

Expression and Regulation of Retinoic Acid Receptor Responders in the Human Placenta

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Abstract

Introduction: Retinoic acid (RA) signaling through its receptors (RARA, RARB, RARG, and the retinoic X receptor RXRA) is essential for healthy placental and fetal development. An important group of genes regulated by RA are the RA receptor responders (RARRES1, 2, and 3). We set out to analyze their expression and regulation in healthy and pathologically altered placentas of preeclampsia (PE) and intrauterine growth restriction (IUGR) as well as in trophoblast cell lines. **Methods:** We performed immunohistochemical staining on placental sections and analyzed gene expression by real-time polymerase chain reaction. Additionally, we performed cell culture experiments and stimulated Swan71 and Jeg-3 cells with different RA derivatives and 2'-deoxy-5-azacytidine (AZA) to induce DNA demethylation. **Results:** RARRES1, 2, and 3 and RARA, RARB, RARG, and RXRA are expressed in the extravillous part of the placenta. RARRES1, RARA, RARG, and RXRA were additionally detected in villous cytotrophoblasts. RARRES gene expression was induced via activation of RARA, RARB, and RARG in trophoblast cells. RARRES1 was overexpressed in villous trophoblasts and the syncytiotrophoblast from PE placentas, but not in IUGR without PE. Promoter methylation was detectable for RARRES1 and RARB based on their sensitivity toward AZA treatment of trophoblast cell lines. **Discussion:** RARRES1, 2 and 3 are expressed in the functional compartments of the human placenta and can be regulated by RA. We hypothesize that the epigenetic suppression of trophoblast RARRES1 and RARB expression and the upregulation of RARRES1 in PE trophoblast cells suggest an involvement of environmental factors (eg, maternal vitamin A intake) in the pathogenesis of this pregnancy complication.

Keywords

placenta, preeclampsia, RARRES1, RARRES2, RARRES3

Introduction

Vitamin A is commonly used as a therapeutic agent for the treatment of psoriasis or in cancer therapy as it is known to induce tumor cell apoptosis and inhibition of cell proliferation.^{1,2} Retinoic acid (RA), the active derivative of vitamin A, is essential for healthy development and growth during embryogenesis and especially controls cellular key processes like development, differentiation, proliferation, and apoptosis.^{3,4} Vitamin A deficiency negatively affects different metabolic pathways, leading to a disruption of organogenesis (e.g. brain, kidney).⁵⁻⁷ RAs function through binding to RA receptors (RARs) and retinoic X receptors (RXRs) which are part of the steroid/thyroid/vitamin D3 nuclear receptor family.⁸ Both RARs and RXRs can form hetero- or homodimers which bind to specific DNA elements and act as ligand-inducible transcription factors.⁹

Furthermore, RAs are important regulators of human trophoblast functions during placentation and throughout gestation.⁴ RAs control trophoblast differentiation as well as hormone production within the syncytiotrophoblast (SCT).¹⁰ Metabolic

signaling pathways are regulated by RA-induced production and release of, for example, human chorionic gonadotropin or human placental lactogen.¹⁰⁻¹² RAs and their transcription factors additionally influence vital processes involved in trophoblast invasion, fusion, proliferation, and differentiation.¹²⁻¹⁵

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Retinoic acid receptor responders (*RARRES1*, 2 and 3) are a group of RA-inducible genes which were identified after stimulation of skin cell explants with the RARB-/G-specific retinoid Tazarotene (TAZA).² *RARRES1* (also known as Tazarotene-induced gene 1 (TIG1), Latexin-like (LXNL), or Phorbol Ester-induced gene 1 (PERG-1)) is a transmembrane protein with a glycosylation-signal and a hyaluronic acid binding motive.² *RARRES1* is proposed as a tumor suppressor gene, as it is often found downregulated in various carcinomas.^{16,17} It was shown that *RARRES1* is capable of inducing apoptosis and inhibiting proliferation and invasion of cancer cells.¹⁸⁻²⁰ *RARRES2* (also known as Tazarotene-induced gene 2 (TIG2), HP10433 or Chemerin) on the other hand, is predominantly found in its secreted form.²¹ It is a chemoattractant, which is expressed in immune cells as well as adipose tissue and induces the chemotactic recruitment of immune cells to inflammatory areas.²¹ High *RARRES2* serum levels were shown to be associated with, for example, psoriasis, diabetes, or cardiovascular disease.²²⁻²⁴ While *RARRES2* serum levels significantly increase during pregnancy,²⁵ little is known about its role during human placentation. In cancer, *RARRES2* facilitates cell migration and invasion and is correlated with inflammation.²⁶⁻²⁸ In contrast, *RARRES3* was shown to be a tumor-suppressor protein, which influences tumor cell metastasis by regulating cell adhesion and differentiation.²⁹ Its expression is downregulated in breast and colorectal carcinomas and loss of *RARRES3* correlates with the presence of lung or nodal metastasis.^{29,30}

RARRES1, 2, and 3 were previously shown to be directly regulated by the binding of RAR/RXR heterodimers to RARE consensus sequences of the respective promoter region.^{2,24,31} Furthermore, genes linked with retinoid signaling, like RARB, cellular retinol-binding protein 1, and *RARRES1*, were associated with DNA methylation in various carcinomas, and correlation of RARB and *RARRES1* methylation was demonstrated.^{32,33} While there are no CpGs (Cytosine nucleotides followed by guanine) within the RARE consensus sequence, DNA methylation of the surrounding CpG islands seems to influence transcriptional regulation of *RARRES* and *RAR* gene expression.³³⁻³⁵ Interestingly, hypermethylation of RARB promoter regions were shown in term placental samples, indicating a diminished RA signaling in late gestation.³⁶ However, little is known about the transcriptional regulation of *RARRES1*, 2, and 3 in human trophoblast cell lines and the effect of DNA methylation on this signaling pathway.

Tumorigenesis and placentation share similar processes for the induction of invasion, proliferation, and inhibition of apoptosis, including the transcriptional regulation of those mechanisms.³⁷ It is believed that failures in these pathways could lead to placental dysfunctions like intrauterine growth restriction (IUGR) or pre-eclampsia (PE).³⁸ Alternatively, pathologic induction of invasion and proliferation is conceived as a risk factor for the development of malignant transformations (e.g. choriocarcinoma³⁹).

In order to investigate the impact of RA signaling during placentation, we set out to analyze the expression and regulation of *RARRES1*, 2, and 3 and their transcription factors RARA, RARB, RARG, and RXRA in healthy and pathologically altered placental tissue (PE/IUGR) and various trophoblast-like cell lines.

