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

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Abbreviated Title: EG-VEGF mediates PPARγ effects during pregnancy
Key terms: Human pregnancy, PPARγ knockout, EG-VEGF, trophoblast invasion.

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Abstract

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 controls the same processes as those described for PPARγ, suggesting potential regulation of EG-VEGF by PPARγ. EG-VEGF exerts its functions via prokineticin receptor 1 (PROKR1) and 2 (PROKR2). This study sought to investigate whether EG-VEGF mediates part of PPARγ effects on placental development. Three approaches were used i) *in vitro*, using human primary isolated cytotrophoblasts, and the extravillous trophoblast cell line (HTR-8/SVneo), ii) *ex vivo*, using human placental explants (n= 46 placentas), and iii) *in vivo*, using gravid wild type, PPARγ+/− and PPARγ −/− mice. Major processes of placental development, 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. Both in human trophoblast cell and placental explants, we demonstrated that rosiglitazone, a PPARγ agonist, increased i) EG-VEGF secretion, ii) EG-VEGF and its receptors mRNA and protein expression, iii) placental vascularization, via PROKR1 and PROKR2, and iv) 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.
Introduction

During early pregnancy two key processes of placental development control its growth and the establishment of the fetomaternal circulation (FC) (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 FC 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 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 at 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−/− embryonic lethality came from the “Sox2Cre/PPARγ12/12” 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 recently shown 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, ie, 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 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-1 (PROKR1) and PROKR2 (30, 32) and controls both villi growth and the establishment of the fetomaternal circulation (6-8, 23). Within the villi, EG-VEGF increases endothelial proliferation, migration, tube-like formation and permeability, as well as trophoblast proliferation and survival (6-8). At the extra-villous 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 to 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, an i) *in vitro* approach using human primary isolated cytotrophoblasts, and the EVT cell line (HTR-8/SVneo), ii) *ex vivo* approach using the human placental explant model, and iii) *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 8–10 weeks of gestation (wg) were used. Human tissues were collected at the Grenoble University Hospital. Collection and processing were approved by the local hospital ethical committees and informed patient consent was obtained in all cases.

Mouse models

Wild type (WT), PPARγ–/– and PPARγ+/– placentas were collected from female PPARγ+/– mice mated with male PPARγ+/– mice as previously described. (40). Animal experimentations were approved by the relevant commission of the Canton of Vaud (Switzerland). Because PPARγ–/– mice die at 9.5 dpc, gravid mice were sacrificed 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 following primers GACCCAGCTCTACAACAGGC (F) and GGGACAGACCTCACTAACCCTAACT (R), which amplified a product of 700 bp derived from the PPARγ wild-type allele. Placentas were used for RT-PCR analyses for EG-VEGF PROKR1 and PROKR2 expression. For each group, 4 different PPARγ+/– gravid mice were used and for each gravid mouse all placentas were collected for immunohistochemistry and RT-qPCR analysis.

