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# Regulation of the Human Endogenous Retroviral Syncytin-1 and Cell–Cell Fusion by the Nuclear Hormone Receptors PPARγ/RXRα in Placentogenesis

Matthias Ruebner,[1] Manuela Langbein,[1] Pamela L. Strissel,[1] Christine Henke,[1] Doreen Schmidt,[1] Tamme W. Goecke,[1] Florian Faschingbauer,[1] Ralf L. Schild,[2] Matthias W. Beckmann,[1] and Reiner Strick[1*]

[1] *Laboratory for Molecular Medicine, Department of Gynaecology and Obstetrics, University-Clinic Erlangen, Universitaetsstr. 21-23, D-91054 Erlangen, Germany*

[2] *Department of Obstetrics and Gynecology, Diakonische Dienste Henriettenstift, Hannover, Germany*

## ABSTRACT

Cytotrophoblast (CT) cell fusion into a syncytiotrophoblast is obligatory for placentation and mediated by the human endogenous retrovirus (HERV)-W envelope gene Syncytin-1. Abnormal placentation is associated with preeclampsia (PE), HELLP and intrauterine growth restriction (IUGR). In placentogenesis, the MAP-kinase p38α regulates PPARγ/RXRα signaling and target genes, like leptin, resistin, ABCG2, and hCG. The aim of this study was to analyze PPARγ/RXRα signaling and target gene regulation using primary CT cultures, the trophoblastic cell line BeWo and placental tissues from patients with normal and abnormal placentation. CT from four different human control placentae and BeWo cells demonstrated that Syncytin-1, other signaling members and CT cell fusions were regulated with PPARγ/RXRα activators troglitazone and 9-*cis* retinoic acid, via protein kinase A and p38α inhibition. Significant discordant regulations between CTs and BeWo were found. Two PPARγ/RXRα-response-elements from upstream regulatory elements and the 5′LTR of HERV-W were confirmed with DNA-protein binding assays using nuclear extracts and recombinant PPARγ/RXRα proteins. These promoter elements were validated with luciferase assays in the presence of PPARγ/RXRα modulators. Furthermore, troglitazone or 9-*cis* retinoic acid treatment of siRNA-PPARγ and siRNA-RXRα transfected BeWo cells proved the requirement of these proteins for Syncytin-1 regulation. Thirty primary abnormal placentae from PE, HELLP and IUGR patients compared to 10 controls showed significant deregulation of leptin RNA and protein, p38α, phospho-p38α, PPARγ, ABCG2, INSL4 and Syncytin-1. Our study characterized PPARγ/RXRα signaling in human CT and cell fusions identifying Syncytin-1 as a new target gene. Based on these results, a disturbed PPARγ/RXRα pathway could contribute to pathological human pregnancies. J. Cell. Biochem. 113: 2383–2396, 2012. 

**KEY WORDS:** PLACENTA; TROPHOBLAST; PPARγ; RXRα; LEPTIN; HERV; SYNCYTIN-1; CELL FUSION; PLACENTAL SYNDROMES; PREECLAMPSIA

During the first 11 days of pregnancy, placental cytotrophoblasts (CTs) differentiate along two pathways: the invasive and the fusion pathways. Extravillous CT (EVCT) are essential for invasion into uterine stromal tissues and for remodeling of spiral arteries to generate the utero-placental circulation [Pijnenborg et al., 1983]. During the fusion pathway, villous CT (VCT) fuse to form a multinuclear syncytiotrophoblast layer (SCT), which facilitates the maternal-fetal exchange of nutrients, gas and waste products. Throughout pregnancy, enlargement and maintenance of the SCT occurs by fusion of single CT into the existing SCT [Potgens et al., 2002]. In addition, the SCT is important for steroid and peptide hormone synthesis including human chorionic gonadotrophin (hCG), human placental lactogen (hPL), leptin and immunologic functions [Jameson and Hollenberg, 1993; Masuzaki et al., 1997].

Additional supporting information may be found in the online version of this article.

Matthias Ruebner and Manuela Langbein contributed equally to this work.

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*Correspondence to: R. Strick, PhD, Laboratory for Molecular Medicine, Department of Gynaecology and Obstetrics, University-Clinic Erlangen, Universitaetsstr. 21-23, D-91054 Erlangen, Germany.
E-mail: reiner.strick@uk-erlangen.de

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One member of the human insulin-family, the early placenta insulin-like peptide (EPIL, INSL4) is highly expressed in placenta and is 10-fold higher expressed in SCT as compared to CT [Laurent et al., 1998], supporting an important role in differentiation. The envelope gene of the human endogenous retrovirus (HERV)-W, Syncytin-1, on chromosome 7q21.2, is a key factor for mediating cell fusion of CT [Mi et al., 2000; Frendo et al., 2003]. Syncytin-1 is a membranous glycoprotein expressed in both VCT and EVCT as well as in the SCT layer. Trophoblastic cell lines, such as BeWo were derived from rare malignant choriocarinomas and used as a model system for CT and cell fusion [Orendi et al., 2011].

The p38α (MAPK14) kinase is essential for trophoblast development and placental vascularization in mice and initiation of human trophoblast differentiation [Daoud et al., 2005]. In CT, p38α was shown to directly regulate the peroxisome-proliferator-activated receptor γ (PPARγ), a member of the superfamily of nuclear receptors regulating glucose homeostasis, adipocyte differentiation, lipid storage, and release [Lehrke and Lazar, 2005; Schild et al., 2006]. In similarity to p38α knock-out mice, PPARγ (−/−) mice showed varying degrees of deficient vascularization and growth restriction [Allen et al., 2000; Mudgett et al., 2000]. PPARγ is specifically expressed in VCT, EVCT, and SCT, and plays a key role in human placentogenesis influencing differentiation of both EVCT and VCT [Tarrade et al., 2001]. The p38α inhibitor SB203580 acts through reversible binding of the ATP pocket leading to PPARγ inhibition [Badger et al., 1996]. On the other hand, PPARγ is directly activated by oxidized and nitrated fatty acids, like 15-deoxy-D12,14-prostaglandin J2 (15Δ-PGJ2), 9-hydroxyoctadecadienoic acid (9-HODE) and 13-hydroxyoctadecadienoic acid (13-HODE), the latter two being oxidized fatty acids present in low-density lipoproteins [Forman et al., 1995; Nagy et al., 1998]. The synthetic PPARγ binding ligands rosiglitazone (Avandia®), pioglitazone (Actos®) and troglitazone (Rezulin®) are thiazolidinedione derivatives used for the treatment of type 2 diabetes, but have several adverse side effects [Willson et al., 1996] and are partly withdrawn from the market. Indirect PPARγ activation has also been demonstrated especially through cross-talk of other signaling pathways, for example, via stimulation of adenylate cyclase and PKA phosphorylation by forskolin [Lazennec et al., 2000]. Interestingly, forskolin is a known inducer of Syncytin-1 and cell fusions as mediated by cAMP and the cAMP responsive binding element protein in primary CT cultures, choriocarcinoma and endometrial carcinoma cells, and therefore is linked with cell differentiation [Strauss et al., 1992; Strick et al., 2007].

The PPARs, like many non-steroid members of the nuclear receptor family, function as obligate heterodimers with retinoid X receptors (RXR) [Chawla et al., 2001]. Crystallization studies showed that PPARγ and RXRα form a non-symmetric complex, where some of these protein/protein interfaces involved the DNA-binding domains of PPARγ and RXRα  The PPARγ ligand binding domain was shown to cooperate with both PPARγ and RXRα DNA-binding domains to enhance response-element binding [Chandra et al., 2008]. Heterodimers bind to the PPAR-response-element (PPRE) composed of two direct-repeat sequences, separated by one or two nucleotides in the promoter region of target genes. PPARγ has been shown to bind the 5′ half of the PPRE whereas RXR binds the 3′ half. Interestingly, direct activation of RXRα following 9-*cis*-retinoic-acid ligand binding, PPARγ1 and γ2 represses or activates transcription of target genes by forming heterodimers with RXRα, or by each binding as homodimers to the response elements (PPRE/RXRE) [Jpenberg et al., 1997]. Additionally, nuclear receptor co-activators, including members of the steroid receptor coactivator (SRC) family, contain LXXLL motifs that dock to the ligand binding domain [McKenna and O'Malley, 2002].

Specific PPARγ target genes for placental development include leptin, resistin [Masuzaki et al., 1997; Yura et al., 2003], hCG [Tarrade et al., 2001] and the xenobiotic transporter ABCG2 [Szatmari et al., 2006]. In early pregnancy, maternal leptin levels correlate with hCG, rise towards the end of pregnancy and then decrease at birth [Hardie et al., 1997]. Besides the classical function of hCG to maintain steroid hormone production in the corpus luteum of early pregnancy, it represents a biochemical differentiation marker, which is secreted by SCT and correlates with CT cell–cell fusion [Masuzaki et al., 1997; Tarrade et al., 2001]. Suppression of ABCG2 using siRNA was shown to result in increased apoptosis and decreased expression of cell fusion markers like hCG and Syncytin-1 [Evseenko et al., 2007], supporting a critical role for ABCG2 in placental development.

There are several pathological pregnancy syndromes, characterized by abnormal placental development and function, which can also result in a systemic maternal syndrome. Preeclampsia (PE) is a common pregnancy disorder characterized by hypertension, proteinuria and edema and can lead to maternal and fetal morbidity and mortality [Wilson et al., 2003]. Another rare but severe pregnancy complication is the development of a hemolysis elevated liver enzymes and low platelets (HELLP) syndrome [Sibai, 2004]. Intrauterine growth restriction (IUGR) represents impaired fetal growth and development with an incidence from 4% to 7% live births and can occur alone or in combination with PE and HELLP [Wilson et al., 2003]. Leptin deregulation was shown in PE and diabetic pregnancies where leptin mRNA expression was elevated in placental tissue [Lea et al., 2000]. In term placental tissues from PE, PE/IUGR, HELLP/IUGR and IUGR patients, lower Syncytin-1 expression levels were found using quantitative real time PCR and protein levels. Interestingly, low Syncytin-1 expression levels were also confirmed with cultured primary CT from these same placentae and impaired cell fusion was observed [Lee et al., 2001; Langbein et al., 2008; Ruebner et al., 2010].

Due to a strong association of PPARγ/RXRα signaling during placental development, especially SCT differentiation, the aim of this study was to examine PPARγ/RXRα signaling and cell fusion using a primary CT culture system and the BeWo trophoblast cell line. Gene expression differences from cell cultures were compared to abnormal placentae from PE, HELLP, IUGR patients and healthy controls to determine, if deregulated PPARγ/RXRα signaling plays a role in these placental disorders.

