PPARγ controls pregnancy outcome through activation of EG-VEGF: new insights into the mechanism of placental development

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Garnier V, Traoulsi W, Salomon A, Brouillet S, Fournier T, Winkler C, Desvergne B, Hoffmann P, Zhou QY, Coniglio C, Onnis V, Benharouga M, Feige JJ, Alfaidy N. PPARγ controls pregnancy outcome through activation of EG-VEGF: new insights into the mechanism of placental development. Am J Physiol Endocrinol Metab 309: E357–E369, 2015. First published June 16, 2015; doi:10.1152/ajpendo.00093.2015.—PPARγ-deficient mice die at E9.5 due to placental abnormalities. The mechanism by which this occurs is unknown. We demonstrated that the new endocrine factor EG-VEGF mediates part of PPARγ effects on placental development. Three approaches were used: 1) in vitro, using human primary isolated cytотrophoblasts and the extravillous trophoblast cell line (HTR-8/SVneo); 2) ex vivo, using human placental explants (n = 46 placentas); and 3) in vivo, using gravid wild-type PPARγ+/− and PPARγ−/− mice. Major processes of placental development that are known to be controlled by PPARγ, such as trophoblast proliferation, migration, and invasion, were assessed in the absence or presence of PROKR1 and PROKR2 antagonists. In both human trophoblast cell and placental explants, we demonstrated that rosiglitazone, a PPARγ agonist, 1) increased EG-VEGF secretion, 2) increased placental EG-VEGF and its receptors mRNA and protein expression, 3) increased placental vascularization via PROKR1 and PROKR2, and 4) inhibited trophoblast migration and invasion via PROKR2. In the PPARγ−/− mouse placentas, EG-VEGF levels were significantly decreased, supporting an in vivo control of EG-VEGF/PROKRs system during pregnancy. The present data reveal EG-VEGF as a new mediator of PPARγ effects during pregnancy and bring new insights into the fine mechanism of trophoblast invasion.

human pregnancy; peroxisome proliferator-activated receptor-γ knockout; endocrine gland-derived vascular endothelial growth factor, trophoblast invasion

DURING EARLY PREGNANCY, two key processes of placental development control its growth and the establishment of the fetomaternal circulation (24). The former one occurs early on during pregnancy to ensure the growth and the branching of the newly formed vasculature (11). The second one establishes the fetomaternal circulation and is achieved by specialized placental cells, the cytotrophoblasts (CT). By the end of the first trimester of pregnancy, CT that are present at the anchoring villi generate multilayered columns of highly invasive cells called extravillous trophoblasts (EVT). EVT invade and transform maternal spiral arteries from high- to low-resistant vessels (38). Failure in these processes is known to be associated with the development of pregnancy pathologies such as early pregnancy loss, fetal growth restriction (FGR), and preeclampsia (PE) (43).

Among the factors reported to control these processes is the nuclear receptor peroxisome proliferator-activated receptor-γ (PPARγ) (16). PPARγ is abundantly expressed in the CT and syncytiotrophoblast (ST) of the human placenta as well as in the labyrinthine zone of the rodent placenta (4, 17). DNA binding of PPARγ to its PPAR response elements requires heterodimerization with another nuclear receptor, the retinoid X receptor (RXR) (41). Genetic studies showed that PPARγ-deficient mice die on day 9.5 of gestation because of placental abnormalities with defects in trophoblast differentiation and in vascular development (4, 26). The demonstration that placental defects were the unique cause of PPARγ-deficient mice came from the “Sox2Cre/PPARγfl/fl” mouse model, a fully viable PPARγ-null mouse generated by specific and total epiblastic gene deletion (35). Nevertheless, the mechanism by which PPARγ controls these processes is still unknown.

We have shown recently that a specific placental angiogenic factor named EG-VEGF (endocrine gland-derived vascular endothelial growth factor), or prokineticin-1, controls the same processes as those described for PPARγ, i.e., inhibition of trophoblast invasion during the first trimester of pregnancy and involvement in the development of placental vasculature (9, 23), suggesting that some of PPARγ’s effects might be mediated by EG-VEGF.

EG-VEGF is highly expressed in the ST layer during early pregnancy (21), acts via two G protein-coupled receptors, prokineticin receptor (PROKR) 1 and PROKR2 (30, 32), and controls both villi growth and the establishment of the fetomaternal circulation (6–8, 23). Within the villi, EG-VEGF in-
creases endothelial proliferation, migration, tube-like formation, and permeability as well as trophoblast proliferation and survival (6–8). At the extravillous side, EG-VEGF is rather an inhibitor of early trophoblast invasion (23). In relation to pregnancy pathologies, we have demonstrated that EG-VEGF levels were increased both in PE and FGR, suggesting that EG-VEGF deregulations might be associated with the development of pregnancy pathologies (7, 8, 23).

