samples (N=6 pregnancies; 3 pooled samples/genotype/pregnancy) using the Trizol method with removal of any contaminants by means of DNase treatment (RNase-free DNase Set, Qiagen) and RNeasy Mini-eltue cleanup kit
(Qiagen). Samples were reverse transcribed to cDNA using TaqMan Reverse Transcription Reagents (Applied Biosystems). Expression of genes of interest were measured using qRT-PCR (Eppendorf Realplex²) with SYBER Green (iTaq SYBER Green Supermix with ROX, Bio-Rad Laboratories) using 40 cycles of 95°C (15 sec) and 60°C (1 min) followed by melting curve analysis. Primers were designed for murine Gcm1, Syncytin A (SynA), Syncytin B (SynB), vascular endothelial growth factor A (Vegfa), soluble fms-like tyrosine kinase 1 (sFlt1), placental growth factor (Pgf), along with three housekeeping genes Beta-actin (BAct), glyceraldehyde-3-phosphate dehydrogenase (Gapdh) and TAT box binding protein (Tbp). All primer sequences (Table S1) were subjected to BLAST analysis to ensure specificity and were tested for efficiency. Expression data was analyzed using the \( \Delta \Delta -Ct \) method with gene expression in the \( Gcm1^{+/+} \) placenta normalized to the geometric mean of the three housekeeping genes and expressed relative to the expression in the wildtype littermate sample.

**Protein Expression**

Frozen placental samples (N=6 pregnancies; 3 pooled samples/genotype/pregnancy) were ground into a fine powder using a mortar and pestle in the presence of liquid nitrogen. Samples were then lysed and homogenized in the presence of RIPA buffer (Thermo Scientific; Rockford, IL) and protease inhibitors (Complete Mini EDTA-free Inhibitor, Roche Applied Science; Laval, QC) and subjected to centrifugation. Concentrations of VEGFA and PGF were measured in prepared lysates using commercially available ELISA kits (R&D Systems; Burlington, ON) as per manufacturers’ directions. All results were normalized to total protein concentration in each sample, as measured by Bradford Reagent. GCM1 protein was measured by western blot analysis. SDS-polyacrylamide gel electrophoresis was used to separate proteins. Samples were then transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA). After a 1-hour incubation in blocking solution consisting of 5% dry milk and 0.05% Tween 20 in PBS (PBS-T), the membranes were incubated overnight at 4°C with an anti-Gcm1 antibody for mice (sc-69406, 1:500; Santa Cruz Biotechnology, Santa Cruz, CA) or humans (OAAB04228 1:1000; Aviva Systems Biology, San Diego, CA) followed by three 15-minute washes in PBS-T. The membranes were then incubated with secondary rabbit anti-goat IgG (#31402, 1:3000; Pierce Thermo Scientific) or goat anti-rabbit IgG (#31460, 1:3000; Pierce Thermo Scientific; Ottawa, ON) labeled with horseradish peroxidase. After three additional 15-minute washes with PBS-T, the blots were developed using a chemiluminescence HRP detection (Immun-Star Western C Chemiluminescence kit; BioRad Laboratories; Mississauga, ON), and visualized using Versa-Doc Imaging System (BioRad Laboratories). Uniformity of loading of protein extracts was determined by probing with an anti-β-actin antibody (sc-1616, 1:1000; Santa Cruz). Relative intensities of bands were determined by densitometry using Quantity One 1-D Analysis software (BioRad Laboratories).

**Transmission Electron Microscopy**

Mouse placenta for transmission electron microscopy (EM) were fixed in 2% glutaraldehyde, post fixed in 1% osmium tetroxide in 0.1M sodium cacodylate buffer, dehydrated in a graded ethanol series followed by propylene oxide, and embedded in Quetol-Spurr epoxy resin. Ultra-thin sections (100 nm) were cut and counterstained with uranyl acetate and lead citrate, and imaged by transmission EM (FEI CM100; Hillsboro, OR) at magnifications between x2500 - x25000. Imaging focused on the interhaemal membrane of the placental labyrinth (i.e. the tissue layers separating the maternal and fetal blood spaces) with specific
attention paid to the appearance and integrity of the 2 syncytial membranes (SynT-I and SynT-II) and the sinusoidal trophoblast giant cells (sTGC).