## MATERIALS AND METHODS

### FRACTIONATION AND CULTIVATION OF CYTOTROPHOBLASTS

Human CT were isolated from 4 independent control term placentae and 300,000 viable cells/cm$^2$ were cultured [Kliman

et al., 1986; Langbein et al., 2008; Ruebner et al., 2010]. CT viability was routinely determined over 85% using trypan blue exclusion and 86–90% of the fractionated cells were trophoblastic and 10–14% non-trophoblastic using multiple FACS (FACSCalibur, BD-Biosciences) with HLA-A,B,C+ (10–14%), Cytokeratin 7+ (95–98%) and CD45+ (2.5–4.5%) antibodies (HLA-A,B,C/PE Biolegend, Netherlands; Cytokeratin 7/PE: Santa Cruz Bio, Germany; CD45/FITC: Miltenyi Biotec, Germany) [Ruebner et al., 2010]. Four hours after the cells were seeded, the media was changed and the PPARγ activator troglitazone (TZ; 10 μM; Calbiochem) and the RXRα 9-*cis* retinoic acid (9-*cis*-RA) activator (0.1 μM; Sigma) were added to CT cultures for 72 h and then harvested for gene and protein expression or analyzed for cell fusion. Also tested were forskolin (FK; 40 μM; Sigma) to stimulate the PPARγ/RXRα signaling pathway indirectly through cross-talk via cAMP, the p38α (MAPK14) kinase inhibitor SB203580 (10 μM; Sigma) and direct PPARγ natural ligands including 15Δ-PGJ2, 13-HODE, 9-HODE and 15-HETE (all 10 μM; all from Biozol, Eching). Forskolin at 40 μM was used according to Strick et al. [2007] and Langbein et al. [2008]. TZ, SB203580 and the natural ligands were used at 10 μM according to Schild et al. [2006]. We also performed a drug concentration kinetic for 9-*cis*-RA (10–200 nM) and determined viability by trypan blue cell staining. Our criterion for the drug concentration used was the highest concentration, which demonstrated cell viability >85% along with 18S-rRNA integrity similar to untreated cells using real time PCR. The choriocarcinoma cell line BeWo was cultured in Hams-F12 media (Invitrogen) and treated with the same drug concentrations as CTs.

### PLACENTAL COHORT COLLECTIVES

For gene expression studies, a total of 40 different placentae were analyzed from patients including controls (n = 10), PE (n = 10), HELLP (n = 10), and IUGR (n = 10; Supplemental Table 1). The diagnosis of IUGR, PE and HELLP was based on general accepted criteria previously described in Langbein et al. [2008] and Ruebner et al. [2010]. None of the patients had diabetes or received PPARγ agonists. Human placentae were collected with the approval of the Ethics Committee at the University of Erlangen-Nuremberg after elective Caesarean section and written consent of the patients. Placenta specimens were sampled from the fetal–maternal interface near cord insertion and snap frozen in liquid nitrogen and stored at −80°C until use.

### RNA EXTRACTION AND REAL TIME PCR

RNA extraction, cDNA synthesis, Syncytin-1 absolute quantitative PCR (qPCR) and semi-quantitative real time PCR (sqPCR) for all other genes were performed from CT cell cultures harvested at day 3 or from 50 to 100 mg placenta tissues according to Langbein et al. [2008] and Ruebner et al. [2010]. SYBR-Green based qPCR requires the gene of interest cloned and implements a standard curve [cT (molecules) per concentration], where every cT data of this gene can be calculated as copy number/molecules per concentration of total RNA [Ruebner et al., 2010]. For qPCR, Syncytin-1 was cloned in a TopoTA vector (Invitrogen) and a standard curve was generated ($\gamma = -3.3609$; $t = 35.706$; $R^2 = 0.9919$) [Ruebner et al., 2010]. SqPCR describes the relative expression of the gene of interest relative to an internal control using the comparative $2^{-\Delta\Delta CT}$ method. The following TaqMan assays were used for sqPCR (Applied Biosystems): PPARγ (Hs00234592_m1), RXRα (Hs00172565_m1), p38α (Hs00176247_m1), SRC (Hs00186661_m1), SMRT (Hs00196955_m1), Gcm-1 (Hs00172692_m1), resistin (Hs00220767_m1), leptin (Hs00174877_m1), INSL4 (Hs00171411_m1) and ABCG2 (Hs00184979_m1). Co-amplification of 18S-rRNA and an independent patient cDNA were used as internal controls and for normalization.

### EMSA

Nuclear extracts were prepared using a protocol from Andrews and Faller [1991], which entailed CT culturing for 3 days under the influence of 10 μM TZ to activate PPARγ. Plasmid DNAs for pCMX-mouse (m) PPARγ, pCMX-human (h) RXRα and pCMX-mRXRα (generous gifts from Prof. Dr. Ronald M. Evans, The Salk Institute for Biological Studies, USA) were used in in vitro transcription–translation assays (TNT® Coupled Reticulocyte Lysate Systems; Promega) according to manufactures' instructions. EMSA was according to Strick et al. [2007] using nuclear extracts or in vitro translated PPARγ/RXRα proteins incubated with $^{32}$P-labeled double-stranded oligonucleotides of a known positive control Mucin-1 PPRE, HERV-W PPRE-1, HERV-W PPRE-2 and a mutant HERV-W PPRE-1 (primer sequences Supplemental Table 5).

### LUCIFERASE ASSAYS

Six fragments from the HERV-W 5′LTR promoter and upstream regulatory regions were cloned into the luciferase plasmid pGL3basic (Promega). All primers were designed with a *Kpn*I (TF) and *Hin*dIII (BR) restriction site for cloning. The full-length URE/U3/R/U5 fragment containing PPRE-1-3 was cloned using the following primers (primer sequences Supplemental Table 5). Syn1-UTR-TF and Syn1-LTR-BR; the URE/U3/R containing PPRE-1-2 using Syn1-UTR-TF and Syn1-R-BR; the U3/R/U5 region containing PPRE2 & 3 using Syn1-LTR-TF and Syn1-LTR-BR; the URE containing PPRE-1 using Syn1-UTR-TF and Syn1-UTR-BR; the U3/R containing PPRE-2 using Syn1-LTR-TF and Syn1-R-BR and the U5 region containing PPRE-3 using Syn1-U5-TF and Syn1-LTR-BR. Luciferase assays (Roche) along with direct and indirect activators or inhibitors of PPARγ/RXRα signaling were done in parallel with six independent transfections of BeWo trophoblastic cells using the JetPEI transfection reagent (peqlab, Erlangen) then analyzed 48-h post-transfection (2 μg 5′LTR plasmids for the promoter assay and a transfection control with 2 μg of the β-galactosidase expression-plasmid pSVβGal (Promega). Statistics were performed with a minimum of six measurements per plasmid. All luciferase values for each construct were normalized to pGL3basic (promoter assay) and β-galactosidase (transfection control).

### siRNA MEDIATED KNOCK-DOWN OF PPARγ AND RXRα

Specific siRNA against PPARγ and RXRα (Ambion, Life technologies) were transfected in 70%-confluent BeWo cells with a concentration of 60 nM using HiPerFect (Qiagen) according to manufactures' protocols and Strick et al. [2007]. After 7-h transfection, media was removed. A mock siRNA (Alexa488 scrambled negative siRNA, Qiagen) was used as a control siRNA,

demonstrating >80% transfection efficiency by microscopy. Therefore, these conditions were implemented for all other siRNA experiments. A double transfection with siRNA against PPARγ and RXRα was performed with a concentration of 30 nM for each siRNA. After 48-h siRNA post-transfection when protein levels were significantly reduced, 10 μM TZ or 100 nM 9-*cis*-RA were added to the cells, incubated for an additional 72 h and then the RNA was harvested. All single and double siRNA transfections were performed six times and then statistically normalized to the siRNA transfected PPARγ and RXRα untreated drug controls set to one.

### STAINING OF MULTINUCLEATED FUSED CELLS AND HCG MEASUREMENTS

At day 3 for all CT and BeWo cell cultures, supernatants were assayed in duplicates for the CT to SCT differentiation marker hCG using an Immulite2000 (DPC). Cells were also stained with the specific membrane stain wheat germ agglutinin conjugated with Alexa 594 and the nuclear stain Hoechst 33342 (Molecular Probes) according to Strick et al. [2007] and Ruebner et al. [2010]. Images were acquired using an Olympus BX51 microscope with Olympus color-view and applying the program Cell-F. The fusion index (FI) as well as the average number of nuclei per SCT were calculated according to Langbein et al. [2008] and Ruebner et al. [2010]. Detection and quantification of giant nuclei in BeWo cells after 72-h culturing were also performed according to Hoechst 33342 staining and met the criteria of an over threefold nuclear diameter compared to normal BeWo nuclei.

### ELISA AND IMMUNOBLOTS

For leptin protein quantification, lysates from placentae and primary CT cultures at day 3 (in duplicates) were analyzed using the human leptin duoSet ELISA (R&D, Wiesbaden) according to manufactures' instructions. Syncytin-1 protein expression and quantification was performed with 15 μg of cell lysates and immune blotted with a SU-specific antibody (1:1,000, Abnova, Offenbach, Germany) and a β-actin specific antibody (Sigma) according to Ruebner et al. [2010]. To determine phosphorylated p38α in lysates from placental tissues, a phospho-p38α Immunoassay (R&D, Wiesbaden) was performed according to the manufactures' instructions.

### STATISTICAL ANALYSIS

All data were expressed as a mean ± SEM. Differences were assessed using the independent-sample *t*-test and Mann–Whitney *U*-test (PASW 19.0.0.1, IBM SPSS, Inc.). A *P*-value of $<0.05$ was considered statistically significant.

## RESULTS

### PPARγ/RXRα SIGNALING ENHANCED GENE EXPRESSION OF LEPTIN, hCG, THE NEW TARGET GENE SYNCYTIN-1 AND CELL FUSION IN PRIMARY CT CULTURES

Our previous studies found that cultured control primary CT peak at day 3 in cell fusion, Syncytin-1 gene expression and hCG secretion [Langbein et al., 2008; Ruebner et al., 2010]. Therefore, we employed this assay to study PPARγ/RXRα signaling, target gene regulation and cell fusion. Control placenta CT ($n = 4$) were incubated with direct PPARγ/RXRα activators (TZ, 9-*cis*-RA) or by an indirect PPARγ/RXRα activator and inhibitor (FK, SB203580) then analyzed for gene and protein expression and cell fusion at day 3 (Fig. 1). Results of gene expression studies showed that direct or indirect activation or inhibition of PPARγ/RXRα signaling had no significant effect regarding the members PPARγ, p38α and SRC compared to untreated cultures (Fig. 1A, Supplemental Table 1). Although the PPARγ ligand TZ did not increase leptin gene expression to significant levels, leptin protein rose significantly to twofold (Fig. 1B). Compared to untreated cultures, TZ also induced a significant increase of ABCG2 (2.8-fold), Syncytin-1 (5.2-fold), hCG (3.7-fold) and the cell fusion index (FI; 1.2-fold) (Fig. 1, Supplemental Table 1). Stimulation of PPARγ/RXRα signaling via 9-*cis*-RA resulted in a similar differential gene regulation resulting in inhibition and activation of genes. For example, 9-*cis*-RA significantly down regulated INSL4 (15.4-fold), whereas Syncytin-1 (2.9-fold) and hCG secretion (1.7-fold) were significantly up-regulated. 9-*cis*-RA also showed a significant induction of the cell FI and number of nuclei per SCT. The incubation of control CT with other PPARγ natural ligands, like 15Δ-PGJ2, 13-HODE, 9-HODE and 15-HETE showed on day 3 a significant induction of hCG with 15Δ-PGJ2 (3-fold), of leptin mRNA for 15Δ-PGJ2 (9.2-fold) and 9-HODE (2.1-fold) and for Syncytin-1 with all four ligands (Supplemental Fig. 1). Interestingly, the highest induction for Syncytin-1 was observed with 9-HODE at 19.3-fold compared to control.

