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Induction of the PPARγ-GCM1 syncytialization axis reduces sFLT1 in the preeclamptic placenta

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29 Abstract

Preeclampsia (PE) is a hypertensive disorder of pregnancy that is a major cause of maternal-fetal 30 morbidity and mortality worldwide. Severe PE (sPE) is mediated by pathology of the placental 31 32 villi resulting in repressed PIGF production and hyper-secretion of sFLT1, the net effect being widespread maternal endothelial dysfunction. Villous trophoblast differentiation is under control 33 of the PPARy and GCM1 axis which is dysregulated in sPE. We hypothesized that disruption of 34 trophoblast differentiation via the PPARy-GCM1 axis is a major contribution to excess production 35 of sFLT1 and pharmacological activation of PPARy in the sPE placenta could reduce sFLT1 to 36 normal levels. sPE, age-matched control placentas and first trimester villous explants were used to 37 investigate the molecular relationships between PPARy-GCM1 and sFLT1. We modulated this 38 pathway by pharmacologic activation/inhibition of PPARy using Rosiglitazone and T0070907, 39 40 respectively and through siRNA repression of GCM1. PPARy and GCM1 protein expressions are reduced in the sPE placenta while FLT1 protein and sFLT1 secretion are increased. GCM1 41 reduction in the first trimester explants significantly increased sFLT1 secretion, suggesting GCM1 42 43 as a key player in this pathway. Activation of PPARy restored GCM1 and significantly reduced sFLT1 expression and release in first trimester and sPE placental villi. Functional integrity of the 44 45 PPARy-GCM1 axis in the villous trophoblast is critical for normal pregnancy development and is 46 disrupted in the sPE placenta to favor excessive production of sFLT1. Pharmacologic manipulation

of PPARγ activity has the potential to rescue the anti-angiogenic state of sPE and thereby prolong
pregnancy and deliver improved clinical outcomes.

49 **Keywords:** *PPARy; GCM1; Human placenta; FLT1; Preeclampsia; Pregnancy*

50

51 Introduction

The placenta serves as a critical organ during pregnancy to support fetal growth and 52 development [1-3]. Abnormal placental development is a hallmark of several pregnancy-related 53 54 complications causing significant maternal and/or fetal morbidity and mortality, especially severe fetal forms of fetal growth restriction (sFGR) and preeclampsia (sPE) that result in stillbirth or 55 early preterm delivery [3, 4]. sPE comprises new onset of maternal hypertension after 20 weeks of 56 gestation with systemic endothelial dysfunction and critical end-organ injury involving the 57 kidneys, liver, brain and coagulation system [1, 5]. In sPE, the placenta most commonly exhibits 58 multiple histopathologic features, collectively described as maternal vascular malperfusion [4, 6]. 59 The disease begins with reduced extravillous trophoblast invasion and transformation of the 60 uteroplacental arteries, which results in chronic ischemia of the developing placental villi [6-8]. 61 62 Patients at highest risk of sPE demonstrated bilateral abnormal uterine artery Doppler and low circulating levels of placenta growth factor (PIGF) [9] and subsequently begin to express very high 63 levels of soluble fms-like tyrosine kinase 1 (sFLT1) [10, 11]. In combination, the high sFLT1/PIGF 64 65 ratio is now an established diagnostic test for PE [12, 13].

sFLT1 is a potent anti-angiogenic protein and major contributor to endothelial damage in
PE [14]. sFLT1 is a splice variant of the vascular endothelial growth factor receptor 1 (VEGFR1)
also known as FLT1. sFLT1 competitively binds to the receptor domains of vascular endothelial
growth factor (VEGF) [15] and its dimeric partner PIGF [16], preventing their interaction with the

endothelial cell surface receptors. Our group previously found that first trimester placentas secrete
higher amounts of sFLT1 by tissue weight compared to healthy term placenta, suggesting that
sFLT1 has important roles in early pregnancy [17]. Increased levels of sFLT1 throughout
pregnancy combined with lowered levels of PIGF largely mediates the systemic endothelial
dysfunction observed in PE [18-21].

75 The transcription factor, Glial cell missing 1 (GCM1) regulates villous trophoblast differentiation in human placental villi [22, 23] and analogous labyrinth formation in mice [24, 76 25]. GCM1 thereby modulates the expression of trophoblast-derived proteins involved in the 77 78 maintenance of normal pregnancy and cardiovascular function, especially the promotion of PIGF following syncytial fusion [16]. Prior reports found reduced GCM1 expression in PE placentas 79 [26] with similar repression of the downstream fusogenic partner, syncytin, that is required for 80 syncytiotrophoblast fusion to grow the continuous outer layer covering the placental villi. GCM1 81 is a strong candidate to regulate sFLT1 production in human placental villi, since a previous study 82 83 identified that heterozygous Gcm1 knockout murine placentas secrete significantly higher levels of sFLT1 [27]. In further support of this hypothesis, enhanced expression of GCM1 via anti-viral 84 drugs reduced the expression of sFLT1 in murine placentas [28]. 85

GCM1 and the nuclear steroid receptor, peroxisome proliferator activated receptor gamma (PPAR γ), are critical proteins needed for placental development and pregnancy. PPAR γ and GCM1 work as sequential partners to regulate proper villous trophoblast to syncytiotrophoblast differentiation [23, 27]. PPAR γ functions as an upstream transcriptional regulator of GCM1 [25, 29] through binding at two PPAR γ response elements in the GCM1 promoter [30]. Using the BeWo choriocarcinoma villous trophoblast cell line [30] and a first trimester placenta explant model [22], our group previously identified that pharmacological activation of PPAR γ by Rosiglitazone led to an increase in GCM1 expression and villous trophoblast differentiation. These
findings suggest that this pathway is important for normal trophoblast function and turnover.