**Immunohistochemistry and histomorphometry**

After genotyping, 3 Wt and 3 Gcm1+/− placentas per pregnancy (N=5 pregnancies for each gestational age) were arbitrarily selected for placental histomorphometry. Paraffin-embedded placental sections (5 µm) were de-paraffinized by sequential incubations in Xylene and decreasing ethanol baths (each for 2 x 3 min). Antigen retrieval was performed by heating the sections for 20 min followed by incubation in sodium citrate buffer (10 mM). Following washes in PBS, endogenous peroxidase activity was quenched using 3% hydrogen peroxide and nonspecific binding was limited using Dako protein block (Dako; Burlington ON). The sections were treated with antibodies overnight at 4°C to detect trophoblast (anti-cytokeratin, Z0622, 1:1000; Dako), endothelial basement membranes (anti-CD34, MCA1825, 1:100, AbD Serotec, Raleigh, NC), or proliferating cells (anti-Ki67, RM-9106, 1:200, Thermo Fisher Scientific, Fremont, CA). A biotinylated anti-rat (#712066153, 1:200, Research Diagnostics INC; Flanders, NJ) or anti-rabbit IgG (BA-1000, 1:200; Vector Laboratories; Burlington, ON) secondary antibody was added to the sections for 30 min at room temperature. Additional processing of the sections for the detection of the trophoblast (cytokeratin), fetal vascular endothelial cells (CD34) or proliferating cells (Ki67) was performed according to the instructions provided with the Vectashield Elite ABC kit (Vector Laboratories). Colorimetric detection was achieved using diaminobenzidine as the peroxidase substrate.

Histomorphometry of each placenta using newCAST stereological software (Visiopharm; New York, NY) 4 was performed on midline transverse sections immunohistochemically stained for cytokeratin. Sections from 1-5 placentas per genotype (typically 3) were examined from 5 ICR x Gcm1+/− pregnancies. Placental tissue was outlined using the mask tool and a pre-defined grid superimposed. Spongiotrophoblast, labyrinth, and chorionic plate were identified at grid intersection points at 20x magnification. Proportions of each component were calculated as a percentage of total point counts. Subsequently, proportional counts specific to the components of the labyrinth (including fetal blood space, maternal blood space, sinusoidal trophoblast giant cells, cuboidal cells and other) were performed at 40x on serial sections stained for CD34 to label the fetal endothelial cell basement membrane. Proportions of each component were calculated as a percentage of total labyrinth point counts. The grid sizes and coverage were sufficient to obtain a minimum of 250 total point counts per region of interest (in the placenta or labyrinth) per genotype per pregnancy. Interhaemal membrane thickness was measured on the same midline sections stained for CD34 at 60x as previously described 4. A line grid with 50% coverage was used to obtain a minimum of 250 measurements per genotype per pregnancy. The harmonic mean of the interhaemal membrane thickness was calculated per genotype per pregnancy taking into account 29% shrinkage factor, as calculated based on measurements of red blood cell diameters in fresh blood smears vs. those measured on the processed sections (data not shown). The total number of proliferating cells (Ki67 positive) per field of view was counted on midline sections stained with Ki67. This was performed using Image J freeware (National Institute of Health; Bethesda, MD) on captured images of 20 random fields of view per placenta (40x, newCAST software). Sinusoidal trophoblast giant cells, identified by large nuclear size, were not counted to exclude cells undergoing DNA endoreduplication from the count of proliferating cells.
Transverse sections (5 µm) of human placental biopsies were stained with H&E. Using a similar histomorphological approach described above, newCAST analysis tools were used to mask the entire placental biopsy. Counts of villous tissue and feto-placental vasculature were performed at 20x using a pre-defined point grid with 50% coverage to obtain a minimum of 250 total point counts per placental biopsy. Proportions of feto-placental vasculature were calculated as a percentage of total villous tissue counts.

**Murine feto-placental vascularity**

Feto-placental vascularity was examined in Gcm1+/- murine placentas using two imaging approaches. In one method the feto-placental vasculature was filled with X-ray opaque silicone rubber using our published methods and imaged by micro-computed tomography (micro-CT) to visualize and quantify feto-placental arteries and arterioles ≥ 50 µm in diameter. Automated vascular segmentation of micro-CT datasets as described previously, was used to determine the number of vessel segments and the total vessel length for given vessel diameters (all >50 µm, 50-100 µm, 100-300 µm, and all >300 µm). Micro-CT analysis was performed on 2-3 conceptuses per genotype from 5 pregnancies (total of N = 11 per genotype). To visualize capillaries, liquid plastic was infused into the feto-placental vasculature to prepare vascular corrosion casts using our previously published methods. Feto-placental vascular casts from two conceptuses per genotype from 3 pregnancies were imaged by scanning electron microscopy (FEI XL30; Hillsboro, OR).