Materials and Methods

Patient and Tissue Collection

Human term placentas were obtained from healthy patients (n = 15) or pregnancies complicated by PE (n = 3) and/or idiopathic IUGR (n = 3) after elective cesarean delivery, with “n” representing different patients. The clinical data of all patients are summarized in Supplementary Table 1. The inclusion criteria for PE and IUGR were described earlier by Fahlbusch and Ruebner et al.^{40,41} Placental tissues were formalin-fixed and paraffin-embedded (FFPE) for histological staining or snap frozen in liquid nitrogen for RNA extraction within 1 hour after delivery.

Ethical Approval

Each participant signed a written informed consent. Handling of patients and tissues were approved by the Ethics Committee at the University of Erlangen-Nuremberg (No. 353_15B).

Immunohistochemical Staining

Human FFPE placental sections were deparaffinized using Roti-Histol (Carl Roth, Karlsruhe, Germany) and rehydrated in a series of descending ethanol concentrations. Immunohistochemical staining was performed using the labeled streptavidin-biotin (LSAB) + horseradish peroxidase (HRP) kit (Agilent, Hamburg, Germany) according to the manufacturer's instructions. Mouse anti-human *RARRES1* (1:6000 ab92884; Abcam, Cambridge, UK), rabbit anti-human *RARRES2* (1:100 ab729065; Abcam), rabbit anti-human *RARRES3* (1:50 ab77010; Abcam), rabbit anti-human RAR α (1:250 sc551; Santa Cruz, Heidelberg, Germany), rabbit anti-human RAR β (sc552 1:50; Santa Cruz), rabbit anti-human RAR γ (sc550 1:50; Santa Cruz), and rabbit anti-human RXR α (1:50 ABIN685702; antibodies-online.com, Aachen, Germany) antibodies were used. Nuclei were stained with hematoxylin. Images were obtained using the Nikon Eclipse 80i microscope (Nikon, Duesseldorf, Germany).

Isolation of Primary Trophoblast Cells

Primary trophoblast cells were isolated from 10 individual healthy term placentas as well as placentas complicated by PE (n = 3), PE/IUGR (n = 3), or IUGR (n = 7). The trypsin-DNase-Dispase/Percoll method was used, as previously described.⁴² Before RNA isolation, the primary trophoblast cells were cryopreserved in liquid nitrogen. The percentage of trophoblastic cells (86.6%-90%) was determined by fluorescence activated cell sorting (FACSCalibur; BD Biosciences, Heidelberg, Germany).

Cell Culture

Stimulation experiments were performed using the choriocarcinoma cell line Jeg-3 and the first trimester cell line Swan71.

The Swan71 cells were a kind gift from Dr G. Mor, Department of Obstetrics, Gynecology and Reproductive Sciences, Reproductive Immunology Unit, Yale University School of Medicine, New Haven, USA.⁴³ The cell lines were cultivated in DMEM: F12 phenol-red free (high glucose; Thermo Fischer, Darmstadt, Germany) supplemented with 10% fetal calf serum (FCS; Thermo Fischer), 100 U/mL penicillin, and 100 µg/mL streptomycin (Sigma-Aldrich, Taufkirchen, Germany). Swan71 cells were additionally supplemented with 0.1 mM nonessential amino acids (NEAAs; Sigma-Aldrich). Furthermore, RNA was extracted from BeWo and JAR cells. BeWo cells were grown in DMEM: F12 phenol-red free (high glucose; Thermo Fischer) supplemented with 10% FCS (Thermo Fischer), 100 U/mL penicillin, and 100 µg/mL streptomycin (1% P/S; Sigma-Aldrich). JAR cells were cultivated in RPMI 1640 media (Thermo Fischer) with 10% FCS, 10 mM HEPES (Sigma-Aldrich), 2 mM L-glutamine (Sigma-Aldrich), and 0.1 mM NEAAs (Sigma-Aldrich).

For treatment with 2'-deoxy-5-azacytidine (AZA; Sigma-Aldrich) or RA, cells were seeded at a density of 3×10^4 /mL in a 12-well cell culture plate. Cells were cultivated in 2.5% (Swan71) or 5% (Jeg-3) charcoal-treated fetal bovine serum (Thermo Fisher) 24 hours prior to and during stimulation. Cells were treated with 1.0 µM of all-trans-RA (ATRA; Biomol, Hamburg, Germany) of the RAR β - γ -specific RA derivate TAZA (Sigma-Aldrich) and 200 nM of the RAR α agonist Am580 (Tocris; Lille Cedex, France) for 48 hours. AZA was used in a concentration of 1.0 µM for 72 hours. For a specific inhibition of RA stimulation, different RAR antagonists were used. Therefore, cells were treated with CD2665 (RAR β / γ antagonist) or ER50891 (RAR α antagonist) at concentrations between 0.5 and 8.0 µM for 48 hours. Every 24 hours, the medium was changed and the stimulation was repeated. The specificity of Am580, TAZA, ATRA, and the antagonists were commercially and/or experimentally tested beforehand by others.^{5,44-49} Agonists and antagonists were used as described elsewhere,^{2,50,51} and different concentrations as well as time points were tested by us beforehand (data not shown). We chose to conduct our experiments at 48 hours, as it represents the time point with the highest RA-induced increase of gene expression. All experiments were performed with 6 or 9 biological replicates from different passages of each cell line. The different RAs and antagonists were either solubilized in ethanol or DMSO. Thus, cells were treated with ethanol and/or DMSO in the respective amounts preanalytically. We found no differences in the gene expression of target genes and housekeepers with and without ethanol and/or DMSO. Hence, we used the "no treatment" condition (i.e. without ethanol/DMSO) as a control for our final experiments.

Extraction of RNA

As previously described,⁴¹ RNA was isolated from placental tissues, primary trophoblast cells, and cultivated cell lines (Swan71, Jeg-3, JAR and BeWo) using peqGold Trifast (Peqlab, Erlangen, Germany). Before complementary DNA

(cDNA) synthesis, RNA was treated with DNase I (Roche, Mannheim, Germany). The cDNA was generated using the high-capacity cDNA Reverse Transcription kit (Thermo Fisher) in a thermal cycler (ABI2720) for 2 hours at 37°C.

Quantitative Real-Time Polymerase Chain Reaction

Quantification of RARRES1, 2, and 3 (semiquantitative) as well as RARA, B, and G (full quantitative) cDNA was performed by quantitative real-time polymerase chain reaction (qRT-PCR). Forward and reverse primer of 10 nM and cDNA of 40 ng were used and quantification analyzed by SYBR-green-based technology (SYBR select master mix; Thermo Fischer). The expression was normalized using *18SrRNA* and Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) as reference genes. Due to lack of differences, only 18SrRNA data are presented. Primer sequences are listed in Supplementary Table 2. For RAR and RXR, a full quantitative qRT-PCR analysis using plasmids with the respective region of the gene of interest was performed in order to calculate the molecules per ng cDNA. This allowed the comparison of different genes within 1 cell line. The ng cDNA content was calculated using a standard curve and *RAR/RXR* cDNA plasmids. Semiquantitative analysis was performed using the $\Delta\Delta C_T$ method and values were normalized to the untreated control. All semi- and full quantitative qRT-PCR analyses were performed in duplicates.