Culture models

Placental explants (PEX) 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 weight) were placed into 48-well plates pre-coated with 150 µL per well of diluted Matrigel (Matrigel/DMEM-F12, 1/1) (Becton–Dickinson, Le Pont-de-Clai, 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 µM or 10 µM Rosiglitazone (a PPARγ agonist, Sigma-Aldrich, France) 30min-24h, 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-b-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 10⁶ 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 (3ml~3x10⁶ cells) or 60-mm (8ml~8x10⁶ cells) culture plates (Techno Plastic Products, Switzerland). VCT were incubated overnight in 5% CO₂ at 37°C and washed three times to eliminate non-adherent 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 syncytiotrophoblasts (ST) at 48 h 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, 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 SV40 virus (18). These cells were generously given to us by Dr C. Graham (Kingston, ON, Canada). Cells were used between 24–30 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, France) 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 1μg 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 GoTaq qPCR Master Mix (Promega, Madison, WI, USA). PCR conditions were: step 1, 94°C for 10 min; step 2, 45 cycles consisting of 95°C for 15 s, temperature indicated in Table 2 for 5 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 µm sections as previously described (1). 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% (v/v) 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 previously used and characterized (6, 9, 21-23). CD31/PECAM and cytokeratin were detected using commercial antibodies (Dako, 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 µg/ml for anti-EG-VEGF, 0.84 µg/ml for anti-PROKR1 and 0.84 µg/ml for anti-PROKR2. The tissue sections were subsequently washed three times with PBS and incubated with biotinylated goat anti-rabbit IgGs (1:400 dilution in blocking solution; Sigma-Aldrich, Saint-Quentin Fallavier, France) for 1 h in a humid chamber. After three PBS washes, the slides were incubated with an avidin-biotin complex (Vectastain ABC kit; Vector Laboratories, Burlingame, CA) for 1 h. After a final PBS wash, the immunoreactive proteins were visualized after the addition of 3, 3-diaminobenzidine (Dako, Trappes, France) for 2 min and then counterstained with hematoxylin. To quantify capillary length upon CD31 staining, images were processed for morphometric analysis with ImageJ software. A macro-command was edited to give the total vessel length after binarization, skeletization, and pixel count of the CD31-staining (39). In another set of experiments explants were also used to assess rosiglitazone effect on trophoblast proliferation. To this end placental explant were immunostained with anti-human Ki67 antibody (Dako, France). Proliferation index was determined by assessing the percentage of Ki67 positive cells in control and rosiglitazone treated explants (9).

Western-blot analysis

Placental explants collected from at least six different first trimester placentas were homogenized in RIPA lysis buffer and protease inhibitors as previously described (2). Protein extracts were electrophoretically separated on 0.1% sodium dodecyl sulfate-12% polyacrylamide gels and electrically transferred onto 0.45-µm nitrocellulose membranes. The membranes were blotted with antibodies against CD31 (Abcam, France), anti-PCNA (Proliferating Cell Nuclear Antigen) (DAKO, France), anti-CD68, a marker for macrophages (SANTA CRUZ, France), PROKR1, PROKR2, EG-VEGF (in house antibodies). PROKR1 and PROKR2 antibodies were both used at a final concentration of 0.45 µg/mL. A specific Western-blot protocol was set up to detect EG-VEGF protein (10-17 kD). Briefly, 100 µg of placental proteins were separated on 0.1% SDS-17% polyacrylamide gels, and electrically transferred onto 0.2 µm polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA), for 45 min at 90 V. The blots were washed with PBS-Tween 0.1 % and
incubated overnight in blocking solution (5 % skimmed milk in PBS-T). Subsequently, the membranes were immunoblotted with a rabbit antibody against EG-VEGF (0.48 µg/ml) (Covalab Lyon, France) overnight. The blots were then rinsed with PBS-T and incubated with goat anti-rabbit IgG (1:3000) for 1 hour. After three PBS-T washes, the antibody-antigen complex was detected using the ECL plus detection system (Amersham Pharmacia Biotech). The intensities of immunoreactive bands were measured by scanning the photographic film and analyzing the images on a desktop computer using the Image J software. The Chemidoc analyzing system was also used (Image Lab version 4.0.1). To standardize for sample loading, the blots were subsequently stripped using a commercially available kit following the manufacturer’s instructions (Re-blot; Millipore) and re-probed with an anti-β-actin antibody (Sigma-Aldrich, France) as an internal control for protein loading.

**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 × 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; Scion Corp). 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 pre-coated with 100 µl 1:25 Matrigel (BD Biosciences). 2×10^4 DiI-stained HTR cells were seeded per insert in 500 µl RPMI 1640 medium with 1% FBS. The inserts were placed into 24-well plates containing 750 µl RPMI-1640 medium with 15% FBS. Cells were treated with 10 µM Rosiglitazone, 1 µM PROKR1, or 1µM PROKR2 antagonists or both for 24 h. The chambers were then removed, and fixed with PFA. 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:** 150 µl of ice-cold Matrigel (BD Biosciences, Grenoble, France) was layered into each well of 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. 10 µM Rosiglitazone was added at the time of plating. Daily observations were made under an inverted photomicroscope to document the invasion process.