**Maternal arterial pressure, proteinuria, and plasma sFlt**

Maternal heart rate and arterial blood pressure were measured in awake mice using an automated tail cuff plethysmography system (BP-2000, Visitech Systems, Apex, NC). The tail-cuff system has previously been validated and accurately reflects mean carotid arterial blood pressure as measured by chronic arterial catheter in mice. Non-pregnant measurements were taken on 3 consecutive days prior to breeding and were averaged per female. Following confirmation of a vaginal plug, measurements were obtained longitudinally (N= 10 CD1 females mated with Gcm1+/- males, N=6 CD1 females mated with CD1 males) at E13.5 and at E17.5 (late gestation). All blood pressure measurements were obtained between 9:00 AM and 11:00 AM.

Proteinuria was assessed longitudinally in the same mice across gestation (non-pregnant, E13.5 and E17.5). Urine samples were collect between 9:00 AM and 11:00 AM and stored at -20°C until further processing. Protein concentrations were measured in the urine samples (diluted 10-fold) using the Bradford Reagent and expressed relative to creatinine concentrations, as measured by the Jaffé reaction. At euthanasia, maternal blood was collected from these same mice by cardiac puncture using heparin coated syringes. Following centrifugation (1500 g for 15 minutes at room temperature), plasma was isolated and frozen at -80°C. Concentrations of sFlt were measured in plasma samples (diluted 5-fold) using a commercially available ELISA kit (R&D Systems; Burlington, ON) as per manufacturer’s directions.

**Statistical analysis**

Results for wild type littermates did not significantly differ from those of wild type conceptuses from pregnancy control breedings (i.e. CD1 x CD1 matings) by one-way ANOVA with Tukey’s post-hoc analysis (not shown). Wild type littermates from CD1 x Gcm1+/- matings were therefore used as controls for Gcm1+/- conceptuses. A Student’s t-test was used for variables measured at one gestational age, and a 2-way ANOVA when multiple gestational ages
were studied. Real time PCR data was analyzed using a Wilcoxon sign-ranked test. Maternal blood pressure measurements were analyzed using a 2-way ANOVA using paternal genotype and gestational age as factors. Linear regression analysis was performed on maternal arterial blood pressure at E17.5 in relation to number of heterozygous conceptuses per pregnancy ($R^2=$ correlation coefficient of linear regression). Linear regression analysis was also used to test the correlation between GCM1 protein expression and the proportion of fetal vasculature within villous tissue in the human placenta. Statistical significance was set at $p<0.05$. All data are presented as mean ± standard error of the mean (SEM).

REFERENCES

SUPPLEMENTAL FIGURE LEGENDS

FIGURE S1. Gcm1 and SynB expression in the placental labyrinth at E13.5 and E17.5 in Gcm1+/− (Het) compared to wild type littermate controls (Wt). (A) mRNA expression of Gcm1 and its downstream target SynB was significantly decreased in Het vs. Wt (hashed line at 1.0). (B) Western blot showing lower protein expression of GCM1 in Het vs. Wt. β-actin (BAct) was used as the loading control. * p < 0.05. Number of pregnancies for each gestational age and genotype was N = 6 in (A), N = 2 in (B).

FIGURE S2. Cell proliferation in the labyrinth at E17.5. (A) Ki67 positive cells in 20x view of the labyrinth of Gcm1+/− and (B) wild type littermate controls (Wt) as shown by brown nuclear stain (e.g. nuclei marked by arrows in 60x image in right panel of A). (C) The number of Ki67 positive cells per 20x field of view was significantly greater in Gcm1+/− vs. Wt. Number of pregnancies examined per genotype was N=5. * p<0.05.

FIGURE S3. Images illustrating increased fetal capillary density in the labyrinth at E17.5 in Gcm1+/− vs. wild type littermate controls (Wt). (A, B) Scanning electron micrographs of vascular corrosion casts of Wt and Gcm1+/− feto-placental vasculature showing increased capillary density in Gcm1+/− placenta. (B) Higher magnification view of (A). Representative images from casts of two conceptuses per genotype from 3 pregnancies are shown. (C) Increased fetal capillaries in Gcm1+/− placenta compared Wt. CD34 stains the basement membrane of endothelium brown. Representative images from 3 conceptuses per genotype from 5 pregnancies. Scale bars in (A) are 500 µm and in (B,C) they are 60 µm.
### Table S1. Primers for qRT-PCR

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<tr>
<th>Gene of interest</th>
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<th>Reverse Primer (5'→3') Sequence</th>
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