Because of the similarities of action between EG-VEGF and PPARγ to control placental development, we hypothesized that EG-VEGF might mediate part of PPARγ functions during early pregnancy.

Three approaches were used to verify this hypothesis: 1) an in vitro approach using human primary isolated cytotrophoblasts and the EVT cell line (HTR-8/SVneo), 2) an ex vivo approach using the human placental explant model, and 3) an in vivo approach using wild type, PPARγ+/−, and PPARγ−/− mouse placentas.

**PATIENTS AND METHODS**

**Patients and Tissues**

Placentae were obtained from 46 singleton pregnancies after elective terminations of pregnancies. First-trimester human placentas from 8 to 10 wk of gestation (wg) were used. Human tissues were collected at the Grenoble University Hospital. Collection and processing were approved by the relevant commission of the Canton of Vaud (Switzerland). Because PPARγ−/− mice die at 9.5 days postcoitum (dpc), gravid mice were euthanized at 8.5 dpc. A binocular microscope was used to dissect embryos for DNA sequencing and to discard maternal blood cells. Embryonic genotyping was performed by PCR amplification using the primers GACCCAGCTCTACAACAGGC (forward) and GGGACAGACCTGCTGCTG (reverse) consisting of 95°C for 15 s, with temperature indicated in Table 1 for each PCR experiment. PCR cycling was performed on 106 cells by 10.22±0.33.4 on June 25, 2017 http://ajpendo.physiology.org/ Downloaded from

**Table 1. Primers used for real-time RT-PCR**

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward (5’ to 3’)</th>
<th>Reverse (5’ to 3’)</th>
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<tbody>
<tr>
<td>hEG-VEGF</td>
<td>AGG TCC CCT TCT TCA GGA AAC G</td>
<td>TCC AGG CTG TGC TCA GGA AAA G</td>
</tr>
<tr>
<td>hGAPDH</td>
<td>ACC CAG AAG ACT GTG CAT GG</td>
<td>TTC TAG ACG GCA GGT CAT GT</td>
</tr>
<tr>
<td>mEG-VEGF</td>
<td>TG AGG AAA GUC CAA CAC CAT</td>
<td>CC GGG AAC CTT GAG CAC</td>
</tr>
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hEG-VEGF, human endocrine gland-derived vascular endothelial growth factor; hGAPDH, human GAPDH; mEG-VEGF, murine EG-VEGF.

**Mouse Models**

Wild-type (WT), PPARγ−/−, and PPARγ+/− placentas were collected from female PPARγ+/− mice mated with male PPARγ+/− mice, as described previously (40). Animal experimentation were approved by the relevant commission of the Canton of Vaud (Switzerland). Because PPARγ−/− mice die at 9.5 days postcoitum (dpc), gravid mice were euthanized at 8.5 dpc. A binocular microscope was used to dissect embryos for DNA sequencing and to discard maternal decidua for further molecular analysis of placental tissues. Embryonic genotyping was performed by PCR amplification using the primers GACCCAGCTCTACAACAGGC (forward) and GGGACAGACCTGCTGCTG (reverse), which amplified a product of 700 bp derived from the PPARγ WT allele. Placentas were used for RT-PCR analyses for EG-VEGF PROKR1 and PROKR2 expression. For each group four different PPARγ+/− gravid mice were used, and for each gravid mice all placentas were collected for immunohistochemistry and RT-quantitative (q)PCR analysis.

**Culture Models**

**Placental explant culture and treatment.** Villous explant cultures were established from first-trimester human placentas at 8–10 wg. Small fragments of placental villi (30–40 mg wet wt) were placed into 48-well plates precoated with 150 μl/well of diluted Matrigel (Matrigel/DMEM-F-12, 1:1; Becton-Dickinson, Le Pont-de-Claix, France) and polymerized at 37°C for 30 min. Explants were cultured in DMEM Ham’s F-12 medium (Invitrogen, Cergy Pontoise, France) supplemented with 100 μg/ml streptomycin and 100 U/ml penicillin. After 24 h of culture the medium was changed, and the explants were incubated in the absence or presence of 1 or 10 μM rosiglitazone (a PPARγ agonist, Sigma-Aldrich) for 30 min to 24 h and 4 μM T0070907 (a PPARγ antagonist) for 8 h. For each placenta, three explants were used for each determination. For statistical analysis, the n value represents the number of placentas, not explants. In some experiments, explants were treated with the following compounds: 5,6-dichloro-1-β-d-ribofuranosylbenzimidazole (DRB; 50 μg/ml) a potent RNA polymerase inhibitor for 24 h.