Stimulation of PPARγ/RXRα signaling by PKA via FK, a significant induction was noted for both leptin gene (9.4-fold) and protein expression (2.0-fold), Syncytin-1 (6.8-fold), hCG secretion (5.4-fold) and the number of nuclei per SCT from 4.5 to 7.4 (Fig. 1, Supplemental Table 1). On the other hand, the p38α inhibitor SB203580 showed statistically significant inhibition of the INSL4 gene (154.0-fold), the leptin gene (18.7-fold) and protein expression (8.6-fold); a reduction of Syncytin-1 (3.8-fold), hCG secretion (424.2-fold) and the cell FI (2.6-fold; $P < 0.0001$). Taken together, the above findings identified Syncytin-1 as a new PPARγ/RXRα target gene in primary CT cultures. In addition, the modulation of cell fusion by PPARγ/RXRα pathway signaling points to direct regulation of CT differentiation to SCT, via Syncytin-1 a known mediator of placental CT cell fusion.

### SPECIFIC BINDING OF CT NUCLEAR EXTRACTS, PPARγ AND RXRα PROTEINS TO HERV-W 5′LTR PPREs

From 5′ to 3′, a sequence analysis of the 5′LTR of HERV-W showed three putative PPREs (PPRE-1, -2, -3) with a range of 61–77% homology to the PPRE consensus (Fig. 2A). The PPRE-3 locates 3′ to the TATA-box and was predicted as non-functional regarding transcriptional regulation [Prudhomme et al., 2004; Cheng and Handwerger, 2005]. Therefore, the HERV-W 5́-LTR PPRE-1 and -2, a mutant PPRE-1 and the PPRE of Mucin-1, a known placental PPARγ target gene [Shalom-Barak et al., 2004] were compared using EMSA analysis with nuclear extracts from CT cultures and recombinant PPARγ and RXRα proteins (Fig. 2A). EMSA results demonstrated that the Syncytin-1 PPRE-1 bound specifically to nuclear extracts, but not with a mutant Syncytin-1 PPRE-1 (Fig. 2B). In order to prove

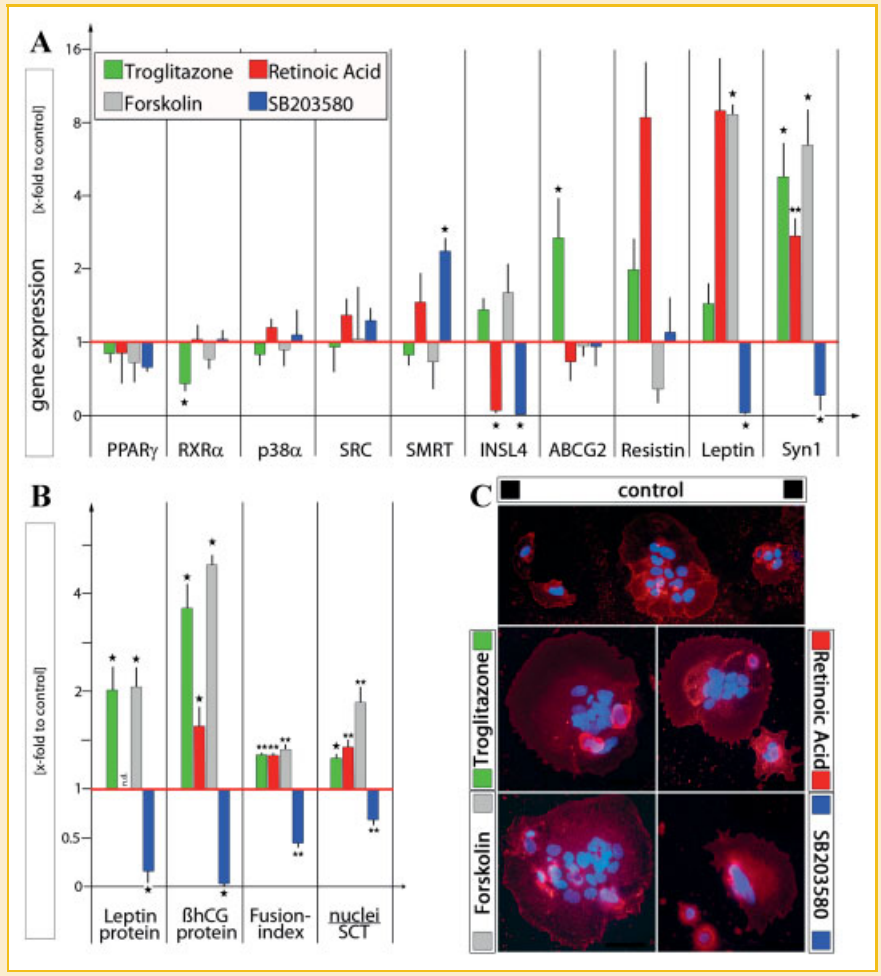



Fig. 1. Gene expression, protein quantification, and cell fusion of cultured primary control CT treated with PPARγ/RXRα inhibitors and activators. A: Histographs represent the mean expression from four different CT cultures (four different placentae) after 72 h in culture in fold-changes compared to control (Y-axis) for each gene indicated below following treatment of CT with inhibitors and activators (top inset). Statistical significances ($^{*}P < 0.05$; $^{**}P < 0.005$) are indicated; B: Histographs representing fold-change of leptin and hCG protein levels, cell fusion index, and nuclei per SCT. Statistical significances ($^{*}P < 0.05$; $^{**}P < 0.005$) are indicated. All values are listed in Supplemental Table 1. C: CT cell cultures on day 3 demonstrating examples of cell fusions corresponding to (B), following no treatment (control), and treatment with the p38α inhibitor SB203580 and three PPARγ/RXRα activators. Cells were stained with cell membrane stain (agglutinin 594 = red) and counterstained with Hoechst 33342 to identify nuclei (blue). Syn1, Syncytin-1. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

that PPARγ/RXRα heterodimers or homodimers could specifically bind to the HERV-W PPRE-1 sequence, in vitro translated mouse and human PPARγ and RXRα proteins were tested. Both single and combined PPARγ and RXRα recombinant proteins demonstrated specific binding to the HERV-W PPRE-1, however, a combination of mouse PPARγ and RXRα did not enhance DNA-binding compared to single protein experiments (Fig. 2C). Using the same EMSA conditions the 5′LTR PPRE-2 of HERV-W also demonstrated specific nuclear extract and recombinant PPARγ/RXRα protein binding (Fig. 2D). In summary, both PPRE-1 and -2 in the 5′LTR of HERV-W bound specifically to CT nuclear extracts and PPARγ and RXRα recombinant proteins.

### DIFFERENTIAL GENE AND CELL FUSION REGULATION WITH PPARγ/RXRα ACTIVATORS AND INHIBITORS IN BeWo CELLS

Similar to primary CT cultures, BeWo trophoblastic cells were also treated with TZ, 9-*cis*-RA, FK and SB203580 and analyzed on day 3 for comparison. TZ significantly stimulated gene expression of RXRα (3.6-fold), SMRT (2.3-fold), INSL4 (6.7-fold), leptin (5.6-fold), the transcriptional activator Gcm-1 (3.7-fold), hCG protein levels (1.4-fold) and the FI (2-fold; Fig. 3). However, for Syncytin-1, we observed a 1.6-fold inhibition with TZ cell treatment (Fig. 3A). 9-*cis*-RA significantly induced Syncytin-1, leptin, INSL4, hCG and the FI (Fig. 3, Supplemental Table 2). SB203580 on the other hand significantly inhibited SRC, INSL4, Leptin, Syncytin-1 and hCG. The activator FK stimulated PPARγ, INSL4, Leptin, Syncytin-1, Gcm-1, hCG, and FI, but inhibited RXRα and SRC. Importantly, as demonstrated above, comparing fractionated CT with BeWo showed many similarities, but also clear differences were found upon drug treatment. For example, TZ significantly inhibited RXRα in CT, but was stimulated in BeWo. TZ also stimulated Syncytin-1 in CT, but was inhibited in BeWo. The significant inhibition of Syncytin-1 with TZ in BeWo was confirmed in two other choriocarcinoma cell lines JAR (fivefold with TZ compared to control; $P = 0.009$) and Jeg3