Statistical Analysis

All data are presented as mean \pm standard error of the mean (SEM). Differences between the subgroups were analyzed using the Mann-Whitney *U* test (SPSS Version 21; IBM Inc., Armonk, USA). *P* values of $\leq .05$ were considered statistically significant.

Results

Expression of RARRES1, 2, and 3 in Different Compartments of the Human Placenta

In order to determine whether the RA responsive genes *RARRES1*, 2, and 3 are expressed in human placental tissues and trophoblastic cell lines (Swan71, Jeg-3, JAR and BeWo), we performed qRT-PCR analysis of RNA isolated from tissues and cell lines (Figure 1A) and immunohistochemical staining of third-trimester placental FFPE sections (Figure 1B).

In comparison to placental biopsies, significantly higher *RARRES1* and *RARRES3* expression was detected in isolated villous cytotrophoblasts (trophobl; Figure 1A, a and c; $P < .05$). Furthermore, the first-trimester cell line Swan71 showed a significantly higher expression of *RARRES1*, 2, and 3 compared to placental tissues (Figure 1A, a-c; $P < .005$). Expression of *RARRES1* was significantly reduced ($P < .005$) in the chorio-carcinoma cell lines Jeg-3, JAR, and BeWo. Similar results were obtained for *RARRES2* expression in Jeg-3 and BeWo cells ($P < .05$) and for *RARRES3* in Jeg-3 and JAR cells ($P < .05$). In contrast, a significantly higher expression was detected for *RARRES2* in the JAR cell line (3-fold, $p < 0.05$) and for

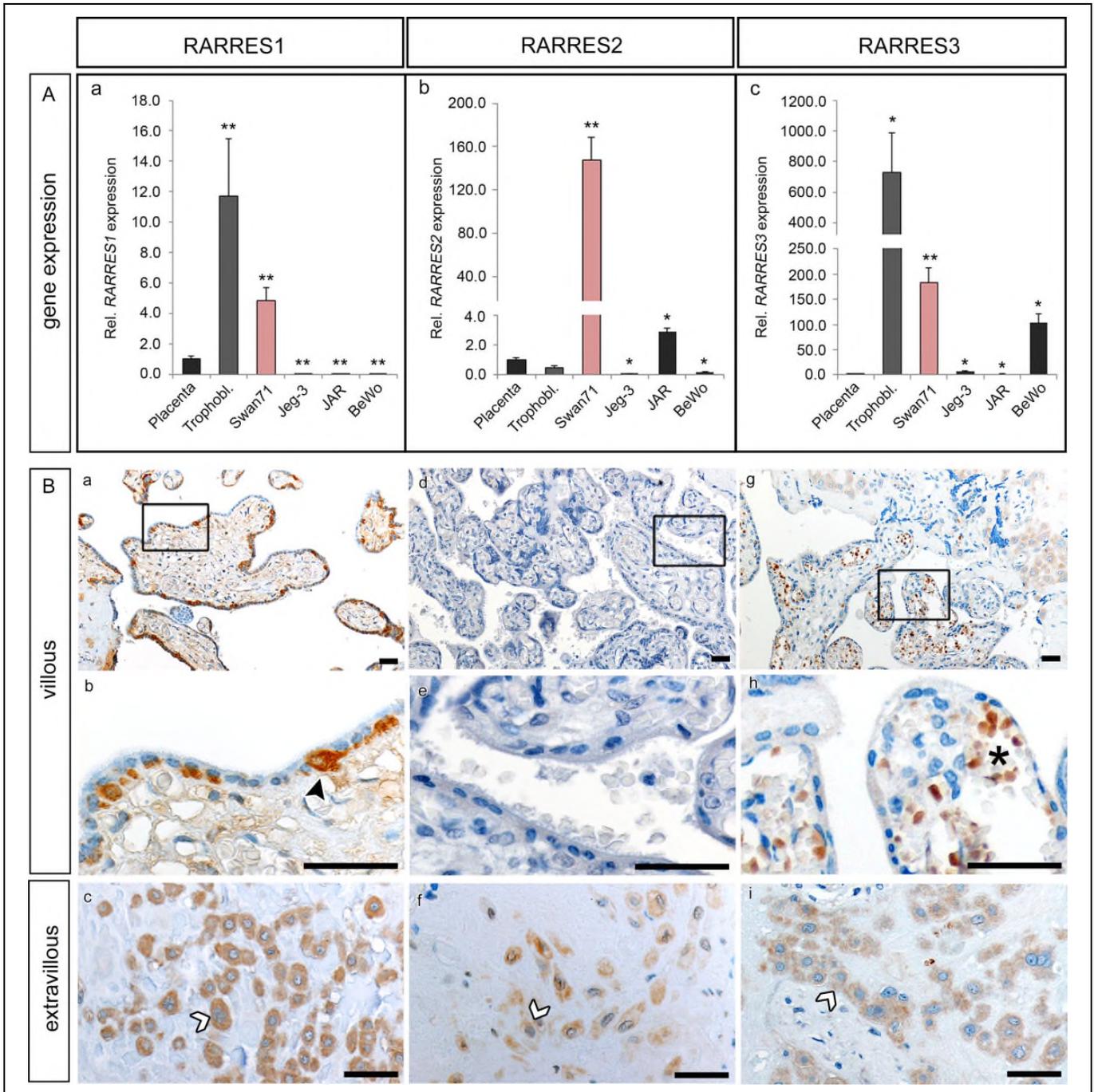


Figure 1. Expression of *RARRES1*, 2, and 3 in the human placenta. A, *RARRES1* (a), *RARRES2* (b), and *RARRES3* (c) gene expression (mean and SEM) was analyzed by qRT-PCR in placental tissue (biopsies from $n = 15$ different placentas), primary trophoblast cells (Trophobl., isolated from $n = 5$ different placentas), the first-trimester cell line Swan71 ($n = 9$), and the choriocarcinoma cell lines Jeg-3 ($n = 6$), JAR ($n = 9$), and BeWo ($n = 6$). The level of significance is indicated as follows: * $P \leq .05$ and ** $P \leq .005$, relative to placental tissue. B, Protein expression of *RARRES1* (a-c), *RARRES2* (d-f), and *RARRES3* (g-i) was analyzed by immunohistochemical staining of placental FFPE sections. *RARRES1*-positive VTs are indicated by black arrowheads (b) and *RARRES1*-, 2-, or 3-positive EVT by white arrowheads (c, f, and i). The asterisk marks a fetal vessel (h). Black bars define 100 μm . FFPE indicates formaldehyde-fixed and paraffin-embedded; qRT-PCR, quantitative real-time polymerase chain reaction; RARRES, retinoic acid receptor responder; SEM, standard error of the mean.