**Isolation and purification of villous cytotrophoblasts.** Villous cytotrophoblast cells (VCT) were isolated from first-trimester chorionic villi by differential trypsin digestion according to previous published protocols (20, 25, 45). After Percoll gradient fractionation, cells were diluted to a concentration of 105 cells/ml in DMEM supplemented with 10% FCS, 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin and plated on 35-mm (3 ml/3×106 cells) or 60-mm (8 ml/8×106 cells) culture plates (Techno Plastic Products). VCT were incubated overnight in 5% CO2 at 37°C and washed three times to eliminate nonadherent cells. VCT cultures were characterized by examining expression of cytokeratin 7, a trophoblast cell marker (95% positive cells), and by the observation of cell aggregates and ST at 48 and 72 h, respectively. After overnight incubation and washes, cells were treated for 24 h with 10 μM rosiglitazone. Conditioned media from primary cultures were collected and centrifuged and the supernatants stored at −20°C until use.

**HTR cell line culture.** The HTR-8/SVneo cells were produced by immortalization of HTR-8 cells, an EVT cell line, with the SV40 virus (18). These cells were generously given to us by Dr. C. Graham (Kingston, ON, Canada). Cells were used between 24 and 30 passages and grown in RPMI 1640 supplemented with 5% fetal bovine serum (FBS), penicillin-streptomycin, and amphotericin B (Invitrogen, Cergy Pontoise, France). Cells were maintained at 37°C in an atmosphere of 5% CO2 in air.

**EG-VEGF ELISA**

EG-VEGF secretion was measured by ELISA (PeproTech) in the collected media. Two separated standard curves were constructed to allow accurate readings of samples at upper and lower ranges of the assay. The intra-assay coefficient of variability (CV) was 6.7%, and the interassay CV was 8.1%. Detection limit was 16 pg/ml.

**RNA Isolation and Real-Time PCR Analysis**

Total RNA was extracted from placental explants or mice placentas using Nucleospin RNA II (Macherey-Nagel). Reverse transcription was performed on 1 μg of total RNA (Invitrogen). Primers used are reported in Table 1. EG-VEGF and GAPDH mRNA expressions were quantified by real-time RT-PCR using a Bio-Rad CFX96 apparatus and GoTag qPCR Master Mix (Promega, Madison, WI). PCR conditions were as follows: step 1, 94°C for 10 min; step 2, 45 cycles consisting of 95°C for 5 s, with temperature indicated in Table 1 for 30 s, and 72°C for 10 s. The results were normalized to GAPDH.

**Immunohistochemistry**

Placental explants from 8 to 10 wg were collected and fixed for 24 h at 4°C in 4% (vol/vol) paraformaldehyde, embedded in
paraffin, and cut into 5- 

Western Blot Analysis

Placental explants collected from at least six different first-trimester placentas were homogenized in RIPA lysis buffer and protease inhibitors, as described previously (2). Protein extracts were electrophoretically separated on 0.1% sodium dodecyl sulfate-12% polyacrylamide gels and electrically transferred onto 0.45- 

adjacent sections were stained using specific antibodies, and the avidin-biotin immunoperoxidase detection method was applied. Endogenous peroxidase activity was quenched by pretreatment with 3% (vol/vol) hydrogen peroxide in methanol for 30 min. Polyclonal rabbit antibodies were used to detect EG-VEGF, PROKR1, and PROKR2 (Covalab, Lyon, France). These antibodies were used previously and characterized (6, 9, 21–23). CD31/PECAM and cytokeratin were detected using commercial antibodies (Dako, Trappes, France). For immunohistochemical detection, antibodies were incubated with the tissue sections for 18 h at 4°C and used at final concentrations of 0.33 

Fig. 1. Peroxisome proliferator-activated receptor-γ’s (PPARγ) effect on endocrine gland-derived vascular endothelial growth factor (EG-VEGF) secretion. A: EG-VEGF secretion levels in placental explants after treatment with 1 and 10 

Wound-Healing Assay

Wound-healing assay was performed using HTR-8 cells. Cells were seeded in complete medium (RPMI 5% FBS) at a density of 2 x 10^5 cells/well into 24-well plates. At confluence, complete medium was replaced by serum-free medium in the absence or presence of
rosiglitazone, PROKR2 antagonist, or rosiglitazone plus PROKR2 antagonist. Cells were scratched with a sterile tip to create an artificial wound and allowed to heal for the next 24 h. Photographs were taken at regular time intervals (0, 9, and 24 h). The size of the wound was measured on photographs from three separate experiments. The closing of the wound was analyzed using Scion Image software (version 4.0.2). The results are presented as percentage of wound closure after 9 h of treatment.