In the past decade, PPARy has emerged as an important player in placental development 95 due to its regulatory roles in multiple cellular pathways including metabolism, nutrient balance, 96 and anti-inflammatory response pathways [31-33]. Murine studies have shown that embryonic 97 98 knockdown of PPAR γ is lethal due to gross cardiovascular and placental abnormalities [34]. Prior studies have shown that hypoxia reduces PPARy expression in the human placenta and murine 99 trophoblast stem cells [35-37]. Therefore, the prolonged hypoxic/ischemic nature of the severe PE 100 101 placenta is likely a cause for the reduced placenta expression of PPARy observed in PE [38-41]. This further imposes an abnormal villous trophoblast structure and poorly-developed feto-102 placental vasculature observed in severely preeclamptic placentas [34]. In a rodent model of PE, 103 decreased activity of PPARy was found to correlate with increased sFLT1 levels [42] and re-104 introducing PPAR γ in PPAR $\gamma^{-/-}$ murine trophoblast stem cells rescued differentiation of the 105 syncytiotrophoblast and labyrinthine trophoblast lineages by GCM1 upregulation [25]. 106

While it is established that PPARy and GCM1 are critical factors for normal placental 107 development, the potential molecular connections between PPARy, GCM1, and sFLT1 in the 108 109 normal and diseased human placenta are unclear. In the current study, we hypothesized that maintenance of the PPARy-GCM1 axis in healthy developing placental villi represses the 110 111 expression and secretion of the anti-angiogenic sFLT1, whereas this axis is disrupted in favor of 112 hyper-secretion of sFLT1. We tested this hypothesis in explanted human placental villi from first trimester or from healthy and severely-preeclamptic women to determine the expression of GCM1 113 114 and production of sFLT1, under conditions that pharmacologically activated or inhibited PPARy 115 via the drugs Rosiglitazone and T0070907, respectively.

116 **Results**

Cultured sPE placentas show increased protein expression of FLT1, increased secretion of 117 118 sFLT1 and reduced protein expression of PPARy and GCM1 compared to PTC controls. sFLT1 secretion into the placenta culture media was measured by ELISA after 48 hours of culture 119 for sPE and gestational age-matched preterm control (PTC) placentas. We observed significantly 120 higher secretion of sFLT1 from sPE placentas compared to PTC (3327±198 pg/mL vs. 2361±198 121 122 pg/mL, p=0.0067, n=5, Fig. 1A). Placental protein expression of FLT1, PPARy, and GCM1 were measured by western blotting. FLT1 protein expression was significantly upregulated in sPE 123 124 placentas comparison to PTC (0.96±0.2 vs. 0.28±0.06 relative expression values, p=0.0167, n=6, Fig. 1B) which mirrored representative immunohistochemical staining patterns showing enhanced 125 localized expression of total-FLT1 (which includes all FLT1 and sFLT1 variants) in the 126 syncytiotrophoblast layer of the sPE placenta (Figure 1E). sPE placentas showed a significant 127 reduction of PPARy protein expression (0.476±0.13 vs. 1.09±0.1, p=0.0042, n=6, Fig. 1C, F) and 128 a significant reduction of GCM1 protein expression (0.56±0.06 vs. 0.99±0.07, p=0.0001, n=14, 129 130 Fig. 1D, F) compared to PTC.

Activation of PPARy by Rosiglitazone induces GCM1 expression and lowers placental 131 132 **sFLT1 secretion in first trimester placental explants.** First trimester villous placental explants 133 were used as a model to understand how sFLT1 changes in response to modulating of the PPARy-GCM1 axis. These tissues were cultured for 18 hours with PPARy agonist, Rosiglitazone (10mM), 134 135 or antagonist, T0070907 (1mM). Successful activation of PPARy by Rosiglitazone was confirmed by a 28±5% increase in PPARy global DNA-binding activity in the Rosiglitazone treated explants 136 (128±5 vs. 100, p<0.05, n=3, Fig. 2A) as measured by an ELISA-based transcription factor binding 137 assay. There was no significance difference in PPARy DNA-binding activity in the T0070907 138

treated explants (Fig. 2A). PPARy and GCM1 protein expressions in first trimester tissues were 139 measured by western blotting. Rosiglitazone significantly increased PPARy protein expression 140 (1.3±0.09 vs 1, p=0.0446, n=4, Fig. 2B, F) and GCM1 protein expression (1.83±0.2 vs. 1, 141 142 p=0.0402, n=4, Fig. 2C, F). Rosiglitazone significantly increased GCM1 mRNA expression (2.35±0.4 vs. 1, p=0.04, n=4) while T0070907 significantly decreased GCM1 mRNA expression 143 (0.55±0.1 vs. 1, p=0.02, n=4) (Fig. 2D). Rosiglitazone significantly decreased sFLT1 secretion 144 145 into the culture media (0.57 ± 0.07 vs. 1, p=0.025, n=3, Fig. 2E). Treatment with T0070907 did not result in a significant change in sFLT1 secretion from the first trimester explants (Fig. 2E). 146