RARRES3 in BeWo cells (100-fold; $P < .05$), when compared to normal third-trimester placental tissue.

Within the analyzed placental FFPE sections, we detected *RARRES1* protein expression in the villous (Figure 1B, a and

b) as well as the extravillous (Figure 1B, c) part of the human placenta. Villous *RARRES1* protein was mainly expressed in villous trophoblasts (VTs), which were neighboring the SCT border (Figure 1B, b, black arrowhead). The *RARRES1*

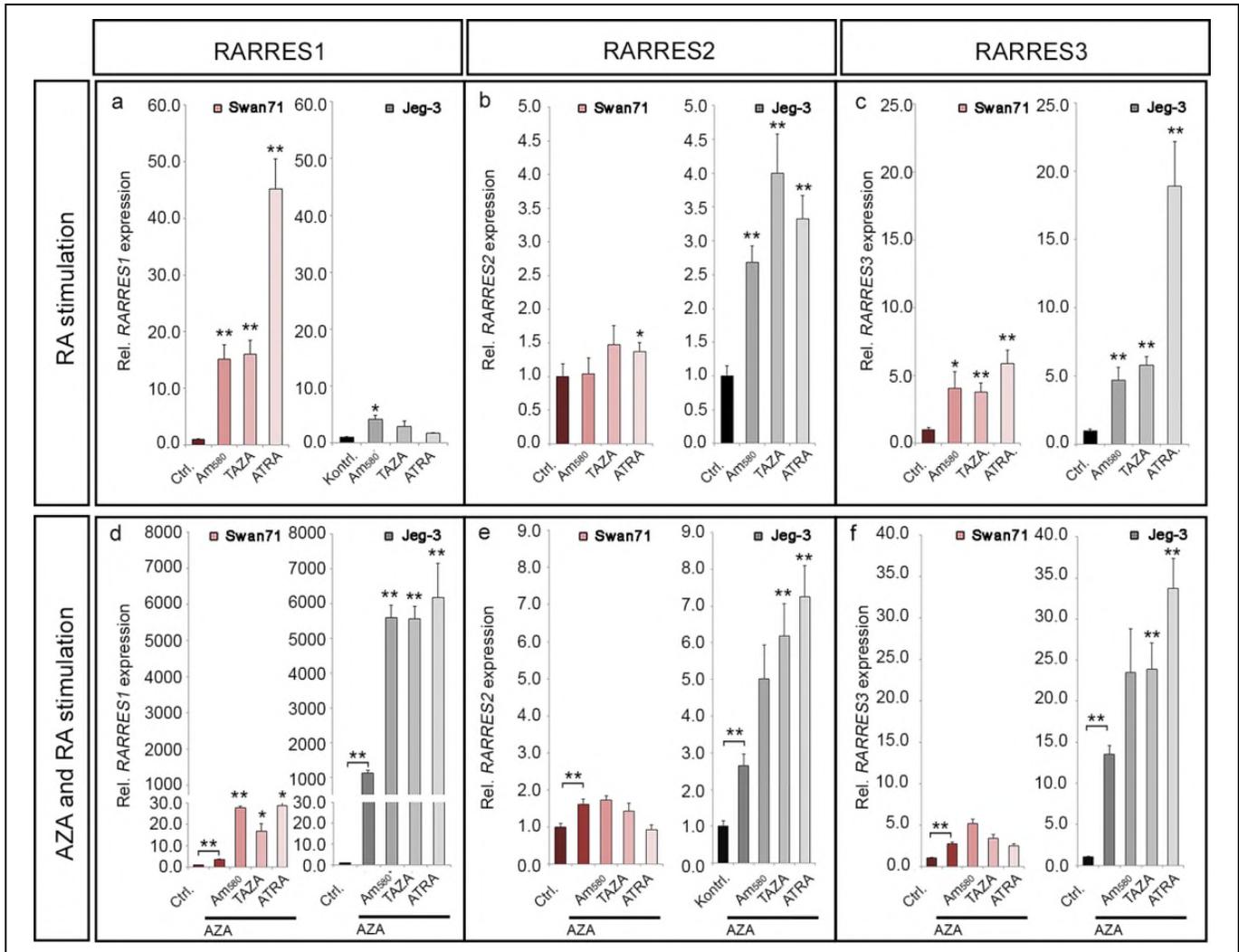


Figure 2. Expression of *RARRES1*, 2, and 3 after RA and AZA treatment. Swan71 and Jeg-3 cells were stimulated with different RAs (Am580, TAZA and ATRA) and gene expression (mean and SEM) of *RARRES1* (a), *RARRES2* (b), and *RARRES3* (c) was analyzed by qRT-PCR. Swan71 and Jeg-3 cells were additionally treated with AZA to induce DNA demethylation and stimulated with RAs to induce gene expression even further (d-f). The graph shows the relative fold change of gene expression normalized to the untreated control (Ctrl.). The level of significance is indicated as following: * $P \leq .05$ and ** $P \leq .005$, relative to Ctrl. or solely AZA treated cells, $n = 6$. ATRA indicates all-trans-retinoic acid; AZA, 2'-deoxy-5-azacytidine; qRT-PCR, quantitative real-time polymerase chain reaction; RA, retinoic acid; RARRES, retinoic acid receptor responder; SEM, standard error of the mean; TAZA, Tazarotene.

staining was cytosolic and membranous for both extravillous trophoblasts (EVTs) and VTs. On the other hand, only EVT showed a *RARRES2*-positive staining (Figure 1B, f). In addition, EVT did express *RARRES3* (Figure 1B, g, and i), while the VTs and the SCT did not show any *RARRES2* or 3 staining (Figure 1B, d, e, and g, h, respectively). Additionally, fetal blood cells were *RARRES3* positive (Figure 1B, h, black asterisk).