**Invasion Assay**

This test was used for HTR-8/SVneo and the explant model. HTR-8 invasion. HTR-8 cells were stained with the Vybrant DiI Cell-Labeling Solution (Invitrogen) for 1 h at 37°C. The top chamber of 8-µm pore size FluoroBlok cell culture inserts (BD Biosciences) were precoated with 100 µl of 1:25 Matrigel (BD Biosciences); 2 × 10³ Dil-stained HTR cells/insert were seeded in 500 µl of RPMI 1640 medium with 1% FBS. The inserts were placed into 24-well plates containing 750 µl of RPMI 1640 medium with 15% FBS. Cells were treated with 10 µM rosiglitazone and with 1 µM PROKR1 or 1 µM PROKR2 antagonists or both PROKR1 and PROKR2 antagonists for 24 h. The chambers were then removed and fixed with paraformaldehyde. The membranes of the chambers were excised and placed on glass slides. The cells that invaded the chamber were visualized under the microscope and counted.

**Placental explants**

One-hundred fifty microliters of ice-cold Matrigel (BD Biosciences, Grenoble, France) was layered into each well of the 48-well plates and allowed to solidify completely at 37°C for 1 h. Placental explants were added and incubated at 37°C for 4 h. Ten micromolars rosiglitazone was added at the time of plating. Daily observations were made under an inverted photomicroscope to document the invasion process.

**PROKR1 and -2 cDNA Cloning and BHK Cell Line Transfection**

Both PROKR1 and PROKR2 cDNAs were cloned from a human lung cDNA library, fused to an HA tag, and inserted into the PCDNA3.1 eucaryotic plasmid. The plasmids HA-PROKR1 and HA-PROKR2 were transfected into BHK-21 (CCL-10; American Type Culture Collection). BHK cells were cultured in DMEM-F-12 culture medium supplemented with 5% FBS and methotrexate (0.4 mg/ml) as a selective agent.

**Validation of PROKR1 and PROKR2 Antagonists Using the Wound-Healing Assay**

BHK cells overexpressing PROKR1 or PROKR2 were subjected to a wound-healing assay (protocol described above) to examine the effects of these antagonists on the cell motility (6, 23). Cells were treated with EG-VEGF in the absence or the presence of PROKR1 antagonist ([12-5-(4-fluorobenzyl)-1-(4-methoxybenzyl)-1,4,5,6-tetrahydro-4,6-dioxo-1,3,5-triazin-2 ylamino-ethyl]guanidine) (1 µM) (12), a PROKR2 antagonist (1 µM), or both (9 h at 37°C) (15). For each cell line tested, the experiment was repeated three times.

**Statistical Analysis**

Statistical comparisons were made using Student’s t-test and one-way ANOVA. All data were checked for normality and equal variance. When normality failed, a nonparametric test followed by Dunn’s or Bonferroni’s test was used. (SigmaPlot and SigmaStat, Jandel Scientific Software). All data are expressed as means ± SE (P < 0.001, 0.01, and 0.05).

**RESULTS**

**PPARγ’s Effect on EG-VEGF Secretion**

Placental explants were incubated in the absence or the presence of rosiglitazone for 24 h. Secreted EG-VEGF was measured in conditioned media by ELISA. Figure 1A shows a dose response effect of rosiglitazone and a time course effect at 30 min, 3 h, 8 h, and 24 h. Rosiglitazone significantly increased EG-VEGF secretion from 8 h of incubation at both concentrations tested (1 and 10 µM). To confirm that the observed effect was specific of rosiglitazone, we treated human placental...
explants by the antagonist T0070907 in the absence or the presence of rosiglitazone. Figure 1B shows that T0070907 significantly reversed rosiglitazone’s effect on EG-VEGF secretion. Importantly, we observed that T0070907 also decreased the basal EG-VEGF secretion, suggesting that EG-VEGF is endogenously regulated by PPARγ. Because EG-VEGF is expressed mainly in the ST layer, we determined the direct effect of rosiglitazone on its secretion by this cell type. Isolated primary cytotrophoblast cells were cultured for 72 h to form a syncytium and then treated with rosiglitazone. Figure 1C shows that rosiglitazone significantly increased EG-VEGF secretion by ST cells.