147 Silencing of GCM1 upregulates FLT1 and sFLT1 in human first trimester explants.

148 We have shown that activating PPARy in the placenta leads to a significant induction of GCM1 149 mRNA and protein expression and a significant reduction of sFLT1 secretion from the first 150 trimester placenta. Since it is already established that PPARy acts as an upstream transcriptional 151 regulator for GCM1 [30], we aimed to determine if GCM1 has a role in this potential pathway for regulation of sFLT1 secretion. We further used first trimester villous explants as a model for 152 153 siRNA-mediated repression of GCM1. GCM1 siRNA caused a significant reduction of GCM1 mRNA expression (0.49±0.09, p=0.031, n=3, Fig. 3A) and reduced GCM1 protein as compared to 154 tissues transfected with the scramble siRNA (Fig. 3C). The GCM1-silenced explants secreted 155 significantly more sFLT1 (1.59±0.13 vs. 1, p=0.0389, n=3, Fig. 3B). A representative 156 immunohistochemistry staining shows higher expression of total-FLT1 protein (FLT1 and sFLT1 157 158 variants) in the syncytiotrophoblast of the GCM1-silenced explants compared to scramble siRNA 159 and no-treatment controls (Fig. 3D).

160 Rosiglitazone restores PPARy and GCM1 expression and downregulates sFLT1 in the severe

161 **preeclamptic placenta.** To test if the PPARγ-GCM1 axis can be modulated in the sPE placenta,

we pre-treated sPE placentas with Rosiglitazone (10μ M) or T0070809 (1μ M) for 24 hours. 162 Rosiglitazone significantly increased PPAR γ protein expression in sPE (1.34±0.04 vs. 1, p=0.051, 163 n=4) and treatment with T0070907 did not have a significant effect on PPARy protein expression. 164 Rosiglitazone restored GCM1 by increasing mRNA expression the sPE placenta (1.28±0.09 vs. 1, 165 p=0.0162, n=9, Fig 4A). Treatment with T0070907 caused a significant reduction of GCM1 166 mRNA expression in the sPE placenta (0.49±0.05 vs. 1, p=0.001, n=9, Fig. 4A). Rosiglitazone 167 significantly decreased sFLT1 mRNA expression in the sPE placenta (0.655±0 vs. 1, p=0.0058, 168 n=7, Fig 4B). Treatment with T0070907 did not cause a significant change in sFLT1 mRNA 169 170 expression in the sPE placenta (Fig. 4B).

171

172 **Discussion**

In this study, we provide novel molecular evidence to demonstrate that FLT1 and its 173 secreted splice variant, sFLT1, are regulated by a PPAR_γ-GCM1 axis in the trophoblast layer 174 covering human placental villi. Under physiologic conditions, this axis mediates orderly 175 syncytiotrophoblast formation via asymmetric divisions of progenitor villous cytotrophoblasts 176 [43], which in turn promotes PIGF synthesis and release into maternal blood, and in tandem, 177 represses sFLT1 [16, 22, 23, 27]. Conversely, under sub-optimal PPARy–GCM1 signaling, the 178 179 placental villi show both structural and molecular defects characterized by defective syncytial fusion, repressed PIGF and aberrant release of sFLT1 into maternal blood [26, 38-41]. sFLT1 plays 180 a major role in PE pathology as it promotes wide-spread endothelial dysfunction which largely 181 182 contributes to the multi-organ dysfunction in the mother [7, 44, 45]. Highly secreted sFLT1 and its anti-angiogenic properties are well characterized in sPE placentas, where syncytial knot 183

formation is one of the hallmarks of the maternal-vascular malformation disease [46, 47]. While FLT1 is known to be increased in tissues during vascular reoxygenation in response to hypoxic/ischemic insult [48] which similarly occurs in the PE placenta [49], the regulatory mechanism(s) of FLT1 and sFLT1 in the placenta remains unclear.