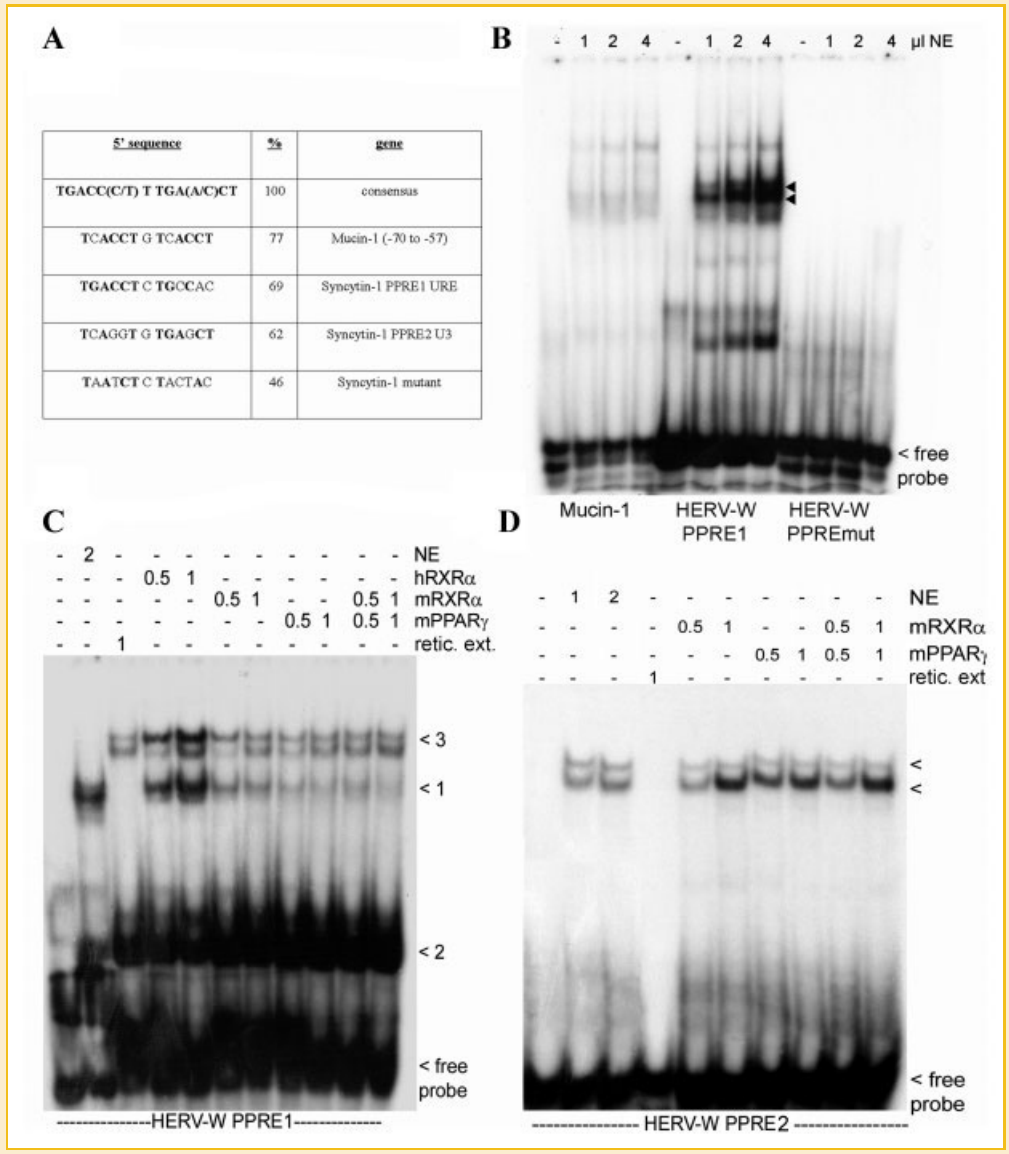



Fig. 2. EMSA analysis of HERV-W PPRE1 and PPRE2. A: DNA 5′ sequences of HERV-W PPREs used in EMSA analysis in B–D. The percent similarity to the PPRE consensus is shown for each fragment. B: EMSA analysis using gene DNA fragments (below) with indicated µl nuclear extracts (NE). DNA specific DNA/protein shifts are indicated with arrows. C: EMSA analysis using the HERV-W PPRE1 gene DNA fragment (below) with µl nuclear extracts (NE), or µl of the recombinant proteins human RXRα, mouse RXRα and mouse PPARγ. The specific DNA/protein shift (1) is indicated to the right. The DNA/protein shifts (2 and 3) represent non-specific DNA interactions. D: EMSA analysis using the HERV-W PPRE2 gene DNA fragment with µl nuclear extracts (NE), or µl of the recombinant proteins human RXRα, mouse RXRα and mouse PPARγ. Two specific DNA/protein shifts are indicated to the right as arrows.

(4.9-fold with TZ compared to control; $P = 0.009$; data not shown). Additionally, 9-*cis*-RA inhibited INSL4 in CT, but was stimulated in BeWo (Figs. 1A and 3A). Another difference was the microscopic detection of giant nuclei in BeWo, but not in CT. These giant nuclei were over threefold the size of normal nuclei in BeWo and were significantly up-regulated with FK and inhibited with 9-*cis*-RA and SB203580 (Fig. 3C).

### HERV-W PROMOTOR AND LUCIFERASE ASSAYS

Based upon our above findings supporting Syncytin-1 as a PPARγ and RXRα target gene in placentogenesis we tested, if the different PPREs in the HERV-W 5′LTR and the adjacent upstream regulatory element (URE) could function in luciferase assays. We tested six different HERV-W 5′LTR and adjacent URE luciferase constructs in BeWo cells with the same activators and inhibitors of PPARγ and RXRα used in our culture analysis (Fig. 4). We predicted that these constructs would determine promoter activity in conjunction with transcriptional gene activation or inhibition via PPREs. Normalized luciferase values showed that the highest basal promoter activity in BeWo cells stemmed from the URE/U3/R compared to the full-length construct ($618.68 \pm 33.52$-fold vs. $250 \pm 7.18$-fold). The finding that the U5 region alone harboring the PPRE-3 conferred non-significant luciferase activity ($11.53 \pm 2.10$) not only supports a lack of innate U5 promoter activity in HERV-W but also inhibitory cooperative

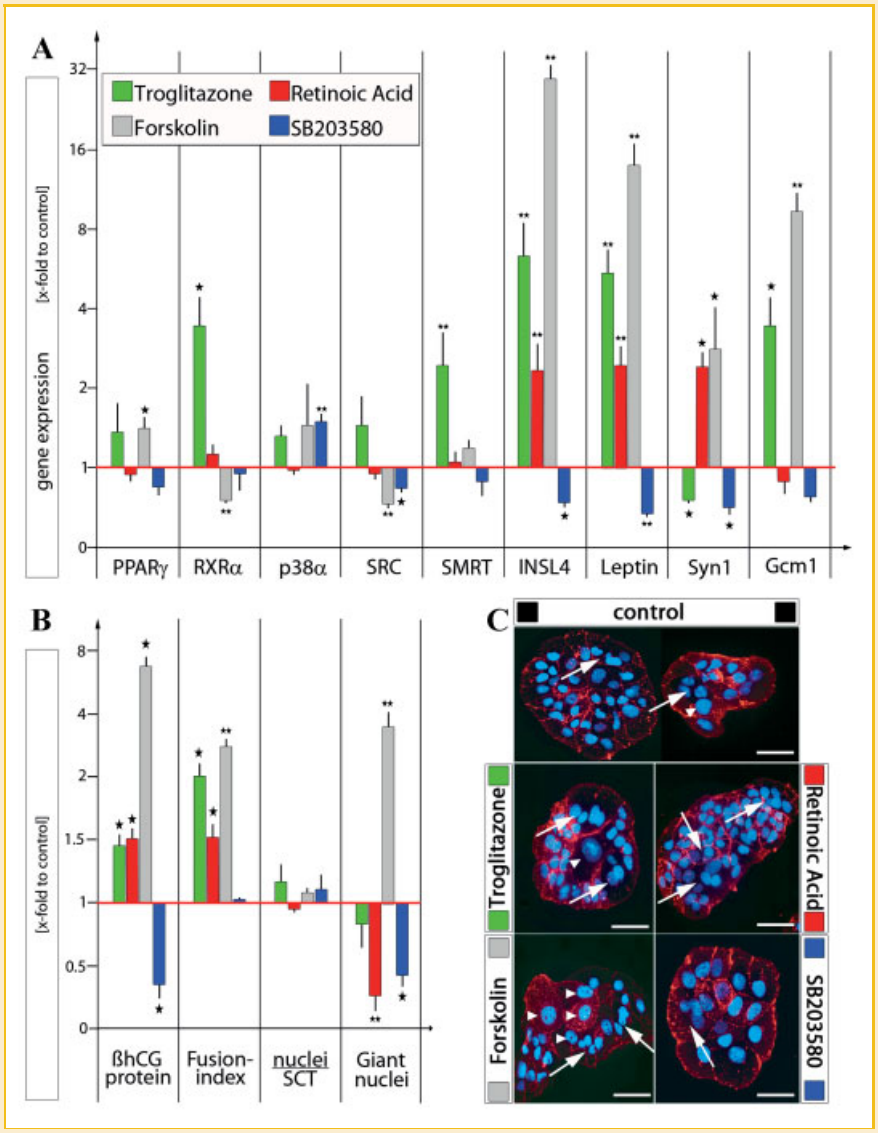



Fig. 3. Gene expression, protein quantification, and cell fusion of cultured BeWo cells treated with PPARγ/RXRα inhibitors and activators. A: Histographs represent expression in fold-changes compared to control (*Y*-axis) for each gene indicated below following treatment of BeWo with inhibitors and activators (top inset). Four independent experiments were performed. Statistical significances ($^{*}P < 0.05$; $^{**}P < 0.005$) are indicated; B: Histograph represents fold-change of hCG-protein levels, cell-fusion index, nuclei per SCT, and giant nuclei. Statistical significances ($^{*}P < 0.05$; $^{**}P < 0.005$) are indicated. All values are listed in Supplemental Table 2. C: BeWo cultures on day 3 demonstrating examples of cell fusions, following no treatment (control), treatment with inhibitor SB203580 and three activators. Cells were stained with cell membrane stain (agglutinin 594 = red) and counterstained with Hoechst 33342 for nuclei (blue). Arrows indicate cell fusions, arrow heads indicate giant nuclei. Syn1, Syncytin-1; Bars = 50 μm. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

properties, for example, possibly with the URE inhibitory domains (MaLR) in the full-length construct (Fig. 4). Since both the 5′LTR U3/R/U5 construct and the U3/R construct were similar in basic luciferase activity (117.08 ± 7.94-fold vs. 73.74 ± 5.46-fold) supports that significant promoter activity can be driven from the U3/R region alone. Analyzing the URE alone showed almost no luciferase activity (4.99 ± 2.77) and thus represents a lack of innate promoter activity. However, comparing luciferase activity in the presence or absence of the URE, for example, URE/U3/R and the U3/R constructs supports positive cooperative interactions via specific regulatory elements in the HERV-W URE possibly the positive regulatory activity 307 bp upstream of the MaLR (Fig. 4).

Regarding luciferase values following transcriptional induction with TZ, 9-*cis*-RA and FK demonstrated that the full-length construct containing all three PPREs had the highest statistically significant inducible luciferase activity compared to basal promoter activity (Fig. 4). Furthermore, treatment with FK resulted in significant luciferase induction among all constructs containing the U3 region, which was contributed not only through indirect induction of PPARγ/RXRα activity via PKA, but also by cAMP activation leading to CREB binding of the FK responsive element (Fig. 4). Separation of PPREs, for example, URE/U3/R with PPRE-1 and -2 and the U3/R/U5 with PPRE-2 and -3 showed lower overall induction with TZ, 9-*cis*-RA and FK compared to the full-length