Expression of *RARRES1*, 2, and 3 can be Induced by RAs and AZA Treatment

As the highest expression for all 3 *RARRES* genes was detected in the Swan71 cell line and the lowest expression in the choriocarcinoma cell line Jeg-3, these cell lines were chosen for further stimulation experiments. Our primary objective was to

analyze whether *RARRES1*, 2, and 3 expression could be increased by treatment with different RAs. On the one hand, cultured cells were treated with the RARA-specific agonist Am580, with the RA derivate TAZA (RARB- and RARG-specific) or with ATRA (RARA-, RARB-, and RARG-specific; Figure 2a-c) and on the other hand additional treatment with AZA was performed to obtain global DNA demethylation (Figure 2d-f). In the Swan71 cell line significant stimulation of *RARRES1* and *RARRES3* expression was obtained after treatment with Am580, TAZA, and ATRA (Figure 2a and c). Expression of *RARRES2* and 3 increased significantly after stimulation of Jeg-3 cells with all RA derivatives (Figure 2b; $P < .005$). No increase was detected in *RARRES1* gene expression. In order to investigate whether the lack of stimulation capacity was caused by DNA methylation, we treated the cells

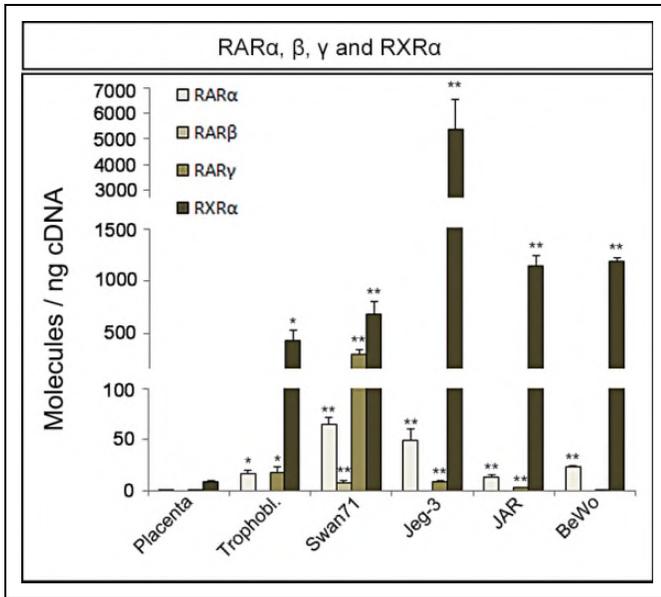


Figure 3. Expression of RAR α , β , γ , and RXR α in the human placenta. Molecules per ng cDNA of RAR α , β , γ , and RXR α were quantified by qRT-PCR analysis. The RNA of placental tissue ($n = 4$), primary trophoblast cells ($n = 6$), Swan71 ($n = 10$), Jeg-3 ($n = 9$), JAR ($n = 9$), and BeWo ($n = 9$) cells was isolated and analyzed. The level of significance is indicated as follows: * $P \leq .05$ and ** $P \leq .005$, relative to placental tissue. cDNA indicates complementary DNA; qRT-PCR, quantitative real-time polymerase chain reaction; RAR, retinoic acid receptor.

with AZA before RA stimulation. Gene expression of all three genes was induced significantly after AZA treatment ($P < .005$; Figure 2d-f). Furthermore, AZA treatment of Jeg-3 cells facilitated the stimulation of *RARRES1* expression by RA (Figure 2d; $P < .005$), which could not be achieved without AZA (Figure 2a).

Retinoic Acid Receptor A, G, and RXRA are Highly Expressed in Placental Tissue

Gene expression of *RARRES1*, 2, and 3 was induced by all 3 RA derivatives (Am580, TAZA, and ATRA) in Swan71 and Jeg-3 cells. In order to investigate whether RAs could stimulate *RARRES* expression via RAR transcription factors, we analyzed the expression of *RARA*, *RARB*, and *RARG* as well as *RXRA* in placental tissue and trophoblastic cell lines (Figure 3) and performed immunohistochemical staining on placental FFPE sections (Figure 4). Analysis of gene expression by qRT-PCR showed a significantly higher expression of *RARA*, *RARG*, and *RXRA* in primary trophoblast cells compared to total placental tissue (Figure 3; $P < .05$). Overall, we detected the highest number of molecules per ng cDNA for *RXRA* (Figure 3). The lowest expression was found for *RARB*, which was even below the lower limit of detection in placental tissue and primary trophoblast cells (Figure 3).

Using immunohistochemical staining, we detected *RARA*, *RARG*, and *RXRA* in the villous as well as the extravillous compartments of the human placenta (Figure 4a-c and g-l). All

3 RARs were expressed in the nuclei of EVT and VTs (Figure 4b, h, and k; black asterisks; and c, i, and l, black arrowheads). Additionally, *RARA* and *RARG* were expressed in several nuclei of the SCT (Figure 4b and h, black arrowheads), while others were negative for the receptors (Figure 4, white arrowheads). In contrast, *RARB* could not be detected in the villous part of the human placenta but showed a cytoplasmic staining in the EVT (Figure 4d-f).

Expression of RARA, RARB, and RARG Significantly Increases After AZA Treatment

Overall, *RARA*, *RARG*, and *RXRA* could be detected in the same placental compartments and cell lines as *RARRES1*, 2, and 3 and thus might function as transcription factors to increase *RARRES* gene expression. However, earlier publications showed that *RARB* is expressed and is an active transcription factor under RA stimulation only.^{34,52,53} Moreover, it is well-known that transcriptional expression of *RARB* is controlled by epigenetic regulation.⁵⁴⁻⁵⁶ Consequently, we set out to analyze *RARA*, *RARB*, *RARG*, and *RXRA* expression under RA and AZA treatment. Interestingly, when stimulated with Am580, the expression of all analyzed receptors increased significantly in Swan71 cells (Figure 5a-d). Nevertheless, the strongest effect could be detected for *RARB* (up to 20-fold increase; Figure 5b; $P < .005$), regardless of the RA derivative used. A slight, but significant increase in *RARA* as well as *RARB* expression could additionally be shown in Jeg-3 cells (Figure 5a and b). This increase in *RARB* expression was more pronounced after AZA treatment (up to 35-fold; Figure 5f). AZA itself induced the expression of *RARA*, *RARB*, and *RARG*, while the expression of *RXRA* decreased significantly in AZA-treated Jeg-3 cells (Figure 5e-h; $P < .05$).

Expression of RARRES1, 2, and 3 can be Blocked by RAR Antagonists

To investigate the specific regulation of *RARRES1*, 2, and 3 expression through *RARA*, B, or G, we performed additional cell culture experiments and used specific receptor antagonists to inhibit RA stimulation. CD2665 is a specific blocker of *RARB* and *RARG*, while ER50891 is a specific *RARA* antagonist. We treated Swan71 and Jeg-3 cells with CD2665 and ER50891 in different concentrations and added the RAs (Am580, TAZA, and ATRA) to increase *RARRES1*, 2, and 3 expression (Figure 6 and Supplemental Figure 1). In Swan71 cells RA-induced gene expression of *RARRES1* and 3 decreased after treatment with CD2665 and ER50891 (Figure 6A and C, a-d). As shown before, *RARRES2* expression could not be increased with Am580, TAZA, or ATRA and, consequently, there was almost no decline detected after inhibition with CD2665 or ER50891 (Figure 6B, a-d). Only the combination of both inhibitors in their highest concentration showed a significant inhibition of *RARRES2* under TAZA treatment (Figure 6 and Supplemental Figure 1B, c). After treatment of Jeg-3 cells with AZA for demethylation, higher concentrations