**PROKR-1 and PROKR-2 cDNA cloning and BHK cell line transfection**
Both PROKR1 and PROKR2 cDNAs were cloned from a human lung cDNA library, fused to a HA tag and inserted into the PCDNA3.1 eucaryotic plasmid. The plasmids HA–PROKR1 or HA–PROKR2 were transfected into BHK-21 (CCL-10; American Type Culture Collection (ATCC)). BHK cells were cultured in DMEM/F12 culture medium supplemented with 5% of FBS (fetal bovine serum) 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 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 [(2-(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), or 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, nonparametric test followed by Dunn’s or Bonferroni were used. (SigmaPlot and SigmaStat, Jandel Scientific Software). All data are expressed as mean ± SEM (***p<0.001; **p<0.01; *p<0.05)
**Results**

**PPARγ 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 24h. Rosiglitazone significantly increased EG-VEGF secretion from 8 h of incubation at both concentrations tested (1 µM 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 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 mainly expressed in the syncytiotrophoblast (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 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 24h of treatment. This effect was transcriptional as it was abolished in the presence of DRB, an inhibitor of transcription (Figure 2B). To confirm the effect of rosiglitazone on EG-VEGF mRNA level, we compared its protein levels by Western Blotting analysis in control versus rosiglitazone-treated explants. Figures 2C and 2D show that rosiglitazone significantly increased EG-VEGF protein expression in the human placental explants.

**Effect of RXR activation on EG-VEGF expression**

As 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 (photographs in c and d) but not PROKR1 (photographs in a and b) expression. Photographs in e and f are 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 proteins family and appear on the blots as two to three glycosylated forms, respectively (Figures 3B and 3C).

**PPARγ effect on the 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 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 effects on trophoblast proliferation was assessed using Ki67 staining. Figure 4A shows representative sections of placental villi (a, c) or placental columns (b, d) that have been incubated in the absence (a, b) or presence of rosiglitazone (c, d) and stained for Ki67. There was a significant increase in the number of Ki67-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 (Figure 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 to the control one (Figure 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 (10µM) 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 to vessels present within the control placental villi. There was a significant increase in the total capillary length within the villi of rosiglitazone treated explants compared to the control ones (Figure 4Da-f). Figure 4E shows a western-blot analysis that compared
CD31 expression in control and treated explant. 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 over-expressing 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 PROKR1 antagonist (Figure 5A and 5B), but not in the presence of PROKR2 antagonist, Figure 5C and 5D. Inversely, in BHK-R2 cells, EG-VEGF effect was abolished in the presence of PROKR2 antagonist (Figure 5E and 5F), but not in the presence of PROKR1 antagonist (Figure 5G and 5H).

**EG-VEGF mediates PPARγ 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 6A, 6C and 6B, 6D show that both PROKR1 and PROKR2 antagonists significantly decreased rosiglitazone effects on CD31 protein expression, respectively. Altogether, these data suggest that EG-VEGF might mediate some of PPARγ-activation effects on the placental endothelium during the first trimester of pregnancy.

**EG-VEGF mediates PPARγ 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 HTR-8/SVneo cell. First, we determined the effect of rosiglitazone on PROKR2 expression, a receptor subtype that mediates EG-VEGF effects on trophoblast invasion (23). Figure 7B shows that rosiglitazone significantly increased PROKR2 protein levels, 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 pre-labeled 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 a part of 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 Figure 7D.