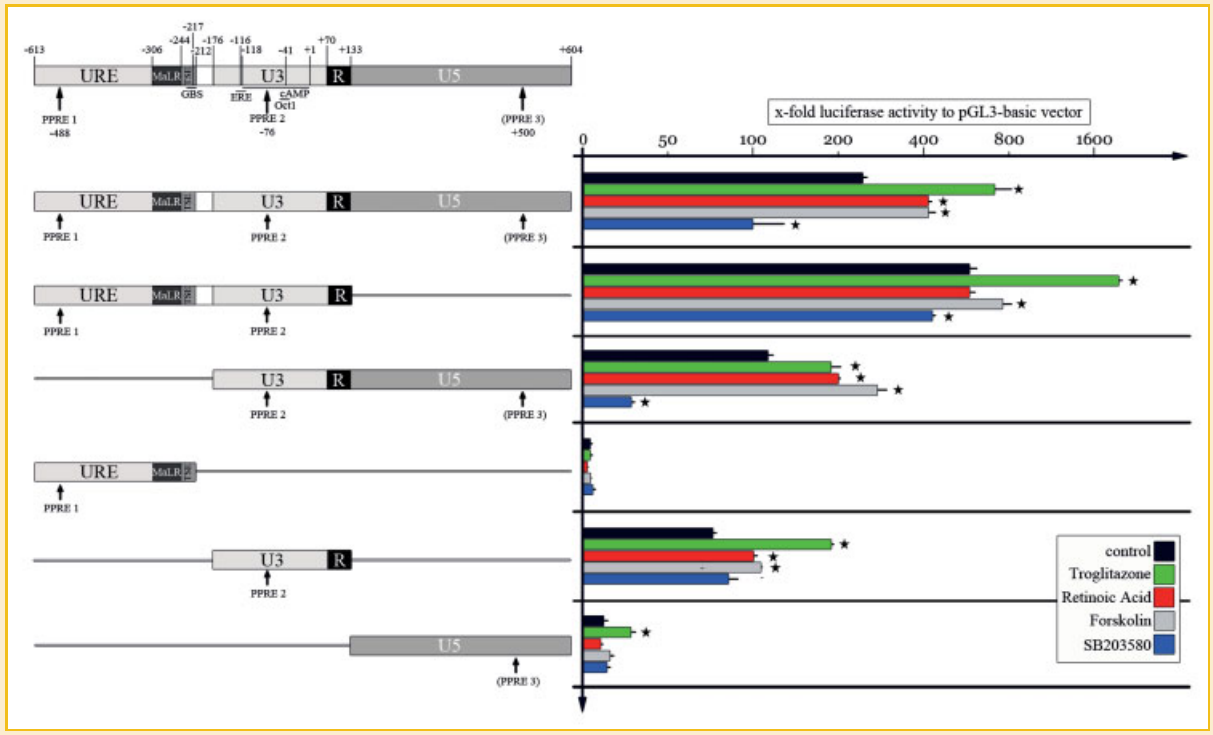


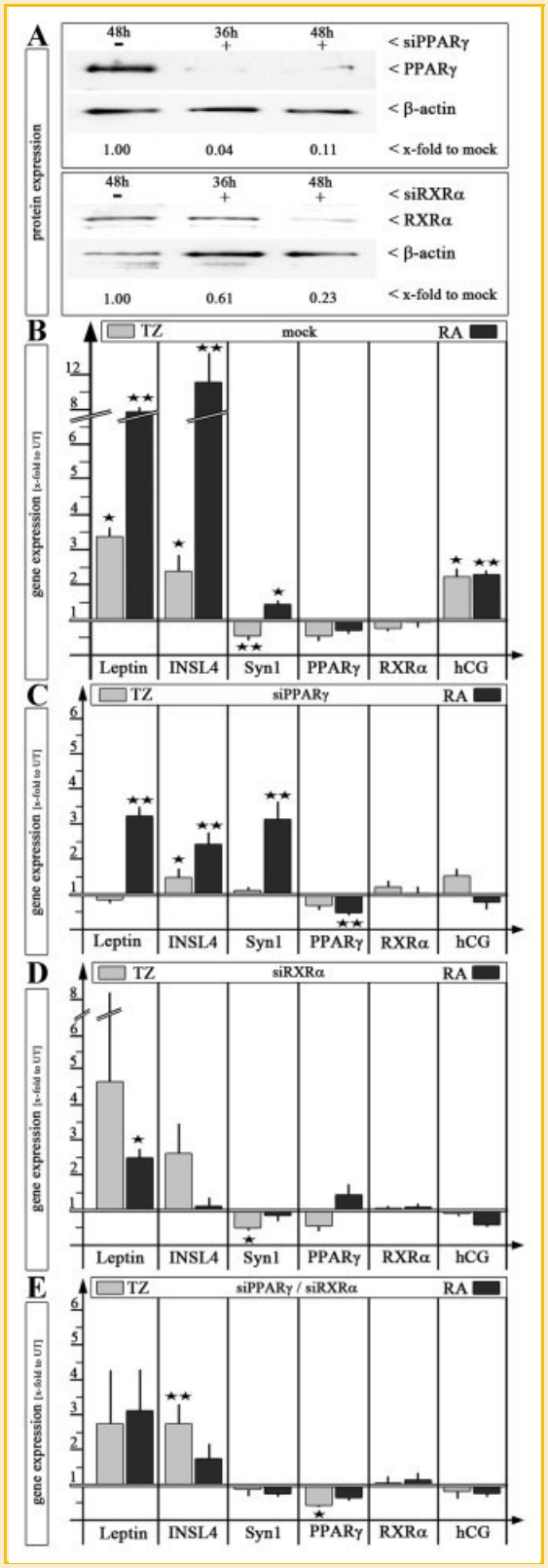

Fig. 4. Luciferase assays of six constructs from the 5′ HERV-W promoter region. URE and 5′LTR (U3/R/U5) of HERV-W construct represents 1217 base pairs in total and different deletion constructs below. PPREs 1–3 are indicated. Right diagram indicates fold-luciferase activity compared to the pGL3-basic vector represented as histographs with each corresponding construct (left) of BeWo transfected cells following +/− treatment with Troglitazone, retinoic acid (9-*cis*-RA), forskolin and SB203580 (right inset). Six independent transfections were performed. Standard error of the mean and statistical significance ($^{*}P < 0.05$) are indicated. MaLR, truncated non-autonomous mammalian apparent LTR retrotransposon; TSE, trophoblast specific enhancer. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

construct URE/U3/R/U5, but led to statistically significant luciferase induction. Interestingly, the URE harboring PPRE-1 alone showed no drug induction. In contrast to all activation studies, treatment of transfected BeWo cells with the p38α inhibitor SB203580 demonstrated statistically significant down-regulation of luciferase activity regarding the full-length, the URE/U3/R and the U3/R/U5; whereas URE, U3/R and U5 alone showed no changes compared to untreated controls (Fig. 4).

### KNOCK-DOWN OF PPARγ AND/OR RXRα GENES USING SPECIFIC siRNA

Knock-down of PPARγ and RXRα in BeWo cells was performed with specific siRNA-PPARγ and siRNA-RXRα to also prove specific Syncytin-1, Leptin, INSL4 and hCG target gene regulation (Fig. 5). A negative siRNA mock transfection control showed the same TZ and 9-*cis*-RA induction patterns for target genes compared to non-transfected BeWo cells (Figs. 3A and 5B). Following 48-h siRNA-PPARγ and siRNA-RXRα inhibited the RNA and protein expression of PPARγ 89% and RXRα 77% in BeWo cells (Fig. 5A and data not shown). Furthermore, we reasoned that TZ or 9-*cis*-RA treatment of PPARγ and RXRα knock-down cells would further prove transcriptional regulation by PPARγ and RXRα. For example, following TZ treatment of PPARγ knock-down cells resulted in no inhibition of Syncytin-1 expression in contrast to controls, supporting a requirement for PPARγ (Fig. 5B,C). However, TZ treatment of RXRα knock-down cells led to a significant reduction of Syncytin-1 expression similar to controls, supporting PPARγ binding (Fig. 5B,D). On the other hand, 9-*cis*-RA treatment of PPARγ knock-down cells resulted in a significant increase of Syncytin-1 expression, supporting a strong involvement of RXR (Fig. 5C). In line with these findings, 9-*cis*-RA treatment of cells lacking RXRα, Syncytin-1 induction was blocked (Fig. 5D). Treatment of cells with TZ or 9-*cis*-RA after the combined knock-down of PPARγ and RXRα Syncytin-1 expression showed no drug regulations demonstrating a specific requirement for both proteins (Fig. 5E).

Other PPARγ and RXRα gene targets like hCG, Leptin and INSL4 also showed a requirement for PPARγ and RXRα in knock-down experiments following TZ or 9-*cis*-RA cell treatment. HCG demonstrated no gene inductions with either TZ or 9-*cis*-RA treatment in all single or double PPARγ and RXRα knock-down experiments, supporting a need for both proteins. On the other hand, Leptin and INSL4 showed additional regulatory differences. For example, INSL4 expression levels remained significantly up-regulated in PPARγ knock-down and in combined PPARγ-RXRα knock-down cells after TZ induction (Fig. 5C,E). Leptin was induced in RXRα knock-down cells after 9-*cis*-RA treatment (Fig. 5D). These findings support homodimerizations or the involvement of other PPAR and RXR family members.




### EXPRESSION OF PPARγ/RXRα SIGNALING MEMBERS AND TARGET GENES IN PATHOLOGICAL PLACENTAL TISSUES

Primary placental tissues from four independent cohorts including control (n = 10), PE (n = 10), HELLP (n = 10), and IUGR (n = 10) patients were analyzed for gene expression of PPARγ/RXRα signaling in order to compare with the results of CT and BeWo experiments (Fig. 6, Supplemental Table 3 for clinical data). In addition to gene expression, we also performed Western analysis using a Syncytin-1 antibody and an ELISA for phospho-p38α and leptin to determine protein changes between the placenta cohorts (Fig. 6B,C, Supplemental Table 4). Regarding signaling members, significantly reduced gene expression levels of PPARγ and p38α in PE (2.1-fold, 1.4-fold), and SMRT in IUGR (1.4-fold) and of the active phosphorylated p38α protein in PE (2.1-fold) and IUGR (3.7-fold) were observed, supporting aberrant signaling (Fig. 6A). On the other hand, the gene expression of all other PPARγ/RXRα signaling members in pathological placentae showed no significant changes compared to controls. At the level of PPARγ/RXRα target gene expression, INSL4 showed significant down regulation in IUGR placentae (2.7-fold; $P = 0.015$) and ABCG2 in HELLP placentae compared to control (1.8-fold; $P = 0.027$). Leptin gene expression was statistically, significantly up-regulated in all three pathological placentae, PE (39-fold), HELLP (14.8-fold) and IUGR (3.3-fold). In addition, leptin protein levels were also significantly increased for PE (2.7-fold) and HELLP (4.5-fold), but were reduced for IUGR by 3.2-fold compared to controls (Fig. 6, Supplemental Table 4). Regarding gene and protein expression of the putative PPARγ/RXRα target gene Syncytin-1, the number of molecules/ng cDNA or normalized Syncytin-1 protein levels demonstrated a significant reduction in all three pathological placentae (PE 2.1-fold; HELLP 1.8-fold; IUGR 2.9-fold) compared to control placentae (Fig. 6, Supplemental Table 4).

## DISCUSSION

During the first trimester, PPARγ is detected in both villous and extravillous CT, and during the third trimester in SCT, pointing to an essential role for PPARγ in placentogenesis and SCT maintenance [Tarrade et al., 2001]. From this present investigation, we determined in human primary CT and BeWo trophoblastic cells that PPARγ/RXRα signaling is directly regulating Syncytin-1 and

Fig. 5. Knock-down of PPARγ and/or RXRα genes using specific siRNA. A: Western immunohybridization of siRNA-PPARγ and siRNA-RXRα transfected BeWo cells using PPARγ or RXRα antibodies at 36 and 48-h post-transfection. Forty-eight hours post-transfection represented the time for drug treatment of cells. A mock siRNA Alexa488 scrambled negative siRNA was used as a transfection control (48 h). The fold-change for each protein normalized to β-actin is shown below. B: Histographs represent the mean expression in fold-changes for six independent transfections for each gene normalized to the untreated (UT) mock transfected BeWo cells set to one following treatment with either TZ or 9-*cis*-RA (Fig. 3A). C–E: Histographs represent the mean expression in fold-changes of six independent transfections for each gene normalized to siRNA transfected control (no TZ or 9-*cis*-RA) set to one. Experiments represent single or double siRNA-PPARγ or siRNA-RXRα transfected BeWo cells treated with TZ or 9-*cis*-RA. Statistical significances ($^{*}P < 0.05$; $^{**}P < 0.005$). Syn1, Syncytin-1.