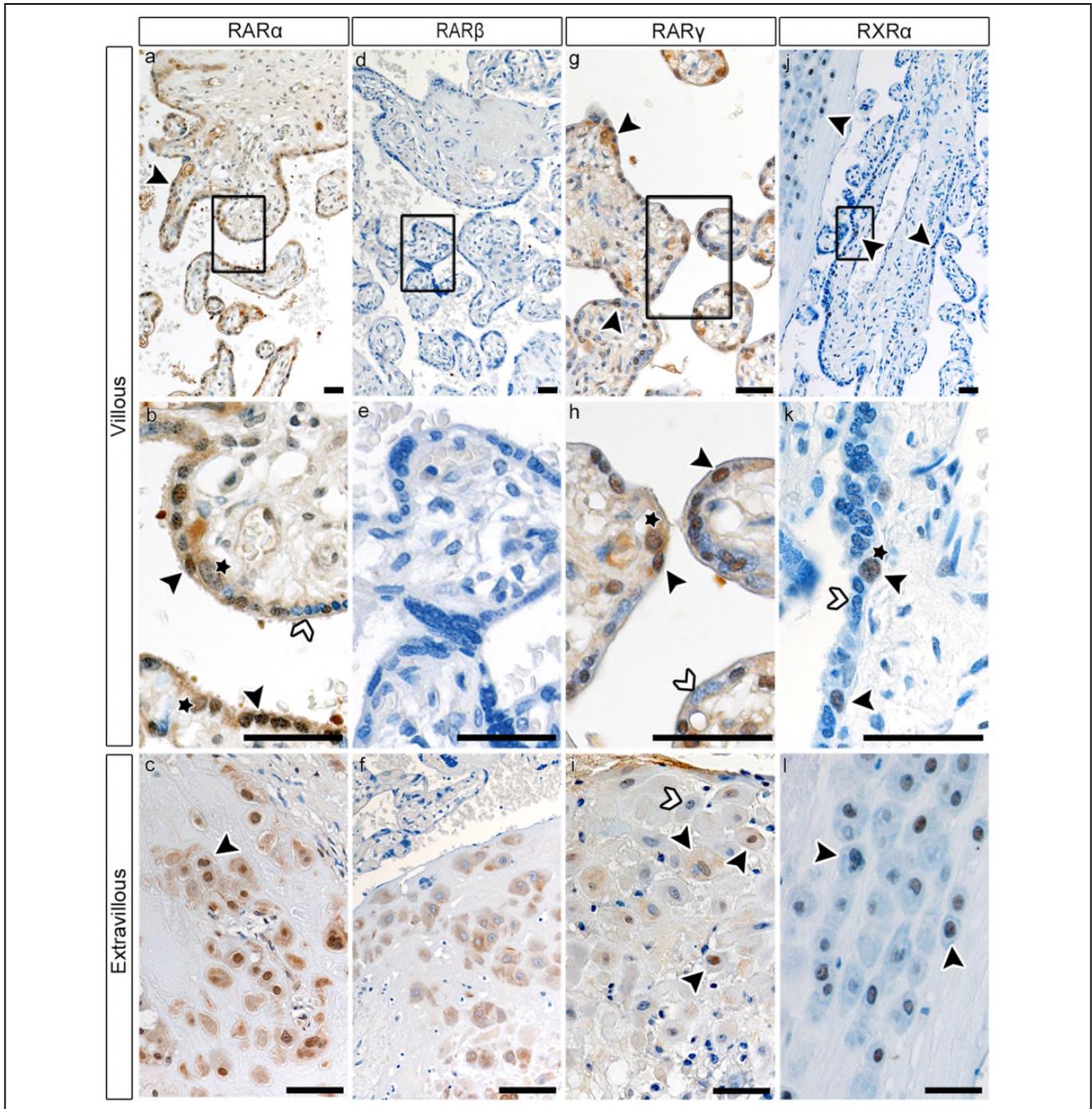


Figure 4. Expression of RAR α , β , γ , and RXR α protein in human placental tissue. Protein expression of RAR α (a-c), RAR β (d-f), RAR γ (g-i), and RXR α (j-l) was analyzed by immunohistochemical staining of placental FFPE sections. Black arrowheads indicate positive nuclei; white arrowheads show negative ones (a-l). Asterisks mark VTs (b, h, and k). Black bars define 100 μ m. FFPE indicates formaldehyde-fixed and paraffin-embedded; RAR, retinoic acid receptor; VT, villous trophoblast.

of the RAR β /G-specific antagonist were necessary to induce a significant reduction in *RARRES1*, 2, or 3 expression (Figure 6A-C and Supplemental Figure 1, g; $P < .005$). This was in concordance with the detected increase in *RARB* and *RARG* expression after AZA treatment (Figure 5f and g). Overall, the stimulation of gene expression could be significantly inhibited using RARA- as well as RAR β /G-specific antagonists.

Expression of RARRES1 Increased in Villous Trophoblasts of PE Placentas

As *RARRES1*, 2, and 3 were found to be expressed in the human placenta and seemed to be influenced by RA stimulation, we were interested in their expression pattern in different placental pathologies. Therefore, we analyzed gene and protein