We observed higher sFLT1 secretion and higher protein expression of FLT1 in the PE 188 189 placenta. Additionally, our immunohistochemistry staining identified that total-FLT1 protein (FLT1 and sFLT1 variants) exclusively localized to the syncytiotrophoblast in both control and 190 191 sPE placenta, and appears to have increased localization in the syncytial knots found in sPE tissue. 192 Our data validates previous findings by Taché et al., who showed a correlation between high syncytiotrophoblast sFLT1 levels and PE disease severity [46]. In addition, Jebbink et al. showed 193 syncytiotrophoblast specific localization of sFLT1 mRNA transcripts and higher sFLT1 mRNA in 194 the PE placenta [20]. Together, these data support the notion that the abnormal syncytiotrophoblast 195 layer in PE may be the major source of high secretion of sFLT1. 196

Our data shows an inverse correlation between high FLT1 expression and sFLT1 secretion 197 and low PPARy and GCM1 expression in the PE placenta. This finding aligns with previous reports 198 in the literature [23, 26, 27]. The relationship between PPARy and sFLT1/FLT1 in the placenta 199 was previously shown in a study by McCarthy et al., where a reduced utero-placental perfusion 200 pressure (RUPP) model was established to mimic PE in pregnant rats [50]. These RUPP animals 201 202 showed significantly elevated levels of sFLT1 [50]. When treated with Rosiglitazone, these animals showed decreased blood pressure and decreased levels of sFLT1 [50]. In a separate study, 203 McCarthy et al. treated pregnant rats with the PPARy antagonist, T0070907, which caused these 204 205 animals to develop PE-like symptoms such as elevated blood pressure and proteinuria accompanied by decreased VEGF and increased plasma levels of sFLT1 [42]. 206

From this finding and our observations that PPARy and GCM1 are inversely correlated 207 with FLT1 expression, we hypothesized that a molecular connection exists between PPARy, 208 GCM1, and FLT1/sFLT1 in the human placenta, via repression of syncytin-mediated syncytial 209 fusion. We used our first trimester placental explant model to test if this pathway can be modulated 210 in the placenta. We used the PPARy agonist, Rosiglitazone, and PPARy antagonist, T0070907, to 211 212 modulate this pathway. T0070907 antagonizes PPARy by selectively binding to the PPARy ligand binding pocket, preventing its activation by another ligand [51]. It is suggested that repressive 213 ligands such as T0070907 do not have an effect on PPARy protein expression levels [51], which 214 215 could explain our finding that PPARy protein expression was not significantly different in first trimester placenta or in sePE placentas when treated with T0070907. Rosiglitazone acts as a 216 chemical ligand for PPARy by increasing its DNA-binding activity to influence gene expression 217 [32] and is also shown to increase PPAR γ transcription [52]. We confirmed that Rosiglitazone 218 caused an increased in PPARy activity in the first trimester explants and this coincided with a 219 significant increase in PPARy protein expression in the first trimester explants and in the sePE 220 placenta. We observed that Rosiglitazone significantly upregulated GCM1 mRNA and 221 significantly reduced sFLT1 secretion into placental media culture in our first trimester explant 222 223 model. These results suggest that the PPAR γ -GCM1 axis has a role in regulating the angiogenic environment of the placenta via sFLT1. 224

We further questioned whether the changes observed in sFLT1 secretion were a direct result from PPAR γ activation or if GCM1 may serve as an intermediate in this pathway to modulate FLT1 expression, since PPAR γ transcriptionally regulates GCM1 through two PPAR γ response elements in the promoter region of GCM1 [30]. We show that siRNA mediated repression of GCM1 in our first trimester villous explants caused a significant upregulation of sFLT1 secretion,

as well, caused an increase in FLT1 protein expression in the syncytiotrophoblast, observed 230 through immunohistochemistry. Our findings align with previous studies that showed a 231 heterozygous knockdown of Gcm1 in the mouse led to increased secretion of sFLT1 [27]. These 232 results suggest that GCM1 may be involved in FLT1 regulation and a decrease of GCM1 233 expression may contribute to the anti-angiogenic state during PE through upregulation of sFLT1. 234 235 We lastly show that this pathway is not unique to the first trimester placenta and can be modulated in the diseased severely preeclamptic placenta. We found that activation of PPARy in sPE placenta 236 increased GCM1 and reduced sFLT1 mRNA expression. 237

238 In the current study, we show for the first time that activation of PPARy can modulate the angiogenic environment of the human placenta by altering expression of GCM1, FLT1 and sFLT1. 239 More studies are needed for better understanding of how this pathway impacts placental function 240 and physiology during the second and third trimesters of pregnancy, when the high anti-angiogenic 241 environment in PE becomes largely problematic. We acknowledge the limitations of our study, 242 such as small sample size which necessitates follow up studies with larger cohorts. We were unable 243 to observe an effect based on mode of delivery however this should be considered as a confounding 244 factor in future studies. To our knowledge, we report novel findings of PPARy, GCM1 and sFLT1 245 246 in the placenta, although we have not clearly demonstrated that a direct or indirect interaction between these molecules exist. We performed *in silico* prediction of DNA-binding sites for GCM1 247 in the promoter region FLT1 using data from HOmo sapiens COmprehensive MOdel COllection 248 (HOCOMOCO) v9 [53]. No putative binding site for GCM1 was found in the proximity of the 249 FLT1 active promoter however there are two putative GCM1 binding sites approximately ~50kb 250 from the FLT1 promoter (Supplemental Figure 3). Further studies should focus on potential long-251 range effects of GCM1 at these regions to regulate FLT1 promoter elements, co-factors or splicing 252

machinery to directly influence gene expression or protein secretion of FLT1 and its splice variants. Moreover, future studies should consider how modulating this pathway could affect overall angiogenic balance, such as through increasing secretion of PIGF and VEGF from the placenta. Detailed molecular studies investigating these targets potentially using both *in vivo* and *in vitro* models with knockdown or knockout of PPAR γ and GCM1 are needed to fully understand this potential mechanism and their effects on overall placental function.