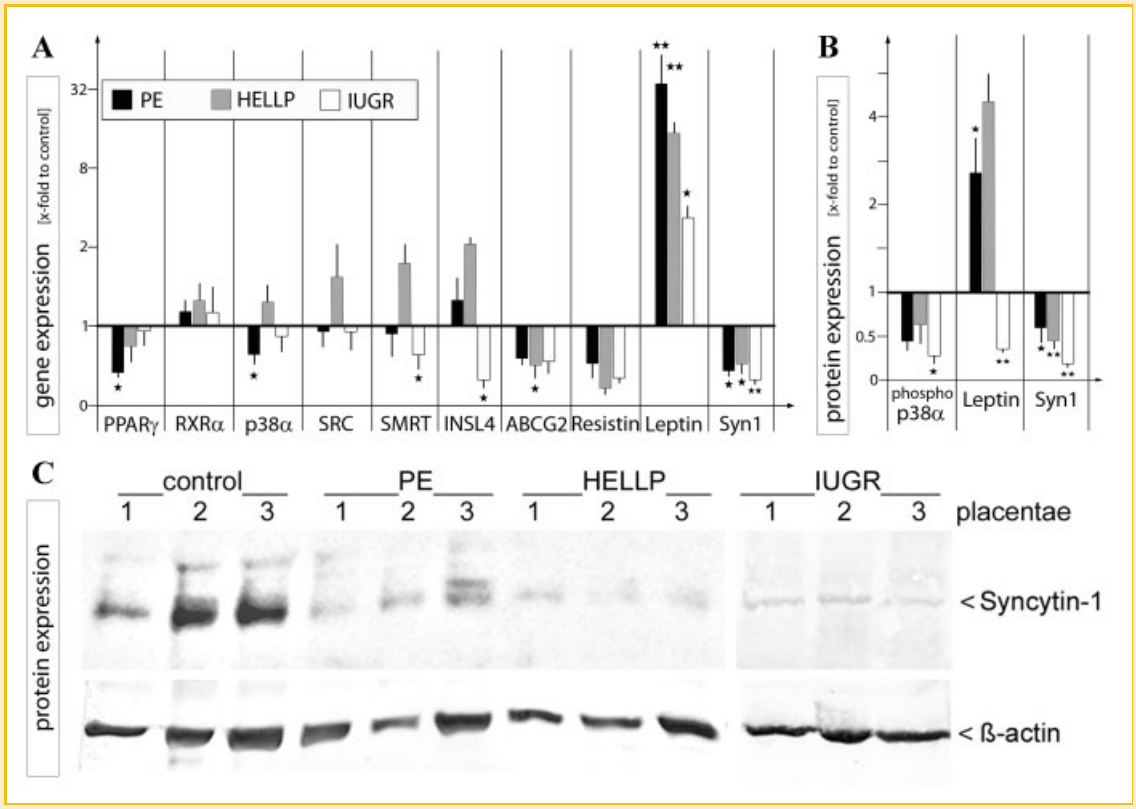



Fig. 6. Gene expression of human placentae from patients with PE (n = 10), HELLP (n = 10), and IUGR (n = 10). A and B: Histographs represent the mean expression in fold-changes for each gene or protein indicated below compared to control placentae (n = 10; *Y*-axis), which were set to a value of one. Statistical significances ($^{*}P < 0.05$; $^{**}P < 0.005$) are indicated. All values are listed in Supplemental Table 4. Syn1, Syncytin-1. C: Example of an immunoblot of Syncytin-1 and ß-actin detected in cell lysates from three of ten different control, PE, HELLP, and IUGR placentae shown in (B).

cell fusion essential for SCT formation. In addition, our findings implicating disturbed PPARγ/RXRα signaling in pathological human pregnancies brings forth new information relating to regulation of placentogenesis and for understanding placental disorders.

A model representing PPARγ/RXRα signaling pathways including the target gene Syncytin-1 is depicted in Figure 7 and is supported by our findings. Regarding normal placentogenesis, direct activation of PPARγ/RXRα signaling in a ligand specific manner with TZ or 9-*cis*-RA was shown to enhance SCT differentiation of fractionated placental CT (Fig. 1). Syncytin-1 gene expression and known PPARγ/RXRα target genes were found specifically up- or down regulated by TZ and 9-*cis*-RA or through cross-talk via p38α MAP kinase or cAMP/PKA pathways. Furthermore, TZ or 9-*cis*-RA treatment of PPARγ and RXRα gene knock-down cells proved the requirement of these proteins for Syncytin-1 regulation (Fig. 5). Upon down regulation of Syncytin-1 expression via p38α inhibition (SB203580) also led to an up-regulation of the co-repressor SMRT, which represses PPARγ transcriptional activity by blocking its downstream action. Strikingly, since TZ and 9-*cis*-RA led to significant increased CT cell fusion (FI) and together with the above findings, supports the idea that upon PPARγ/RXRα transcriptional activation, Syncytin-1 induction contributes to the formation of the placental SCT layer (Fig. 1).

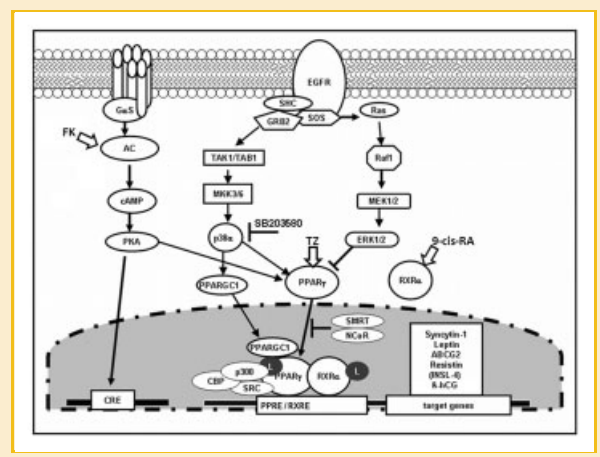



Fig. 7. Model of PPARγ/RXRα cellular signaling. PPARγ/RXRα signaling based upon EGFR activation by EGF resulting in p38α induction and ERK1/2 repression of PPARγ. In addition, activation of PPARγ by adenylate cyclase (AC) induction is also indicated. PPARγ/RXRα target genes analyzed in this study are shown below. FK indirectly activates PPARγ via AC and cAMP induction, SB203580 is a direct inhibitor of p38α (MAPK14) kinase; L, ligands of PPARγ, like TZ as a synthetic activator of PPARγ, as well as natural ligands like 15-HETE, 9- or 13-HODE, and 15Δ-PGJ2; L, ligand of RXRα designated as 9-*cis*-RA, which is a natural activator of RXRα.

FK was previously shown to induce PPARγ indirectly via stimulation of adenylate cyclase and PKA phosphorylation [Lazennec et al., 2000]. Besides PKA, PPARγ can also be phosphorylated by the extracellular receptor kinase–mitogen activated protein kinase and the 5′-AMP-activated protein kinase [Zhang et al., 1996; Leff, 2003]. In addition, FK directly activated CREB binding to the upstream forskolin-responsive domain in the U3 region of the HERV-W 5′LTR and led to increased Syncytin-1 expression and cell fusions in CT and carcinomas [Prudhomme et al., 2004; Strick et al., 2007] (Figs. 1B and 4). In the present study, FK significantly activated PPARγ/RXRα signaling in CT and BeWo cells and resulted in the highest levels of leptin, hCG and Syncytin-1 and an increase in the number of nuclei per fused cell or cell fusion when compared to all other activators (Figs. 1 and 3). The fact that Syncytin-1 evolved to be regulated by separate transcription factors ensures increased expression levels for SCT formation essential for a successful pregnancy.

It is important to note from our study that the comparison of primary CT and BeWo cells after incubation with different PPARγ/RXRα signaling activators and an inhibitor showed several similarities, but also significant antagonistic differences. An example for a significant antagonistic effect between cultured CT and BeWo, JAR and JEG cells was the RXRα and Syncytin-1 expression following treatment with TZ (Figs. 1 and 3). Despite decreased Syncytin-1 levels following TZ treatment in BeWo, the FI still increased significantly. One direct transcription factor of Syncytin-1 Gcm-1 was significantly induced by TZ and FK in BeWo cells (Fig. 3A, Supplemental Table 2). Chang et al. [2005] demonstrated that transfected Gcm-1 was activated by FK and protein kinase A in a reporter assay. FK induced Gcm-1, Syncytin-1 and the FI in BeWo cells, but the fact that TZ induced Gcm-1 and the FI, but decreased Syncytin-1 could indicate that in the presence of TZ other cell fusogenic genes like Syncytin-2 and envP(b) mediated cell fusion [Ruebner et al., 2010; Strick et al., 2011]. It is also noteworthy that the higher spontaneous cell FI of primary CT ($62.5 \pm 5.6\%$) compared to a 9.2-fold lower FI of BeWo cells ($6.7 \pm 1.4\%$) further points to a deregulation of cell fusion. In addition, another difference to CT was the presence of giant nuclei in BeWo cells after FK treatment (Figs. 1C and 3C). The origin or formation of these giant nuclei is presently unknown, but endoreduplication, as found in giant nuclei mouse trophoblasts and tumor cells or endopolyploidy through fusion of nuclei is conceivable [Strick et al., 2011]. In summary, we attribute that these regulatory differences between primary CT and choriocarcinoma cells are due to the tumor phenotype, which may also be important for understanding the etiology of this rare disease.

Additional support for Syncytin-1 representing a new PPARγ/RXRα target gene came from EMSA, siRNA-PPARγ and siRNA-RXRα knockdown experiments and luciferase functional assays. Since HERV-W PPRE-1 and PPRE-2 specific binding occurred with CT nuclear extracts containing active PPARγ and EMSA experiments with recombinant mouse and human PPARγ and RXRα expressed proteins; this points to functional PPARγ/RXRα transcriptional regulation of Syncytin-1 in CT (Fig. 2). Further support for PPARγ and RXRα homo- or heterodimer binding stems from results that recombinant mouse and human RXRα proteins specifically bound alone or in combination. PPRE and RXRE DNA binding motifs consist of two direct repeats similar to "AGGTCA" divided by a one nucleotide spacer [Tan et al., 2005]. Interestingly, the specific DNA binding domain of the hetero-complex PPARγ/RXRα displays half-site selective binding that results in a polar arrangement of their DNA binding domains, with PPARγ residing upstream of RXRα. Both PPARγ and RXRα DNA binding domains interact within the minor groove of the spacer, where PPARγ mediates more nucleotide base and phosphate backbone interactions compared with RXRα. In addition, the PPARγ hinge region also has extensive DNA interactions as well, binding to the upstream AAACT element, so-called extension site [Jpenberg et al., 1997]. Crystallization studies of PPARs showed that full agonists stabilized helix H12 of the ligand binding domain in the active conformation and partial agonists interacted with other regions than helix 12. In addition, helix 12 also regulated the binding of the co-regulators [Gampe et al., 2000; Waku et al., 2009]. Further data showed that RXR binding to the 3′half of PPRE was more stringent than PPARγ binding to the 5′half of PPRE. The PPRE "5′CGGCCAnAGGTCA" resulted in DNA binding, whereas the 5′ to 3′ switch to "5′AGGTCAnCGGCCA" abolished all binding, indicating that especially for RXR binding the 3′half has to be the consensus [Temple et al., 2005]. In contrast to the above finding, our EMSA experiments demonstrated specific protein binding with HERV-W PPRE-1, which has a consensus 3′half, but also with PPRE-2, harboring a non-consensus 3′half (Fig. 2). A consensus prediction for the 3′half of the PPRE was not found using DNA binding experiments in a comparative analysis [Juge-Aubry et al., 1997]. For example, non-consensus 3′half PPREs of human genes resulted in over 70% DNA binding. In contrast to the HERV-W PPRE-1, which had a 100% non-consensus extension site (0/6), the extension site of PPRE-2 was 5/6 with the consensus (AACTt), indicating that the PPARγ hinge region could be more involved in DNA binding of the PPRE-2.