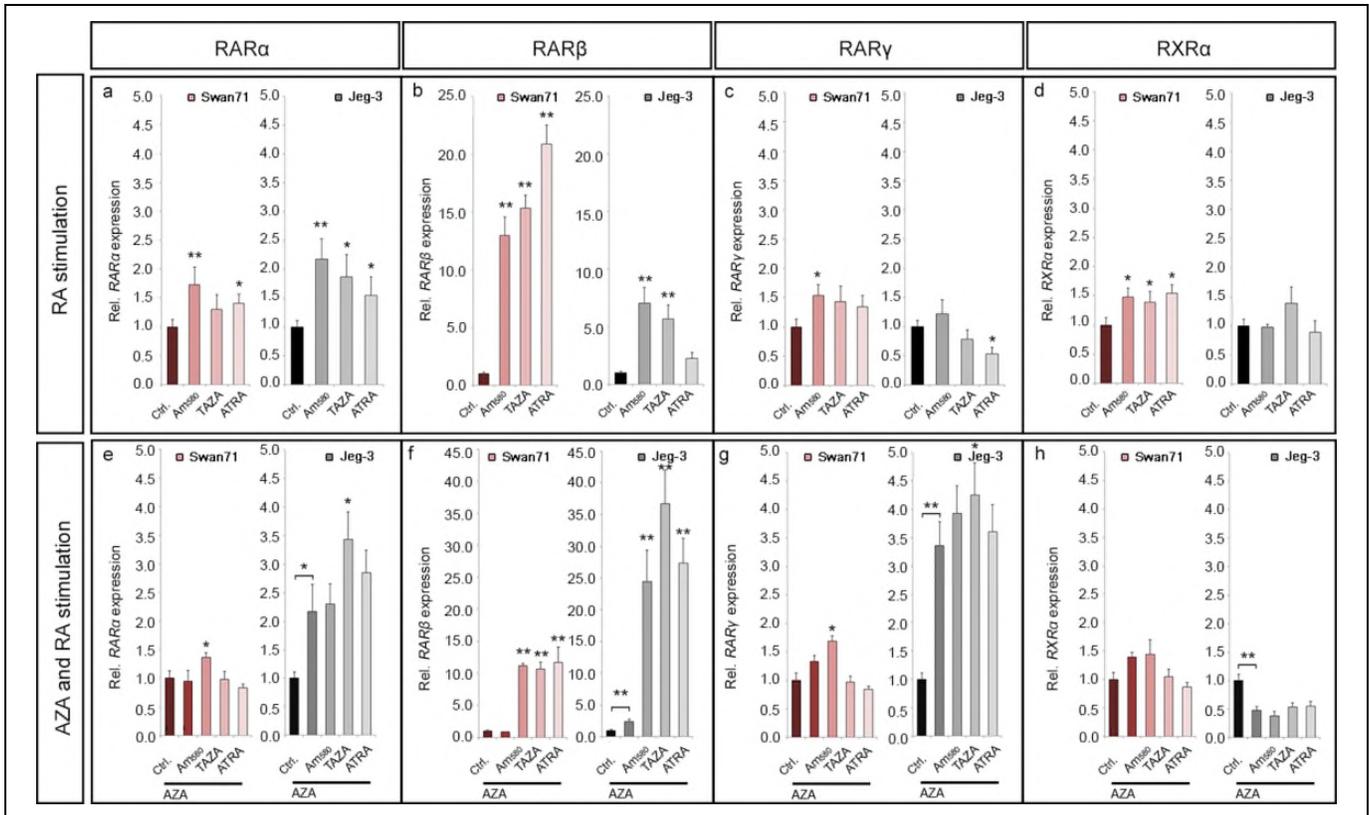


Figure 5. Expression of RAR α , β , γ , and RXR α under RA and AZA treatment. Swan71 and Jeg-3 cells were treated with different RAs alone (Am580, TAZA, and ATRA; a-d) or together with AZA (e-h). Graphs show the relative fold change of gene expression (mean and SEM) to the untreated control (Ctrl.). The level of significance is indicated as follows: * $P \leq .05$ and ** $P \leq .005$, relative to Ctrl. or solely AZA treated cells, $n = 6$. ATRA indicates all-trans-retinoic acid; AZA, 2'-deoxy-5-azacytidine; RA, retinoic acid; RAR, retinoic acid receptor; SEM, standard error of the mean; TAZA, Tazarotene.

expression in placentas from healthy pregnancies and from the pregnancy complications PE and IUGR. Even though we detected a significant decrease in *RARRES2* gene expression in placentas complicated by PE or IUGR, we did not observe a decrease in protein expression by immunohistochemical staining of FFPE sections (data not shown). We also did not detect any differences regarding *RARRES3* gene and protein expression (data not shown). On the other hand, we found an increase in RARRES1 protein expression by immunohistochemical staining of sections from placentas complicated by PE/IUGR when compared to control placentas (Figure 7a-d). In contrast to healthy controls, PE/IUGR placenta sections showed a specifically strong RARRES1 staining within the SCT (Figure 7c). Based on the observation that this increase was only within the villous part of the placenta, we isolated primary villous trophoblast cells from control placentas and placentas complicated by PE and/or IUGR and analyzed *RARRES1* gene expression. *RARRES1* was significantly increased in PE/IUGR (5-fold) and PE (3-fold) trophoblast cells (Figure 7g; $P < .05$), while there was no difference in IUGR alone. Similarly to primary trophoblasts from PE/IUGR placentas, we found a 5-fold higher *RARRES1* expression in the first-trimester cell line Swan71 compared to healthy placental tissue (Figure 1). Thus, we were further interested in the protein expression pattern of

RARRES1 in first-trimester placental tissue. Immunohistochemical staining of first-trimester FFPE sections revealed a strong *RARRES1* expression in the villous (Figure 7e) and extravillous (Figure 7f) compartment. Similarly to PE/IUGR placentas, RARRES1 staining was specifically strong in the SCT (Figure 7e).

Discussion

We observed high expression of *RARRES1* in third-trimester placental tissue, primary villous cytotrophoblasts as well as in the first-trimester cell line Swan71 and determined a significant induction of RARRES1 by different RAs. Thus, we assume that RARRES1 might exert important functions during placental development and for the maintenance of gestation. RARRES1 is known to be significantly downregulated in cancer cell lines or tissues like breast, prostate, or colorectal carcinomas.^{17,57,58} Here, we showed a significant decrease in RARRES1 expression in choriocarcinoma cell lines, which was restored by demethylation via AZA treatment. The RARRES1 promoter is known to be hypermethylated in various cancers.^{59,60} Therefore, we assume that an increased promoter methylation in choriocarcinomas might have contributed to the low RARRES1 expression detected. This might further go in line