**EG-VEGF mediates PPARγ effect on trophoblast migration**
As we showed that EG-VEGF contributes to the rosiglitazone inhibitory effects on trophoblast invasion, we wondered whether it also mediates rosiglitazone effects on trophoblast migration. Hence, we examined EG-VEGF effect on the migration of HTR-8/SVneo cells. Figure 8A shows representative photographs of HTR-8/SVneo monolayers at 0 h 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 Figure 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 to the WT and a trend to a decrease in the PPARγ⁺/⁻. 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 (b,d,f). In the PPARγ⁻/⁻ placenta, EG-VEGF staining was decreased both in the Ec and the decidua (k,m,o). In the Ec strong staining was observed in the Tgc (trophoblast giant cells) of WT placenta that was decreased in the PPARγ⁻/⁻. Photographs in a,c,e and in j, l, n show cytokeration stainings of the WT and PPARγ⁻/⁻ placentas, respectively. Photographs in g, h, i and in p,q,r report negative controls for the EG-VEGF staining.
The present study demonstrates a new regulation of EG-VEGF and its receptors by the major nuclear receptor PPAR\(\gamma\) and bring new insights into the mechanism by which PPAR\(\gamma\) might affect placental development, a main cause of PPAR\(\gamma\)-/- lethality. These statements were based on the following findings:

1. PPAR\(\gamma\) increased EG-VEGF secretion and its mRNA and protein expression both in primary human trophoblast cells and placental explants.
2. PPAR\(\gamma\) increased PROKR2 expression in placental explants.
3. Placental growth controlled by PPAR\(\gamma\) is in part mediated by EG-VEGF, via the activation of PROKR1 and PROKR2.
4. Trophoblast invasion controlled by PPAR\(\gamma\) is in part mediated by EG-VEGF, via PROKR2, and PPAR\(\gamma\)-/- placentas exhibited decreased expression of EG-VEGF compared to WT placentas.

The demonstration that EG-VEGF mediates some of the PPAR\(\gamma\) 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 PPAR\(\gamma\) activation by rosiglitazone reversed adverse nicotine effects on the ovary vascularization by increasing EG-VEGF expression (37).

RXR\(\alpha\) occupies a central position in the nuclear receptor superfamily, though its activation or inhibition by specific ligands did not affect EG-VEGF expression, suggesting that only endogenous PPAR\(\gamma\) 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 PPAR\(\gamma\)/RXR\(\alpha\) system (3).

Most of the studies that have addressed the consequences of PPAR\(\gamma\)-/- on pregnancy outcome have been halted at the report of the mice lethality at 9.5 dpc (4, 26). Because of the difficulty to analyze placenta at this gestational age, only a brief statement on placental vascular system disorganization was reported in PPAR\(\gamma\)-/- mice (4, 26). In vitro studies using human placenta have mainly been focused on the role of PPAR\(\gamma\) in the control of human trophoblast differentiation including trophoblast invasion and syncytiotrophoblast functions (42). No in vitro studies reported the effect of PPAR\(\gamma\) activation on placental vascularization, nor its control of trophoblast invasion in an ex vivo model such as the explant model. In a recent study using the Bewo cell line, PPAR\(\gamma\) 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 tissue that contain all these cell types and showed that rosiglitazone increased the proliferation of cytotrophoblasts at the villi and at the placental column. The discrepancy between our finding and those by Levytska & al might well be due to the type of material used. Here we demonstrated that PPARγ activation increased 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-/- 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 as an important mediator of PPARγ effects during pregnancy.

The EG-VEGF protein family accounts two members, the canonical member, EG-VEGF and its analog BV8 (bombina-variegatea-8) (27). These proteins have been reported to control multiple biological processes (5, 7, 29). Though, the processes that EG-VEGF controls are mainly related 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-/- 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-/- mouse placenta further suggesting that the embryo’s death observed in the PPARγ mice might in part be due 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 et al (33, 34) demonstrated that rosiglitazone administration to the reduced uterine perfusion pressure (RUPP) 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.
Figures and legends

**Figure. 1. PPARγ effect on EG-VEGF secretion.** A. EG-VEGF secretion levels in placental explants after treatment with 1 and 10 µM of rosiglitazone at 30 min, 3h, 8h and 24h incubation (n=6). EG-VEGF levels were measured by ELISA. B. Inactivation of PPARγ by T0070907 (8h) lead to a decrease in EG-VEGF secretion (n=4). C EG-VEGF secretion levels in primary trophoblast cells after treatment with 10 µM of rosiglitazone during 24 h (n=4). Data are presented as mean ± SEM (*p<0.05). Values overwritten with different letters are significantly different from each other. ST: syncytiotrophoblast.