SiRNA knock-down experiments of PPARγ and RXRα demonstrated additional PPAR and RXR regulations at the level of homo- or heterodimers of target genes. Importantly, PPARα, PPARβ and PPARγ as well as RXRα and RXRγ were detected in human placentae, which can form different scenarios of heterodimerizations [Jpenberg et al., 1997; Wang et al., 2002]. TZ treatment of BeWo cells knock-downed for PPARγ by siRNA resulted in no inhibition of Syncytin-1, whereas 9-*cis*-RA induced its expression (Fig. 5C). The following consequences of the latter would have been possible: Heterodimers of other family members like PPARα or PPARβ with activated RXRα or RXRγ or homodimerizations of activated RXR could have been responsible for the Syncytin-1 induction. A combined knock-down of PPARγ and RXRα showed no regulation of Syncytin-1 in the presence of TZ or 9-*cis*-RA (Fig. 5E) indicating the requirement of PPARγ and RXRα for Syncytin-1 regulation. On the other hand, the induction of, for example, INSL4 in knock-down PPARγ cells and following TZ treatment demonstrated the possibility of heterodimerizations of other family members of PPAR with RXR or homodimerizations of RXR (Fig. 5C,E). These knock-down experiments showed that in BeWo cells and probably also in CT different states of the three PPAR and RXRα and RXRγ hetero- and homodimerizations were possible.

Regarding luciferase studies, previous reports have defined the essential HERV-W 5′LTR regions for promoter activity in CT and cell lines [Prudhomme et al., 2004; Cheng and Handwerger, 2005]. Results showed that 436 bp upstream of the 5′LTR were sufficient for high promoter activity in BeWo cells. In addition, a positive regulatory activity was identified in the URE, 307 bp upstream of the MaLR containing a 62 bp negative promoter regulator [Prudhomme et al., 2004] (Fig. 4). Similar results were demonstrated with fractionated human trophoblasts, where mutations of the CCAAT- and Oct-sites in the U3 region almost completely abolished promoter activity [Cheng and Handwerger, 2005]. Interestingly, the mutations of the CCAAT-binding site involved specific nucleotides just 3′ of the PPRE-2. A trophoblast specific enhancer (TSE) was also identified by Cheng and Handwerger [2005], where 147 bp of the URE including 29 bp of the U3 of the 5′LTR were found responsible for promoter activity in BeWo and Jeg3, but not in non-trophoblastic cells. Our study confirmed HERV-W 5′LTR promoter regions in BeWo cells, but also demonstrated that luciferase activity can be modulated via PPARγ activators and inhibitors through PPREs of the HERV-W 5′LTR. The finding that the full-length construct harboring all three PPREs and the URE/U3/R with PPRE-1 and -2 conferred the highest promoter activity when induced by TZ, 9-*cis*-RA, and FK points to transcriptionally active PPREs via PPARγ/RXRα binding (Fig. 4). On the other hand, all three PPREs or PPRE-1 and -2 in reporter constructs demonstrated promoter inhibition via the p38α inhibitor SB203580. In addition, the U5 alone without induction showed no significant innate promoter activity, but demonstrated significant promoter activation with TZ corroborating that the PPRE-3 at +500 has its own regulatory activity. We observed a stimulatory effect of the episomal HERV-W 5′LTR-luciferase gene after TZ in BeWo cells, but a converse effect of endogenous Syncytin-1 in BeWo, JAR and Jeg3 following TZ (Fig. 3A). Furthermore, indirect FK induction proved the proposed FK responsive site in the U3 region by PKA [Prudhomme et al., 2004]. However, the induction of U3/R with FK showed a 51% induction compared to the 5′LTR, and only 11% for the URE/U3/R, indicating for additional FK regulation on the PPARγ/RXRα signaling (Fig. 4). Taken together, our results from CT cultures, EMSA and luciferase experiments prove Syncytin-1 as a PPARγ/RXRα target gene.

Leptin, ABCG2, and INSL4 have been implicated in the etiology of the pregnancy syndromes PE, HELLP, and IUGR. In agreement with other studies, leptin mRNA was significantly up-regulated in all three placental syndromes (Fig. 6) [Lea et al., 2000; Struwe et al., 2010]. Especially, HERV env genes and their receptors were deregulated in placental syndromes resulting in aberrant CT cell fusion to SCT [Chen et al., 2006; Langbein et al., 2008; Ruebner et al., 2010]. Although PPARγ/RXRα signaling has been implicated in PE, HELLP, and IUGR, a previous study showed no differences in gene and protein expression of PPARγ and RXRα between controls and PE or IUGR placentae [Rodie et al., 2005]. Other studies identified reduced p38α phosphorylation in 70% of IUGR [Laviola et al., 2005] and reduced catalytic activity of p38α and phospho-p38α in PE [Webster et al., 2006]. In our investigation, PE placentae showed reduced levels of PPARγ mRNA compared to controls. Additionally, decreased p38α phosphorylation for both PE ($P = 0.069$) and IUGR ($P = 0.002$) could be linked with differential deregulated gene expression of leptin, INSL4 and Syncytin-1 (Fig. 6, Supplemental Table 4). Decreased Syncytin-1 levels could result in an aberrant SCT membrane contributing to the placental pathology and a reduction of nutrient exchange, especially in IUGR [Ruebner et al., 2010]. Importantly, a reduction of both Syncytin-1 and the FI was demonstrated in CT cultures by inhibiting PPARγ signaling (Fig. 1). Regarding aberrant leptin over expression in placental disorders, despite a decrease in p38α activity, we identified from our CT culture experiments that leptin is highly regulated by PPARγ in the presence of several ligands. Thus, a leptin over-compensation in both PE and IUGR could occur as a placental or maternal response due to PPARγ ligands, which were increased in PE CT cultures and patient serum [Johnson et al., 1998]. Addition of 15Δ-PGJ2 and oxidized lipids to CT cultures showed significant increase of leptin and Syncytin-1 (Supplemental Fig. 1). Lastly, INSL4 is regulated by a 3′-LTR of a HERV-element inserted in the promoter and harbors PPRE elements where regulation via PPARγ/RXRα could occur [Bieche et al., 2003] (Figs. 1 and 3).

In conclusion, we propose that Syncytin-1 is regulated by PPARγ/RXRα signaling during placentogenesis to drive SCT formation and maintenance. Furthermore, de-regulation of Syncytin-1 expression could contribute to PE, HELLP, and IUGR, resulting in aberrant cell fusion and a reduced SCT layer affecting the maternal-fetal exchange of nutrients, gas, and waste products. Understanding Syncytin-1 and other HERV-env gene regulations, especially through PPARγ/RXRα signaling is critical for unraveling the underlying basis for placentogenesis, placental syndromes, and choriocarcinomas.

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## REFERENCES

Allen M, Svensson L, Roach M, Hambor J, McNeish J, Gabel CA. 2000. Deficiency of the stress kinase p38alpha results in embryonic lethality: Characterization of the kinase dependence of stress responses of enzyme-deficient embryonic stem cells. J Exp Med 191:859–870.

Andrews NC, Faller DV. 1991. A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. Nucleic Acids Res 19:2499.

Badger AM, Bradbeer JN, Votta B, Lee JC, Adams JL, Griswold DE. 1996. Pharmacological profile of SB 203580, a selective inhibitor of cytokine suppressive binding protein/p38 kinase, in animal models of arthritis, bone resorption, endotoxin shock and immune function. J Pharmacol Exp Ther 279:1453–1461.

Bieche I, Laurent A, Laurendeau I, Duret L, Giovangrandi Y, Frendo JL, Olivi M, Fausser JL, Evain-Brion D, Vidaud M. 2003. Placenta-specific INSL4

expression is mediated by a human endogenous retrovirus element. Biol Reprod 68:1422–1429.

Chandra V, Huang P, Hamuro Y, Raghuram S, Wang Y, Burris TP, Rastinejad F. 2008. Structure of the intact PPAR-gamma-RXR-alpha nuclear receptor complex on DNA. Nature 456:350–356.

Chang CW, Chuang HC, Yu C, Yao TP, Chen H. 2005. Stimulation of GCMa transcriptional activity by cyclic AMP/protein kinase A signaling is attributed to CBP-mediated acetylation of GCMa. Mol Cell Biol 25:8401–8414.

Chawla A, Barak Y, Nagy L, Liao D, Tontonoz P, Evans RM. 2001. PPAR-gamma dependent and independent effects on macrophage-gene expression in lipid metabolism and inflammation. Nat Med 7:48–52.

Chen CP, Wang KG, Chen CY, Yu C, Chuang HC, Chen H. 2006. Altered placental syncytin and its receptor ASCT2 expression in placental development and pre-eclampsia. BJOG 113:152–158.

Cheng YH, Handwerger S. 2005. A placenta-specific enhancer of the human syncytin gene. Biol Reprod 73:500–509.

Daoud G, Amyot M, Rassart E, Masse A, Simoneau L, Lafond J. 2005. ERK1/2 and p38 regulate trophoblasts differentiation in human term placenta. J Physiol 566:409–423.

Evseenko DA, Paxton JW, Keelan JA. 2007. The xenobiotic transporter ABCG2 plays a novel role in differentiation of trophoblast-like BeWo cells. Placenta 28(Suppl A):S116–S120.

Forman BM, Tontonoz P, Chen J, Brun RP, Spiegelman BM, Evans RM. 1995. 15-Deoxy-delta 12, 14-prostaglandin J2 is a ligand for the adipocyte determination factor PPAR gamma. Cell 83:803–812.

Frendo JL, Olivier D, Cheynet V, Blond JL, Bouton O, Vidaud M, Rabreau M, Evain-Brion D, Mallet F. 2003. Direct involvement of HERV-W Env glycoprotein in human trophoblast cell fusion and differentiation. Mol Cell Biol 23:3566–3574.