259

260 **Perspectives**

261	Abnormal trophoblast differentiation and turnover poses a major threat to placental function in
262	preeclampsia (PE) and is driven by dysregulation of the PPARγ-GCM1 syncytialization axis. We
263	identify that high FLT1 expression localizes to the damaged syncytial membrane in the
264	preeclamptic placenta. Silencing of GCM1 in the human placenta significantly upregulates sFLT1
265	secretion and causes intense FLT1 localization to the GCM1-deficient syncytiotrophoblast, further
266	suggesting that dysregulation of villous trophoblast turnover is responsible for the high FLT1 and
267	pathologic levels sFLT1 observed in PE. Our data position PPARy proximal to GCM1 to regulate
268	its expression, as we show that induction of PPAR γ restores GCM1 while providing a physiologic
269	brake on sFLT1 production, and further affords the PPAR γ -GCM1 axis a therapeutic target status.
270	Our study highlights the importance of understanding the molecular control of villous trophoblast
271	turnover as a key for improving placental and maternal health in PE. Future directions should
272	investigate the detailed workings of how GCM1 acts to repress FLT1 and sFLT1 in the placenta.

273

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- 288 None.
- 289
- 290 Methods
- 291 The authors declare that all supporting data are available within the article and its online
- 292 supplementary files.
- 293 Tissue collection
- First trimester (10–12 weeks of gestation) placental tissues (n = 4) were obtained with written
- informed consent from healthy pregnant women undergoing elective termination of pregnancy.

The Institutional Review Board (IRB) of Wayne State University approved all consent forms and 296 protocols used in this study, which abide by the NIH research guidelines. Term placental samples 297 were obtained either by the Research Centre for Women's and Infants' Health (RCWIH) BioBank 298 program of Mount Sinai Hospital in Toronto, Canada, in accordance with the policies of the Mount 299 Sinai Hospital Research Ethics Board or Women's Health Center at Spectrum Hospital in Grand 300 301 Rapids, MI. All placentas collected were approved by the IRB waiver of parental consent. Specimens were collected from age-matched idiopathic preterm without histological evidence of 302 chorioamnionitis not complicated by PE (PTC) (n = 14; gestational age = 31-39 weeks), and 303 304 pregnancies complicated by severe PE (n = 14; gestational age = 31-37 weeks) and were delivered either by Cesarean section or vaginal birth. Inclusion criteria for severe PE was in accordance with 305 current guidelines including blood pressure > 160/110 mm Hg on two occasions longer than 6 hr 306 apart, evidence of end-organ damage including proteinuria, with or without fetal growth restriction 307 [54]. 308

309 Explant culture

For term tissues, a standardized random sampling protocol was applied dissecting random four 310 1 cm³ cuboidal sections to avoid sampling bias. The collected tissues were washed and transported 311 to the laboratory in ice cold HBSS (Hank's Balanced Salt Solution) and processed within a 312 maximum of 2 hr after delivery. On arrival, tissues were rinsed in chilled HBSS to remove residual 313 blood and further dissected under a stereomicroscope to remove placental membranes and generate 314 20-30mg pieces of villous tissues for culture. First trimester explants were cultured according to 315 our previously published floating villous explant protocol [55]. Individual clusters of villous trees 316 317 were dissected under a stereomicroscope. Post dissection, the explants were cultured overnight in 500 uL of Dulbecco's modified Eagle's medium/Ham's F-12 nutrient mixture (DMEM/F-12; 1:1; 318

Life Technologies; Grand Island, NY) containing 10% fetal bovine serum (FBS; Life 319 Technologies) and 1% GibcoTM antibiotic-antimycotic. Term explants were maintained overnight 320 at 8% O2 with 5% CO2 at 37°C [56]. After an overnight culture, the tissues were treated with 321 10µM Rosiglitazone (Selleckchem) or 1µM T0070907 (R&D Systems) dissolved in dimethyl 322 323 sulfoxide (DMSO, Sigma Life Sciences) for 18-24 hours. DMSO alone was used as a vehicle control. Comparison of DMSO to NT (not treated placental tissues) was performed to ensure there 324 325 was not an effect from DMSO. After the culture period for each treatment, replicates were snap frozen for protein and RNA extraction and stored at -80°C. The media was also collected, snap 326 frozen and stored at -80°C. In a few samples, an extra replicate was immediately fixed in 4% 327 paraformaldehyde for immunohistochemistry. This was included in this study as a qualitative 328 assessment to complement RNA/Protein expression findings. 329