**Figure. 2. PPARγ effect on EG-VEGF placental expression.** A. Quantification of EG-VEGF mRNA levels in placental explants in response to rosiglitazone 10 µM (n=6). B. reports EG-VEGF levels of secretion by placental explants treated or not with rosiglitazone 10 µM, 50µg/mL DRB (inhibitor of transcription), or both. EG-VEGF levels were measured by ELISA (*p<0.05). Values overwritten with different letters are significantly different from each other. C. shows a representative Western Blot analysis of EG-VEGF expression after 24 h of treatment with 10 µM of rosiglitazone (n=6). Standardization of protein signals was done with antibodies against β-actin. Quantification of the intensity of the bands is illustrated on the graph shown in D. Data represent the mean ± SEM. VCT: villous cytotrophoblast, ST syncytiotrophoblast (*p<0.05; **p<0.01).

**Figure. 3. PPARγ effect on PROKR1 and PROKR2 expression.** A. reports photographs of placental explant (8 wg) sections immunostained with anti-PROKR1 antibody (a, b) or anti PROKR2 antibody (c, d) in the absence or the presence of 10 µM rosiglitazone (n=4). B. reports representative Western Blot analysis that compares PROKR1 expression between placental explants treated or not with 10 µM rosiglitazone (n=6 different placentas). Quantification of the intensity of the bands is shown below. C. Representative Western Blot of PROKR2 expression after overnight of treatment with 10 µM of rosiglitazone. Standardization of protein signals was done with antibodies against β-actin. Quantification of the intensity of the bands is illustrated below (*p<0.05). VCT: villous cytotrophoblast, ST: syncytiotrophoblast, H: highly glycosylated form, I : intermediate glycosylated form, U : unglycosylated form.
Figure 4. PPARγ increases trophoblast proliferation and placental vascularization.

A shows Ki-67 staining in placental explants treated or not with rosiglitazone (10µM). Photographs in a and b show Ki67 staining in control placental villi and placental column, respectively. Photographs in c and d show the stainings under rosiglitazone treatment. B shows the percentage of Ki67 positive cytotrophoblast cells quantified in three independent experiments (n=6 control and n=6 rosiglitazone). Data represent the percentage of triplicates *p < 0.05. Scale bar: 50μm. CVT: villous cytotrophoblast; St: Syncytiotrophoblast; Pro-Evt: proliferative extravillous trophoblasts. C shows western blotting analysis of PCNA expression in control and rosiglitazone treated placental explants (n=6 control and n=6 rosiglitazone). D shows representative photographs of CD31 staining in placental explants treated (a) or not with rosiglitazone (10 µM) (b). a’ and b’ are skeletonisation of photographs in a and b, respectively. The black color represents the vascular network. Fig. 4Df represents the percentage area occupied by capillaries. Six placental explants photographs were analyzed for each condition. E. reports representative western blot analysis that compares CD31 protein levels in control placental explants versus explants treated with 10µM of rosiglitazone. Quantification of the intensity of the bands is illustrated below. Bv: blood vessel.

Figure 5: Characterization of PROKR1 and PROKR2 inhibitory effects in BHK cells overexpressing PROKR1 receptor or PROKR2. Panel A shows photographs of wounded BHK-R1 monolayers, at 0, and 9 hours post-wounding in the absence or the presence of PROKR1-antagonist. The plots in panel B show percentages of wound closure after 9h of treatment with EG-VEGF in the absence or the presence of PROKR1-antagonist (1 µM). Bars with different letters are significantly different from each other (*p<0.05, n=3). Panel C shows photographs of wounded BHK-R1 monolayers, at 0, and 9 hours post-wounding in the absence or the presence of PROKR2-antagonist. The plots in panel D show percentages of wound closure after 9h 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). Panel E shows photographs of wounded BHK-R2 monolayers, at 0, and 9 hours post-wounding in the absence or the presence of PROKR2-antagonist. The plots in panel F show percentages of wound closure after 9h 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). Panel G shows photographs of wounded BHK-R2 monolayers, at 0, and 9 hours postwounding in the absence or the presence of PROKR1-antagonist. The plots in panel H show percentages of wound closure after 9h of treatment with EG-VEGF in the absence or the presence of PROKR1-antagonist (1 µM). Bars with different letters are significantly different from each other (*p<0.05, n=3).