**PPARγ’s Effect on EG-VEGF Expression**

To determine whether PPARγ increased EG-VEGF expression in the human placenta, we determined the effect of rosiglitazone on its expression at the mRNA level. Figure 2A shows that rosiglitazone significantly increased EG-VEGF mRNA after 24 h of treatment. This effect was transcriptional since it was abolished in the presence of DRB, an inhibitor of transcription (Fig. 2B). To confirm the effect of rosiglitazone on EG-VEGF mRNA level, we compared its protein levels by Western blotting analysis in control vs. rosiglitazone-treated explants. Figure 2, C and D, shows that rosiglitazone significantly increased EG-VEGF protein expression in the human placental explants.

**Effect of RXR Activation on EG-VEGF Expression**

Because one of the four possible types of regulation by the PPARγ system is its heterodimerization with RXRα (3), we wondered whether RXRα activation had any effect on EG-VEGF expression. Placental explants were incubated in the absence or the presence of Ro25, a pan-RXR agonist (4 μM), and/or Ro26, a pan-RXR antagonist (4 μM). There was no effect independent of synergistic effect of these compounds on EG-VEGF secretion (data not shown).

**Effect of PPARγ on PROKR1 and PROKR2 Expression**

Because of the significant effect of PPARγ activation on EG-VEGF expression in placental explants, we wondered whether this key transcription factor also regulates the expression of EG-VEGF receptors PROKR1 and PROKR2. Figure 3 shows the effect of PPARγ activation on PROKR1 and PROKR2 expression at the protein level in the placental explants. Figure 3A shows that PPARγ activation increased PROKR2 (images c and d) but not PROKR1 (images a and b) expression. Figure 3A, images e and f, shows negative controls for PROKR1 and PROKR2 stainings, respectively. This was substantiated by Western blotting analysis, where we observed a significant increase in PROKR2 but not PROKR1 expression. PROKR1 and PROKR2 belong to GPCR protein family and appear on the blots as two to three glycosylated forms, respectively (Fig. 3, B and C).

**PPARγ’s Effect on Villi Growth**

To get more insights on the role of PPARγ activation on the growth of placental villi, we determined the effect of PPARγ activation on trophoblast proliferation within the villi. It was particularly relevant to study PPARγ activation’s effects on trophoblast proliferation in a system in which the villous tissue architecture is maintained. Placental explants in culture preserve the topology of intact villi and
closely mimic the formation of anchoring villi occurring in vivo (10, 23). PPARγ activation’s effects on trophoblast proliferation were assessed using Ki-67 staining. Figure 4A shows representative sections of placental villi (images a and c) or placental columns (images b and d) that have been incubated in the absence (images a and b) or presence of rosiglitazone (images c and d) and stained for Ki-67. There was a significant increase in the number of Ki-67-positive cytotrophoblasts in chorionic villi but also a significant increase in the number of proliferative CT in the placental columns. The quantification of three independent experiments indicated significant increases in the proliferation rate within the chorionic villi and the placental columns (Fig. 4B). To further quantify the effect of rosiglitazone on the proliferation process, we compared the levels of PCNA expression between control and rosiglitazone-treated explants. There was a significant increase in PCNA expression in the rosiglitazone condition compared with the control one (Fig. 4C).

Because the microvascular system within the placental villi drives the growth and the development of this unit during the first trimester of pregnancy (13, 14, 47), we wondered whether PPARγ activation also affected this aspect of placental growth. Figure 4D shows placental explants that have been incubated in the absence or presence of rosiglitazone (10 μM) and stained for an endothelial cell marker, CD31/PECAM. Rosiglitazone treatment markedly increased CD31 staining, suggesting an increase in the vascularization within the placental villi. In the rosiglitazone-treated placental explants, we observed larger blood vessels compared with vessels present within the control placental villi. There was a significant increase in the total

![Fig. 4](http://ajpendo.physiology.org/)

**Fig. 4.** PPARγ increases trophoblast proliferation and placental vascularization. A: Ki-67 staining in placental explants treated or not with Rosi (10 μM). Images a and b show Ki-67 staining in control placental villi and placental column, respectively. Images c and d show the staining under Rosi treatment. B: %Ki-67-positive cytotrophoblast cells quantified in 3 independent experiments (n = 6 control and n = 6 Rosi). Data represent %triplicates. Scale bar, 50 μm. C: Western blotting analysis of PCNA expression in control and Rosi-treated placental explants (n = 6 control and n = 6 Rosi). D: Representative images of CD31 staining in placental explants treated (image a) or not (image b) with Rosi (10 μM). Images c and d are the skeletonization of images a and b, respectively. Black color represents the vascular network. Image f (bar graph) represents the %area occupied by capillaries. Six placental explant images were analyzed for each condition. E: Representative Western blot analysis that compares CD31 protein levels in control placental explants vs. explants treated with 10 μM Rosi. Quantification of the intensity of the bands is illustrated below. Pro-Evt, proliferative extravillous trophoblasts; Bv, blood vessel. *P < 0.05; **P < 0.01; ***P < 0.001.
capillary length within the villi of rosiglitazone-treated explants compared with the control ones (Fig. 4D, images a–f). Figure 4E shows a Western blot analysis that compared CD31 expression in control and treated explants. There was a significant increase in CD31 protein levels in the treated explants, confirming the in situ increase observed in placental sections.