330 Protein extraction and immunoblotting

Protein extraction from tissues (20–30 mg) was performed as previously described [57]. Protein 331 concentration was determined with BCATM protein assay reagent (Thermo Fisher Scientific, 332 Rockford, IL) according to the manufacturer's instructions. Equal protein amounts (35 µg) were 333 denatured (8 min, 95°C) in Laemmli sample buffer (Bio-Rad Laboratories; Hercules, CA) and 334 separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis, with subsequent semi-335 336 dry transfer (Trans-Blot®; Bio-Rad Laboratories) to a polyvinylidene difluoride membrane. The membranes were blocked with 5% nonfat dry milk in 1× Tris-buffered saline containing 0.05% 337 Tween-20 and were incubated overnight at 4°C with anti-GCM1 (1:5,000; Aviva, San Diego, CA), 338 anti-FLT1 (1:1000, Abcam), and anti-PPARy (1:1,000; Cell Signaling Technology) primary 339 antibodies. Subsequently, membranes were incubated with horseradish peroxidase-conjugated 340 secondary antibodies for 1 hr at room temperature and were developed with Western Lightning® 341

ECL Pro (PerkinElmer, Waltham, MA). Signals were visualized using a ChemiDoc[™] Imaging
System (Bio-Rad Laboratories) and Image Lab Version 5.1 software (Bio-Rad Laboratories).
Densities of immunoreactive bands were measured as arbitrary units by the ImageJ software (NIH,
Bethesda, MD). Protein levels were normalized to a housekeeping protein β-actin (1:4,000;
Abcam). Protein expression values are reported as relative to β-actin.

347 ELISA

The media collected from PE, PTC, and first trimester placental explant cultures was assayed for 348 levels of sFLT1 using the Human VEGFR1/Flt-1 DuoSet kit (R&D Systems, Minneapolis, MN) 349 according to the manufacturer's instruction. Culture media was centrifuged at 4,500 x g for 10 350 minutes at 4°C to pellet all cell/tissue debris and the supernatant was used for ELISA analysis. The 351 352 optical density of the final-colored reaction product was measured at 450 nm using a SoftMax Pro5 or a multispectral UV/VIS (Bio-Tek, VT) plate reader. A standard curve was used to calculate 353 protein content, and this was normalized over wet weight of the explant to obtain the amount of 354 protein secreted per milligram of explant tissue. 355

356 **RNA extraction and qPCR analysis**

The tissue was lysed in Qiazol and RNA was extracted using RNeasy Plus Universal Mini kit (Qiagen, Germany) as per the manufacturer's protocol. The extracted RNA was quantified using Nanodrop and 1µg was reverse transcribed using iScript RT synthesis kit (Bio-Rad Laboratories, CA). Real-time PCR was performed on the Bio-Rad CFX384 real time system in triplicates in 10uL total reaction volume containing 10 ng of template cDNA, 5µL of SYBR-green master mix (LuminoCT, Sigma-Aldrich, MO) and 500nM of primers. The primers used for assessing the expression levels of target and housekeeping genes are outlined in Table 1. Data was analyzedusing the delta-delta CT method as described in [58].

365 **PPARy Transcription Factor Assay**

Nuclear proteins were isolated from first trimester tissue after treatment with Rosiglitazone 366 (10µM), T0070907 (1nM) and DMSO using a Nuclear Extract Kit (ActiveMotif, Carlsbad, 367 California). The assay was performed using PPARy binding assay (TransAM, ActiveMotif, 368 Carlsbad, California) following the manufacturer's protocol. Briefly, 3µg of nuclear proteins from 369 370 treatment and control groups were used. The proteins from each group were added to the provided 96 well plate (in triplicates) and volumes were adjusted to 10uL using the complete lysis buffer 371 from the kit. 5ug of given positive control and complete binding buffer containing 40pmol of the 372 373 consensus site (from the kit) was then added to each well. The plate was incubated for 1 hour followed by 3 washes with the 200uL of 1X wash buffer. 100uL of the supplied PPARy antibody 374 was then added to all wells and the plate was incubated again for 1 hour at RT. After the incubation, 375 the wells were washed again 4 times and 100uL of developing solution was then added to each 376 well and incubated for 5 mins. The reaction was stopped using 100uL of Stop solution and the 377 absorbance was read at 665nm. The absorbance for the blank wells was subtracted from all the 378 readings and then values from Rosiglitazone and T0070907 samples were normalized to the values 379 from DMSO vehicle for comparison between the treatments. 380

381 Immunohistochemistry

Immunostainings of placental villi were performed as described in [59]. Briefly, the sections were
deparaffinized and rehydrated, followed by antigen retrieval using Dako Target retrieval solution
(Agilent-DAKO, USA). The intrinsic peroxidase activity was then quenched by incubating the

sections with 3% Hydrogen peroxide (Fisher Scientific, MA) for 30 mins at RT, followed by a 385 wash with 1X PBS. The sections were then incubated overnight at 4°C with anti-FLT1 (Santa 386 Cruz, TX) or 10µg/ml nonimmune Rabbit IgG (Jackson Immunoresearch, PA) (used as a negative 387 control). The following day, the slides were washed 3 times (5 minutes/wash) with 1X PBS 388 containing 0.1% Tween 20. The samples were then incubated for 30 min with a peroxidase-389 390 conjugated polymer coupled to anti-rabbit IgG (EnVision Systems Peroxidase, Agilent-DAKO, USA). The peroxidase was visualized with 3,3-diaminobenzidine (DAB, Agilent-DAKO, USA) 391 and hydrogen peroxide for 5 min. Tissues were counterstained with hematoxylin, dehydrated and 392 393 were cover slipped. The staining was visualized using Nikon Eclipse 90i epifluorescence microscope (Nikon Inc., Japan) and the images were analyzed using ImageJ software. 394