Figure 6. EG-VEGF mediates PPARγ effect on placental vascularization.
A and B respectively report representative western blot analyses that compare CD31 protein levels in control placental explants versus explants treated with 10µM of rosiglitazone, in the absence or presence of PROKR1-antagonist (1 µM), PROKR2-antagonist (1 µM) or the both during 24 h. Quantification of the intensity of the bands is illustrated in C and D, respectively (n=6). Standardization of protein signals was done with antibodies against β-actin protein (*p <0.05). Values overwritten with different letters are significantly different from each other.

**Figure. 7. EG-VEGF mediates PPARγ effect on trophoblast invasion.** A. shows rosiglitazone effect on EVCT invasion placental explants (n=4). B. Representative Western Blot of PROKR2 expression after 24 h of treatment with 10 µM of rosiglitazone in HTR-8/SVneo cell (n=6). Standardization of protein signals was done with antibodies against β-actin. Quantification of the intensity of the bands is illustrated below (**p<0.01). C. reports representative photographs of HTR-8/SVneo invasion that have been treated or not with 10 µM of rosiglitazone, in the absence or the presence of PROKR2-antagonist (1 µM). Quantification of HTR-8/SVneo invasion is reported in D (*p<0.05) (n=6). Values overwritten with different letters are significantly different from each other.

**Figure. 8. EG-VEGF mediates PPARγ effect on trophoblast migration.** Panel A shows photographs of wounded HTR-8/SVneo monolayers, at 0, and 9 hours post-wounding. The plots in panel B show percentages of wound closure after 9 h of treatment with rosiglitazone in the absence or the presence of PROKR2-antagonist (1 µM) (n=6). Bars with different letters are significantly different from each other (*p<0.05).

**Figure. 9. EG-VEGF mRNA and protein expression in placental tissues from WT, PPARγ+/− and PPARγ−/−.** Panel A reports a graph of the quantification of EG-VEGF mRNA in placentas collected from WT, PPARγ+/− and PPARγ−/− (*p<0.05). GAPDH mRNA was used as an internal control. Results are reported as the mean ± SEM. Panel B reports photographs with stainings for cytokeratin (CK7) (a, c,e) and (j, l, n) in WT (wild type) and PPARγ−/−, respectively collected at day 8.5 of gestation. Photographs in (b, d, f) and in (k, m,o) report stainings for EG-VEGF in WT and PPARγ−/−, respectively. Negative controls for EG-VEGF stainings are shown on the right side of the each panel. For each type of mice 6 placentas were collected from 4 different mice. Ec: ectoplacental cone; De: decidua, Tgc : trophoblast giant cells. Photographs: a,b,j,g,j,k and p were taken at x50 magnification. Photographs: c,d,h,l,m,and q were taken at x100 magnification. Photographs: e,f,i,n,o, and r were taken at x200 magnification. Scale bar is 50µM.

**Acknowledgements:** We thank Férdéric Sergent for his technical assistance. We thank the staff of the University of Lausanne, Center for Integrative Genomics, Switzerland Central Animal Facility for their help with the animal work. We also thank the department of obstetrics and gynecology of Grenoble hospital for placental material.
We acknowledge the following sources of funding: Institut National de la Santé et de la Recherche Médicale (U1036), University Joseph Fourier, Commissariat à l’Energie Atomique (DSV/irRTSV/BCI), the region Rhônes Alpes, Ligue Internatinal contre le cancer.