In Vitro Validation of PROKR1 and PROKR2 Antagonists

EG-VEGF has been shown to inhibit the migration of different cell types (6, 19, 36); we used the wound-healing assay to verify the specificity of PROKR1 and PROKR2 antagonists in BHK cells overexpressing either PROKR1 (BHK-R1) or PROKR2 (BHK-R2) proteins. In BHK-R1 cells, we demonstrated that EG-VEGF decreased BHK migration and that this effect was abolished in the presence of the PROKR1 antagonist (Fig. 5, A and B) but not in the presence of the PROKR2 antagonist (Fig. 5, C and D). Inversely, in BHK-R2 cells, the EG-VEGF effect was abolished in the presence of the PROKR2 antagonist (Fig. 5, E and F) but not in the presence of the PROKR1 antagonist (Fig. 5, G and H).

EG-VEGF Mediates PPARγ’s Effect on Placental Vascularization

To determine whether EG-VEGF intervenes in the control of PPARγ-mediated CD31 increase, we tested the effect of rosiglitazone in the absence or the presence of EG-VEGF receptor antagonists. Figure 6, A and C and B and D, show

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Fig. 5. Characterization of PROKR1 and PROKR2 inhibitory effects in BHK cells overexpressing PROKR1 receptor or PROKR2. A: images of wounded BHK-R1 monolayers at 0 and 9 h postwounding in the absence or presence of PROKR1 antagonist. B: plots showing percentages of wound closure after 9 h of treatment with EG-VEGF in the absence or presence of PROKR1 antagonist (1 μM). Bars with different letters are significantly different from each other (P < 0.05; n = 3). C: images of wounded BHK-R1 monolayers at 0 and 9 h postwounding in the absence or the presence of PROKR2 antagonist. D: plots showing percentages of wound closure after 9 h of treatment with EG-VEGF in the absence or presence of PROKR2 antagonist (1 μM). Bars with different letters are significantly different from each other (P < 0.05; n = 3). E: images of wounded BHK-R2 monolayers at 0 and 9 h postwounding in the absence or the presence of PROKR2 antagonist. F: plots show percentages of wound closure after 9 h of treatment with EG-VEGF in the absence or the presence of PROKR2 antagonist (1 μM). Bars with different letters are significantly different from each other (P < 0.05; n = 3).

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*Fig. 4* shows a Western blot analysis that compared CD31 expression in control and treated explants. There was a significant increase in CD31 protein levels in the treated explants, confirming the in situ increase observed in placental sections.
that both PROKR1 and PROKR2 antagonists, respectively, decreased rosiglitazone’s effects on CD31 protein expression significantly. Altogether, these data suggest that EG-VEGF might mediate some of PPARγ’s activation effects on the placental endothelium during the first trimester of pregnancy.

**EG-VEGF Mediates PPARγ’s Effect on Trophoblast Invasion**

Previous studies have shown that PPARγ activation controls trophoblast invasion using in vitro models (46); however, a demonstration of this regulation in an ex vivo model such as the explant model has not been performed. Using the placental explant model, we examined the effect of rosiglitazone on trophoblast invasion. Figure 7A shows that rosiglitazone significantly inhibits invasion of EVT into the matrigel within 72 h of culture. This result confirms the inhibition of the invasive process upon PPARγ activation in trophoblast cells.

Because trophoblast invasion plays a major role in the process that establishes the fetomaternal circulation, we hypothesized that this might also involve angiogenic factors such as EG-VEGF. To verify this hypothesis, we used the HTR-8/SVneo cell. First, we determined the effect of rosiglitazone on PROKR2 expression, a receptor subtype that mediates EG-VEGF’s effects on trophoblast invasion (23). Figure 7B shows that rosiglitazone increased PROKR2 protein levels significantly, confirming the effect observed in whole placental explants. To demonstrate whether EG-VEGF is involved in the PPARγ effect on trophoblast invasion, we determined the percentage of invasion of prelabeled HTR-8/SVneo cells through Matrigel using the BD FluoroBlok cell culture inserts. The effect of rosiglitazone was also tested in the presence of the PROKR2 antagonist. Figure 7C shows that rosiglitazone significantly decreased HTR-8/SVneo cell invasion and that this effect was reversed in the presence of the PROKR2 antagonist, suggesting that part of the rosiglitazone effect on HTR-8/SVneo invasion might be mediated by the EG-VEGF signaling pathway. Quantification of the number of invading cells in each condition is reported in Fig. 7D.