395 siRNA-mediated GCM1 suppression

For silencing, Silencer[™] Select Pre-Designed siRNA assays (Thermo Fischer) were used. For 396 GCM1 specific knockdown, assay ID s16199 was used and a Cy™3-labeled scramble sequence 397 was used a negative control (AM4621, ThermoFisher). A non-silencing control was additionally 398 incorporated as a technical control to exclude any effects of the electroporation procedure used for 399 silencing. The tissues were electroporated using the P3 Primary Cell 4D-NucleofectorTM X Kit L 400 and the NucleofactorTM 2b device (Lonza, Switzerland) following the manufacturer's kit protocol. 401 First trimester villous explants were cultured overnight in Dulbecco's modified Eagle's 402 medium/Ham's F-12 nutrient mixture (DMEM/F-12; 1:1; Life Technologies; Grand Island, NY) 403 containing 10% fetal bovine serum (FBS; Life Technologies) and 1% Gibco[™] antibiotic-404 antimycotic at 8% O₂. On day 2, the explants were placed in the cuvette (2 explants/cuvette) along 405 406 with 100uL of electroporation solution (10uL of silencing probe mix + 90uL of electroporation buffer). The program U017 was used for electroporation after which the explants were taken out 407

and cultured in fresh media for 48 hours. After the culture period, one set of explants were
processed for immunohistochemistry by fixing in 4% paraformaldehyde and another set was
frozen in 700uL of Qiazol to be used for RNA expression studies. The media was collected and
frozen to be used later for sFLT1 analysis using ELISA.

412 Statistical Analysis

All statistical analysis was performed with GraphPad Prism 7.0 software. Raw mRNA and protein 413 expressions were normalized to respective housekeeping genes or protein. ELISA data was 414 415 normalized based on semi dry-tissue weight. Relative expression/secretion values from untreated tissues (Figure 1) were analyzed by student's t-test after determination if samples are normally 416 distributed and an F-test was applied to determine variances between groups which was then used 417 418 in the parameters for the t-test. Raw mRNA and protein expression values from tissues treated with either DMSO, Rosiglitazone and T0070907, or GCM1-siRNA and scramble siRNA (Figures 419 2-4) were normalized to respective housekeeping genes or protein. Relative expression or secretion 420 values for each tissue sets were subsequently normalized to respective DMSO (vehicle control, set 421 equal to 1) or scramble siRNA control (set equal to 1) represented by a dotted line on the graphs. 422 Groups were then analyzed by student's t-test, after determination if samples are normally 423 distributed and an F-test was applied to determine variances between groups which was then used 424 in the parameters for the t-test. p < 0.05 is considered significant and is indicated with (*) on each 425 426 graph. Data is reported as Mean±S.E.M [60]. All sample numbers are reported as per group, for example, n=6 designates 6 samples per treatment/group. 427

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- 576 **Novelty and Significance**
- 577 What Is New?
- Strong FLT1 production occurs in dysregulated areas of syncytiotrophoblast, accompanied
- 579 by low expression of PPARγ and GCM1 which regulate villous trophoblast syncytialization
- 580 in the preeclamptic placenta.
- Silencing of GCM1 in the first trimester placenta leads to an induction of sFLT1.
- We are the first to show that induction of PPARγ increases GCM1 and reduces sFLT1
- 583 expression in the placenta.
- 584 What Is Relevant?
- Restoring the PPARγ-GCM1 syncytialization axis provides a physiological 'break' on sFLT1
- 586 production from severely preeclamptic placenta.
- 587 <mark>Summary</mark>
- 588 Aberrant villous trophoblast turnover contributes to pathologic levels of sFLT1 and this can be
- 589 halted by restoration of the PPARγ-GCM1 syncytialization axis in the preeclamptic placenta.
- 590 Tables
- 591 Table 1. qPCR Primer Sequences

Gene Name	Gene Symbol	Sequence
Cytochrome - C 1	Cycl	5'-CAT CAT CAA CAT CTT GAG CC-3'

		5'-CAG ATA GCC AAG GAT GTG TG-3'
Tyrosine 3-monooxygenase	Ywhah	5'- CCG CCA GGA CAA ACC AGT AT -3'
		5'- ACT TTT GGT ACA TTG TGG CTT CAA -3'
TATA Box Binding Protein	Tbp	5'-CAC ATC ACA GCT CCC CAC CA-3'
		5'-TGC ACA GGA GCC AAG AGT GAA-3'
Glial cell missing 1	Gcm1	5'-TGA ACA CAG CAC CTT CCT C-3'
		5'-CCA CTG TAA CTA CCA GGC AAT-3'
Soluble fms-like tyrosine kinase 1	sFlt1	5'- CCT CAA ATG ATC CAC CTG CCT-3'
		5'- CAG GAA GCA CCA TAC CTC CTG -3'