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

References


Figure 1

A

Normalized EG-VEGF secretion levels (to control)

B

Normalized EG-VEGF secretion level (to control)

C

Normalized EG-VEGF secretion level in ST (to control)
Figure 2

A

EG-VEGF/GAPDH mRNA
(normalized to E
-actin)

Control Rosiglitazone
10μM

B

EG-VEGF secretion level
(normalized to control)

Control Rosiglitazone DRB Rosi/DRB

C

EG-VEGF secretion level
(normalized to control)

Control Rosiglitazone (10μM)

D

EG-VEGF protein level
(normalized to β-actin)

Control Rosiglitazone
Figure 3

A

PROKR1

PROKR2

B

C

55 Kd

55 Kd

PROKR1 protein level
(normalized to β-actin)

Control
Rosiglitazone 10μM

PROKR2 protein level
(normalized to β-actin)

Control
Rosiglitazone 10μM

Negative controls

Rosiglitazone 10μM

Control Rosiglitazone

PROKR1

PROKR2

Control Rosiglitazone
**Figure 4**

**A**

**Villi**

- **Control**
  - a) stroma
  - b) VCT
  - c) ST
  - d) Pro Evt

- **Rosiglitazone**
  - e) stroma
  - f) VCT
  - g) ST
  - h) Pro Evt

**Placental column**

- **Control**
  - i) stroma
  - j) VCT
  - k) ST
  - l) Pro Evt

- **Rosiglitazone**
  - m) stroma
  - n) VCT
  - o) ST
  - p) Pro Evt

**B**

% area occupied by capillaries

- **Control**
  - Ctl
  - Rosi

- **Rosiglitazone**
  - Ctl
  - Rosi

**Negative control**

- ** CTL**
  - CD31
  - PCNA
  - β-actin

**E**

- **CTL**
  - CD31 protein level
  - β-actin

- **Control**
  - Rosiglitazone 10μM
Figure 6

A

130 kD

CD31

β-actin

Control Rosi

PROKR1 antagonist

PROKR1 antagonist + Rosi

B

130 kD

CD31

β-actin

control Rosi

PROKR2 antagonist

PROKR2 antagonist + Rosi

C

CD31 protein level (arbitrary units)

Control Rosiglitazone PROKR1 antagonist Rosi + PROKR21 antagonist

D

CD31 protein level (arbitrary units)

Control Rosiglitazone PROKR2 antagonist Rosi + PROKR2 antagonist
**Figure 7**

**A**
- Control
- Rosiglitazone 10μM

24 h
- Placental villi
- Matrigel

72 h
- EVT
- Matrigel

**B**
- PROKR2 protein level
- β-actin
- Control
- Rosiglitazone 10μM

**C**
- Control
- PROKR2 antagonist
- Rosiglitazone
- Rosi + PROKR2 antagonist

**D**
- Invasive cells level (normalized to control)
- Control
- PROKR2 antagonist
- Rosiglitazone
- PROKR2 antagonist + Rosi

*Note: The images and graphs show differences in protein levels and cell invasion due to the treatment with Rosiglitazone and its antagonist.*
Figure 8

A

\( T0 \)
- Control
- Rosiglitazone
- PROKR2 Antagonist
- PROKR2 Antagonist + Rosi

\( T9h \)
- Control
- Rosiglitazone
- PROKR2 Antagonist
- PROKR2 Antagonist + Rosi

B

\begin{itemize}
  \item \textbf{a}
  \item \textbf{b}
\end{itemize}

% wound closure in HTR8/SVneo
Figure 9

A

EG-VEGF/GAPDH mRNA (arbitrary units)

WT +/- -/-

ns *

B

WT

PPARγ

PPARγ +/-

WT

Cytokeratin EG-VEGF

WT +/- -/-

Cytokeratin EG-VEGF
<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 GAT GG</td>
<td>TTC TAG ACG GCA GGT CAG GT</td>
</tr>
<tr>
<td>mEG-VEGF</td>
<td>TG AGG AAA CGC CAA CAC CAT</td>
<td>CC GGG AAC CTG GAG CAC</td>
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