**EG-VEGF Mediates PPARγ’s Effect on Trophoblast Migration**

Since we showed that EG-VEGF contributes to rosiglitazone’s inhibitory effects on trophoblast invasion, we wondered whether it also mediates rosiglitazone’s effects on trophoblast migration. Hence, we examined EG-VEGF’s effect on the migration of HTR-8/SVneo cells. Figure 8A shows representative photographs of HTR-8/SVneo monolayers at 0 and 9 h after wounding with a pipet tip and subsequent incubation in the absence or presence of rosiglitazone, PROKR2 antagonist, or rosiglitazone plus PROKR2 antagonist. At 9 h of culture, the wound in the control condition was closed at 30%. Rosiglitazone inhibits this process that was reversed in the presence of PROKR2 antagonists. Quantification of three independent experiments is reported in the Fig. 8B.

**EG-VEGF Expression Is Decreased in PPARγ-Knockout Mice**

PPARγ knockout mice (PPARγ−/−) die at 9.5 dpc from failure in placental development and vascularization (4, 26). We compared the levels of expression of EG-VEGF in placentas collected at 8.5 dpc from WT (PPARγ+/+), PPARγ+/−, and PPARγ−/− mice. Figure 9A shows comparisons of the mRNA levels of EG-VEGF in the three groups. There was a significant decrease in EG-VEGF mRNA levels in the PPARγ−/− compared with the WT mice and a trend toward a decrease in the PPARγ+/− mice. We then examined EG-VEGF expression at the protein level by immunohistochemistry. Figure 9B shows representative stainings for EG-VEGF in WT and PPARγ−/− placentas collected at 8.5 dpc. In the WT placentas, EG-VEGF protein was highly expressed in the ectoplacental cone (Ec) and decidua (Fig. 9B, images b, d, and f). In the PPARγ−/− placenta, EG-VEGF staining was decreased both in the Ec and the decidua (images k, m, and o). In the Ec, strong staining was observed in the trophoblast giant cells (Tgc) of the WT placenta that

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Fig. 6. EG-VEGF mediates PPARγ’s effect on placental vascularization. A and B: representative Western blot analyses that compare CD31 protein levels in control placental explants (A) vs. explants treated with 10 μM Rosi (B) in the absence or presence of PROKR1 antagonist (1 μM), PROKR2-antagonist (1 μM), or both during 24 h. C and D: quantification of the intensity of the bands is illustrated (n = 6). Standardization of protein signals was done with antibodies against β-actin protein (P < 0.05). Values overwitten with different letters are significantly different from each other.
was decreased in the PPARγ−/−. Fig. 9B, images a, c, and e and images j, l, and n show cytokeration stainings of the WT and PPARγ−/− placentas, respectively. Figure 9B, images g–i and p–r, shows negative controls for the EG-VEGF staining.

DISCUSSION

The present study demonstrates a new regulation of EG-VEGF and its receptors by the major nuclear receptor PPARγ and brings new insights into the mechanism by which PPARγ might affect placental development, a main cause of PPARγ−/− lethality. These statements were based on the following findings: 1) PPARγ increased EG-VEGF secretion and its mRNA and protein expression in both primary human trophoblast cells and placental explants, 2) PPARγ increased PROKR2 expression in placental explants, 3) placental growth controlled by PPARγ is in part mediated by EG-VEGF via the activation of PROKR1 and PROKR2, 4) trophoblast invasion controlled by PPARγ is in part mediated by EG-VEGF via PROKR2, and 5) PPARγ−/− placentas exhibited decreased expression of EG-VEGF compared with WT placentas.

The demonstration that EG-VEGF mediates some of the PPARγ effects is not unexpected, as both proteins have been reported to play key roles in placental development during early pregnancy and to control similar processes (4, 7, 45). Interestingly, it has been reported that PPARγ activation by rosiglitazone reversed adverse nicotine effects on the ovary vascularization by increasing EG-VEGF expression (37).