592

593 **Figures**

Figure 1. Placentas from women with sPE exhibit higher sFLT1 secretion and FLT1 594 expression accompanied with lower expressions of PPARy and GCM1. sFLT1 secretion was 595 significantly higher secreted by sPE compared to gestational-age matched control (PTC) 596 597 $(2361.3\pm198pg \text{ vs. } 3326.8\pm178.8pg, n=5, p=0.0067)$ (A). This finding corresponds with higher expression of FLT1 protein in sPE compared to control (n=6, p=0.0167) (B). Immunostaining for 598 total-FLT1 (FLT1 and sFLT1 variants) shows light staining in the syncytiotrophoblast of PTC 599 600 tissues compared to a more intense signal in sPE placenta (E). Immunoblotting assessment revealed sPE placentas exhibit lower protein expression of PPAR γ (n=6, p=0.0042) and GCM1 601 (n=14, p=0.0001) compared to PTC (C, D). Representative western blots are shown in (F). 602 (Relative mRNA and protein expression were determine by normalization to housekeeping genes 603 or protein, followed by a student's t-test to determine significant differences between groups, * 604 p < 0.05. ** p < 0.01, Rosi=Rosiglitazone, T007=T0070907, bar plots and data reported are 605

606 presented mean \pm SEM, PE = preeclampsia, PTC = preterm healthy control, SN = Syncytial

607 *Knots, scale bar* = 50 μ m, *bar plots are presented as mean* \pm *SEM.*).



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Figure 2. Rosiglitazone increases PPAR γ activity and GCM1 mRNA expression while reducing sFLT1 secretion in the first trimester placenta. Treatment with Rosiglitazone caused a significant increase in PPAR γ activity (p<0.05, n=3) (A). T0070907 did not cause a significant change in PPAR γ activity (A). Rosiglitazone significantly upregulated PPAR γ (p=0.0446, n=4) and GCM1 (p=0.0402, n=4) protein expression (B, C, F). T0070907 did not cause a significant change in PPAR γ or GCM1 protein expression (B, C, F). Rosiglitazone significantly upregulated GCM1 mRNA expression (p=0.0433, n=4) (D). T0070907 significantly reduced GCM1 mRNA

expression (p=0.02, n=4) (D). Rosiglitazone also caused a significant reduction in sFLT1 616 secretion (p=0.025, n=3) (E). Antagonizing PPARy by T0070907 did not cause a statistically 617 significant change in sFLT1 secretion (E). (Relative mRNA and protein expression were determine 618 by normalization to housekeeping genes or protein. Relative expression values for individual tissue 619 620 sets were normalized to DMSO (vehicle control, dotted line, set equal to 1) and subsequent statistical analysis was performed by student's t-test to determine significant differences between 621 groups,* p<0.05, ns=p>0.05, NT=not treated, Rosi=Rosiglitazone, T007=T0070907, bar plots 622 623 and data reported are reported as mean \pm SEM).



Figure 3. GCM1 reduction increases total-FLT1 expression and sFLT1 secretion in first trimester explants. In our first trimester explant model, siRNA-mediated silencing of GCM1 caused a significant reduction of GCM1 mRNA expression (p=0.031, n=3) (A). Similarly, western blot shows GCM1 protein expression appeared to be decreased in the GCM1-silenced tissues in comparison to the scramble siRNA and no-treatment controls (C). sFLT1 secretion was significantly increased in the GCM1-silenced explants (p=0.0389, n=3) (B). Similarly, placental expression of total-FLT1 protein (FLT1 and sFLT1 variants) was induced in the

syncytiotrophoblast of the first trimester explants, after GCM1 knockdown (**D**). (*Relative mRNA* expression was normalized to housekeeping genes. sFLT1 secretion (pg/mL) data were normalized based on tissue weight. Relative expression/secretion values for individual tissue sets were normalized to the scramble siRNA control (dotted line, set equal to 1) and subsequent statistical analysis was performed by student's t-test was performed to determine significant differences between groups, NT=not treated, * p<0.05, ns=p>0.05, bar plots are reported as mean ± SEM).



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expression significantly reduced in the sPE placenta after treatment with T0070907 (p=0.001, n=9)
(C). sFLT1 mRNA was significantly reduced in the sPE placenta after Rosiglitazone treatment.
No significant change in sFLT1 mRNA expression was observed after exposure to T0070907 (B).

646 (Relative mRNA/protein expressions were determined by normalization to respective 647 housekeeping genes/protein. Relative expression values for individual tissue sets were normalized 648 to DMSO (vehicle control, dotted line, set equal to 1) and subsequent statistical analysis was 649 performed by student's t-test to determine significant differences between groups, *p<0.05, 650 *p<0.01, ns=p>0.05, NT= not treated, Rosi=Rosiglitazone, T007=T0070907, bar plots are 651 reported as mean ± SEM).



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