Gampe RT, Jr., Montana VG, Lambert MH, Miller AB, Bledsoe RK, Milburn MV, Kliewer SA, Willson TM, Xu HE. 2000. Asymmetry in the PPARgamma/RXRalpha crystal structure reveals the molecular basis of heterodimerization among nuclear receptors. Mol Cell 5:545–555.

Hardie L, Trayhurn P, Abramovich D, Fowler P. 1997. Circulating leptin in women: A longitudinal study in the menstrual cycle and during pregnancy. Clin Endocrinol (Oxf) 47:101–106.

Jameson JL, Hollenberg AN. 1993. Regulation of chorionic gonadotropin gene expression. Endocr Rev 14:203–221.

Johnson RD, Polakoski KL, Huang X, Sadovsky Y, Nelson DM. 1998. The release of 15-hydroxyeicosatetraenoic acid by human placental trophoblast is increased in preeclampsia. Am J Obstet Gynecol 178:54–58.

Jpenberg AI, Jeannin E, Wahli W, Desvergne B. 1997. Polarity and specific sequence requirements of peroxisome proliferator-activated receptor (PPAR)/retinoid X receptor heterodimer binding to DNA. A functional analysis of the malic enzyme gene PPAR response element. J Biol Chem 272:20108–20117.

Juge-Aubry C, Pernin A, Favez T, Burger AG, Wahli W, Meier CA, Desvergne B. 1997. DNA binding properties of peroxisome proliferator-activated receptor subtypes on various natural peroxisome proliferator response elements. Importance of the 5′-flanking region. J Biol Chem 272:25252–25259.

Kliman HJ, Nestler JE, Sermasi E, Sanger JM, Strauss JF III. 1986. Purification, characterization, and in vitro differentiation of cytotrophoblasts from human term placentae. Endocrinology 118:1567–1582.

Langbein M, Strick R, Strissel PL, Vogt N, Parsch H, Beckmann MW, Schild RL. 2008. Impaired cytotrophoblast cell-cell fusion is associated with reduced Syncytin and increased apoptosis in patients with placental dysfunction. Mol Reprod Dev 75:175–183.

Laurent A, Rouillac C, Delezoide AL, Giovangrandi Y, Vekemans M, Bellet D, Abitbol M, Vidaud M. 1998. Insulin-like 4 (INSL4) gene expression in human embryonic and trophoblastic tissues. Mol Reprod Dev 51:123–129.

Laviola L, Perrini S, Belsanti G, Natalicchio A, Montrone C, Leonardini A, Vimercati A, Scioscia M, Selvaggi L, Giorgino R, Greco P, Giorgino F. 2005. Intrauterine growth restriction in humans is associated with abnormalities in placental insulin-like growth factor signaling. Endocrinology 146:1498–1505.

Lazennec G, Canaple L, Saugy D, Wahli W. 2000. Activation of peroxisome proliferator-activated receptors (PPARs) by their ligands and protein kinase A activators. Mol Endocrinol 14:1962–1975.

Lea RG, Howe D, Hannah LT, Bonneau O, Hunter L, Hoggard N. 2000. Placental leptin in normal, diabetic and fetal growth-retarded pregnancies. Mol Hum Reprod 6:763–769.

Lee X, Keith JC, Jr., Stumm N, Moutsatsos I, McCoy JM, Crum CP, Genest D, Chin D, Ehrenfels C, Pijnenborg R, van Assche FA, Mi S. 2001. Downregulation of placental syncytin expression and abnormal protein localization in pre-eclampsia. Placenta 22:808–812.

Leff T. 2003. AMP-activated protein kinase regulates gene expression by direct phosphorylation of nuclear proteins. Biochem Soc Trans 31:224–227.

Lehrke M, Lazar MA. 2005. The many faces of PPARgamma. Cell 123:993–999.

Masuzaki H, Ogawa Y, Sagawa N, Hosoda K, Matsumoto T, Mise H, Nishimura H, Yoshimasa Y, Tanaka I, Mori T, Nakao K. 1997. Nonadipose tissue production of leptin: Leptin as a novel placenta-derived hormone in humans. Nat Med 3:1029–1033.

McKenna NJ, O'Malley BW. 2002. Combinatorial control of gene expression by nuclear receptors and coregulators. Cell 108:465–474.

Mi S, Lee X, Li X, Veldman GM, Finnerty H, Racie L, LaVallie E, Tang XY, Edouard P, Howes S, Keith JC, Jr., McCoy JM. 2000. Syncytin is a captive retroviral envelope protein involved in human placental morphogenesis. Nature 403:785–789.

Mudgett JS, Ding J, Guh-Siesel L, Chartrain NA, Yang L, Gopal S, Shen MM. 2000. Essential role for p38alpha mitogen-activated protein kinase in placental angiogenesis. Proc Natl Acad Sci USA 97:10454–10459.

Nagy L, Tontonoz P, Alvarez JG, Chen H, Evans RM. 1998. Oxidized LDL regulates macrophage gene expression through ligand activation of PPARgamma. Cell 93:229–240.

Orendi K, Kivity V, Sammar M, Grimpel Y, Gonen R, Meiri H, Lubzens E, Huppertz B. 2011. Placental and trophoblastic in vitro models to study preventive and therapeutic agents for preeclampsia. Placenta 32(Suppl): S49–S54.

Pijnenborg R, Bland JM, Robertson WB, Brosens I. 1983. Uteroplacental arterial changes related to interstitial trophoblast migration in early human pregnancy. Placenta 4:397–413.

Potgens AJ, Schmitz U, Bose P, Versmold A, Kaufmann P, Frank HG. 2002. Mechanisms of syncytial fusion: A review. Placenta 23(Suppl A):S107–S113.

Prudhomme S, Oriol G, Mallet F. 2004. A retroviral promoter and a cellular enhancer define a bipartite element which controls env ERVWE1 placental expression. J Virol 78:12157–12168.

Rodie VA, Young A, Jordan F, Sattar N, Greer IA, Freeman DJ. 2005. Human placental peroxisome proliferator-activated receptor delta and gamma expression in healthy pregnancy and in preeclampsia and intrauterine growth restriction. J Soc Gynecol Investig 12:320–329.

Ruebner M, Strissel PL, Langbein M, Fahlbusch F, Wachter DL, Faschingbauer F, Beckmann MW, Strick R. 2010. Impaired cell fusion and differentiation in placentae from patients with intrauterine growth restriction correlate with reduced levels of HERV envelope genes. J Mol Med (Berl) 88:1143–1156.

Schild RL, Sonnenberg-Hirche CM, Schaiff WT, Bildirici I, Nelson DM, Sadovsky Y. 2006. The kinase p38 regulates peroxisome proliferator activated receptor-gamma in human trophoblasts. Placenta 27:191–199.

Shalom-Barak T, Nicholas JM, Wang Y, Zhang X, Ong ES, Young TH, Gendler SJ, Evans RM, Barak Y. 2004. Peroxisome proliferator-activated receptor

gamma controls Muc1 transcription in trophoblasts. Mol Cell Biol 24:10661–10669.

Sibai BM. 2004. Diagnosis, controversies, and management of the syndrome of hemolysis, elevated liver enzymes, and low platelet count. Obstet Gynecol 103:981–991.

Strauss JF III, Kido S, Sayegh R, Sakuragi N, Gafvels ME. 1992. The cAMP signalling system and human trophoblast function. Placenta 13:389–403.

Strick R, Ackermann S, Langbein M, Swiatek J, Schubert SW, Hashemolhosseini S, Koscheck T, Fasching PA, Schild RL, Beckmann MW, Strissel PL. 2007. Proliferation and cell-cell fusion of endometrial carcinoma are induced by the human endogenous retroviral Syncytin-1 and regulated by TGF-beta. J Mol Med (Berl) 85:23–38.

Strick R, Beckmann MW, Strissel PL. 2011. Cell-cell fusions and human endogenous retroviruses in cancer. In: Larsson LI, editor. Cell fusions. Dordrecht, Heidelberg, London, New York: Springer Verlag+Business Media. pp 395–426.

Struwe E, Berzl G, Schild R, Blessing H, Drexel L, Hauck B, Tzschoppe A, Weidinger M, Sachs M, Scheler C, Schleussner E, Dotsch J. 2010. Microarray analysis of placental tissue in intrauterine growth restriction. Clin Endocrinol (Oxf) 72:241–247.

Szatmari I, Vamosi G, Brazda P, Balint BL, Benko S, Szeles L, Jeney V, Ozvegy-Laczka C, Szanto A, Barta E, Balla J, Sarkadi B, Nagy L. 2006. Peroxisome proliferator-activated receptor gamma-regulated ABCG2 expression confers cytoprotection to human dendritic cells. J Biol Chem 281:23812–23823.

Tan NS, Michalik L, Desvergne B, Wahli W. 2005. Multiple expression control mechanisms of peroxisome proliferator-activated receptors and their target genes. J Steroid Biochem Mol Biol 93:99–105.

Tarrade A, Schoonjans K, Guibourdenche J, Bidart JM, Vidaud M, Auwerx J, Rochette-Egly C, Evain-Brion D. 2001. PPAR gamma/RXR alpha heterodimers are involved in human CG beta synthesis and human trophoblast differentiation. Endocrinology 142:4504–4514.

Temple KA, Cohen RN, Wondisford SR, Yu C, Deplewski D, Wondisford FE. 2005. An intact DNA-binding domain is not required for peroxisome proliferator-activated receptor gamma (PPARgamma) binding and activation on some PPAR response elements. J Biol Chem 280:3529–3540.

Waku T, Shiraki T, Oyama T, Morikawa K. 2009. Atomic structure of mutant PPARgamma LBD complexed with 15d-PGJ2: Novel modulation mechanism of PPARgamma/RXRalpha function by covalently bound ligands. FEBS Lett 583:320–324.

Wang Q, Fujii H, Knipp GT. 2002. Expression of PPAR and RXR isoforms in the developing rat and human term placentas. Placenta 23:661–671.

Webster RP, Brockman D, Myatt L. 2006. Nitration of p38 MAPK in the placenta: Association of nitration with reduced catalytic activity of p38 MAPK in pre-eclampsia. Mol Hum Reprod 12:677–685.

Willson TM, Lehmann JM, Kliewer SA. 1996. Discovery of ligands for the nuclear peroxisome proliferator-activated receptors. Ann N Y Acad Sci 804:276–283.

Wilson ML, Goodwin TM, Pan VL, Ingles SA. 2003. Molecular epidemiology of preeclampsia. Obstet Gynecol Surv 58:39–66.

Yura S, Sagawa N, Itoh H, Kakui K, Nuamah MA, Korita D, Takemura M, Fujii S. 2003. Resistin is expressed in the human placenta. J Clin Endocrinol Metab 88:1394–1397.

Zhang B, Berger J, Zhou G, Elbrecht A, Biswas S, White-Carrington S, Szalkowski D, Moller DE. 1996. Insulin- and mitogen-activated protein kinase-mediated phosphorylation and activation of peroxisome proliferator-activated receptor gamma. J Biol Chem 271:31771–31774.