RXRα occupies a central position in the nuclear receptor superfamily, although its activation or inhibition by specific ligands did not affect EG-VEGF expression, suggesting that only endogenous PPARγ ligands and not RXR ligands are potential activators of EG-VEGF in the placenta. Such mode of activation has been described as one of the four possible types of regulation proposed for the PPARγ/RXRα system (3).
EG-VEGF MEDIATES PPARγ EFFECTS DURING PREGNANCY

Fig. 8. EG-VEGF mediates PPARγ’s effect on trophoblast migration. A: images of wounded HTR-8/SVneo monolayers at 0 (T0) and 9 h (T9h) postwounding. B: plots show percentages of wound closure after 9 h of treatment with Rosi in the absence or presence of PROKR2 antagonist (1 μM; n = 6). Bars with different letters are significantly different from each other (P < 0.05).

Most of the studies that have addressed the consequences of PPARγ<sup>−/−</sup> on pregnancy outcome have been halted at the report of the mice lethality at 9.5 dpc (4, 26). Because of the difficulty in analyzing placenta at this gestational age, only a brief statement on placental system disorganization was reported in PPARγ<sup>−/−</sup> mice (4, 26). In vitro studies using human placenta have been focused mainly on the role of PPARγ in the control of human trophoblast differentiation, including trophoblast invasion and syncytiotrophoblast functions (42). No in vitro studies reported the effect of PPARγ activation on placental vascularization or its control of trophoblast invasion in an ex vivo model such as the explant model. In a recent study using the BeWo cell line, PPARγ inactivation increased their proliferation, whereas its activation did not have any effect (28). BeWo cells originate from choriocarcinoma, a cell type that is more often used to mimic syncytiotrophoblast formation rather than cytotrophoblast proliferation, the cell type that represents the proliferative unit of first-trimester placentas. In this study we used explant tissues that contain all of these cell types and showed that rosiglitazone increased the proliferation of cytotrophoblasts at the villi and at the placental column. The discrepancy between our findings and those of Levytska et al. (28) might well be due to the type of material used. Here, we demonstrated that PPARγ activation increases placental growth via an increase in trophoblast proliferation and vascularization and controls precocious trophoblast invasion. Importantly, we demonstrated that part of these effects is mediated by EG-VEGF. These data further support the hypothesis that PPARγ<sup>−/−</sup> placental defects might well be due to deregulations in the EG-VEGF/PROKR functions and validate the role of EG-VEGF during early pregnancy. Increased trophoblast proliferation and villi vascularization as well as decreased EVT invasion upon PPARγ activation are three processes that we previously showed to be controlled by EG-VEGF (7, 8); hence, EG-VEGF might well be considered an important mediator of PPARγ’s effects during pregnancy.

The EG-VEGF protein family consists of two members, the canonical member EG-VEGF and its analog BV8 (bombinavariegata-8) (27). These proteins have been reported to control multiple biological processes (5, 7, 29), although the processes that EG-VEGF controls are related mainly to the success of pregnancy (6–8, 23). Importantly, recent data reported that the levels of circulating EG-VEGF were correlated to the quality of embryonic cohorts used in assisted reproductive technologies as well as to the rates of successful pregnancies (8, 31, 44). Hence, one can speculate that the failure in placental development observed in the PPARγ<sup>−/−</sup> mice might well have occurred during the first stages of placental development. In vivo data showed a significant decrease in EG-VEGF levels in the PPARγ<sup>−/−</sup> mouse placenta, further suggesting that the embryo’s death observed in the PPARγ mice might be due in part to a failure in EG-VEGF signaling.

The expression of EG-VEGF receptors has also been reported to be increased in pathological human pregnancies such as PE and FGR (8, 23). In PE, recent studies from McCarthy and colleagues (33, 34) have demonstrated that rosiglitazone administration to the reduced uterine perfusion pressure rat model of PE ameliorated key hallmarks of the disease, suggesting potential beneficial effects of PPARγ activation in the treatment of this pathology. One can then speculate that the
increased levels of EG-VEGF in pathological pregnancies might well be the consequence of a prior activation by the PPARγ system. Further animal and clinical studies are required to verify this hypothesis.

In conclusion, we demonstrated a new regulatory pathway involving a key placental transcription factor and a recently discovered angiogenic factor in the placenta. These findings will contribute to the understanding of the mechanisms behind placental defects responsible for the PPARγ−/− embryo’s death and will advance our comprehensions of the fine mechanisms that regulate placental development, leading to successful pregnancy outcomes.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

V.G., W.T., A.S., S.B., C.W., C.C., V.O., and M.B. performed experiments; S.B. prepared figures; T.F., Q.-Y.Z., and N.A. conception and design of research; B.D. and P.H. interpreted results of experiments; J.-J.F. approved final version of manuscript.
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