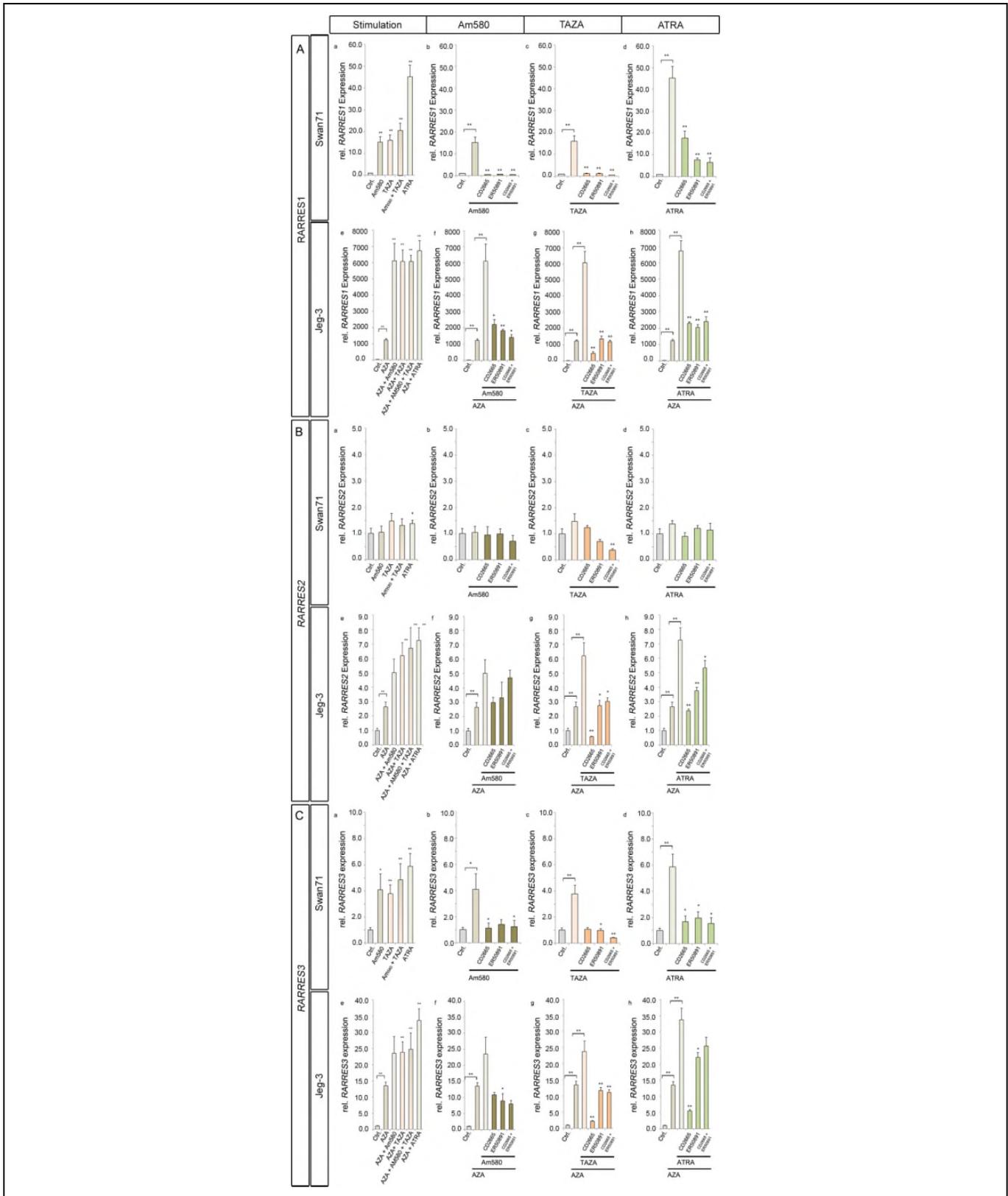


Figure 6. The RAR-dependent expression of *RARRES1*, *2*, and *3*. Swan71 cells were cultivated and treated with RAR antagonists (CD2665: RAR/B/G antagonist; ER50891: RARA antagonist) and agonists (Am580, TAZA, and ATRA; A, B, and C, a-d). CD2665 and ER50891 were used in the most effective concentration of 2.0 μ M. Additionally, Jeg-3 cells were pretreated with AZA and cocultured with CD2665 (8.0 μ M) or ER50891 (2.0 μ M) as well as Am580, TAZA, and ATRA (A, B, and C, e-h). The relative gene expression of *RARRES1* (A), *RARRES2* (B), and *RARRES3* (C) was quantified by qRT-PCR (mean and SEM). If not marked different, the level of significance is indicated as follows: * $P < .05$ and ** $P < .005$, relative to untreated Ctrl. or solely AZA treated cells, $n = 6$. ATRA indicates all-trans-retinoic acid; AZA, 2'-deoxy-5-azacytidine; qRT-PCR, quantitative real-time polymerase chain reaction; RA, retinoic acid; RAR, retinoic acid receptor; *RARRES*, retinoic acid receptor responder; SEM, standard error of the mean; TAZA, Tazarotene.

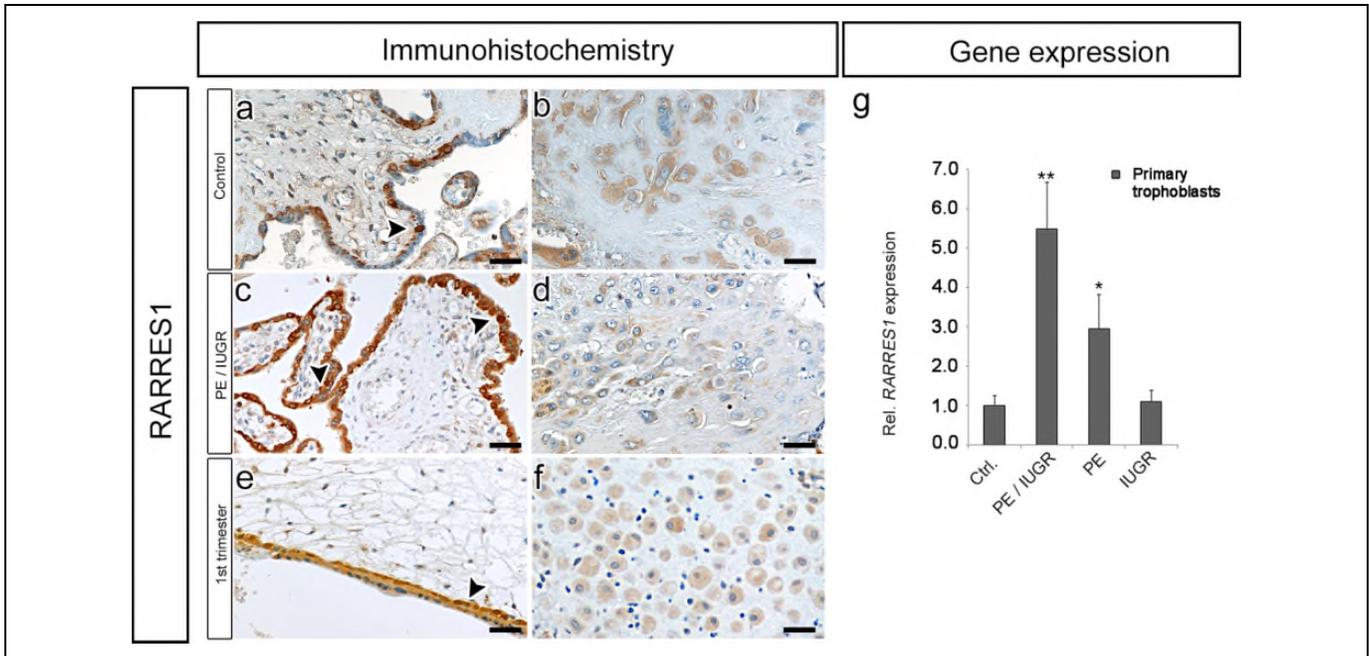


Figure 7. Expression of *RARRES1* in control and PE/IUGR placentas. Immunohistochemical staining of *RARRES1* was performed on FFPE sections from healthy control (a and b), PE/IUGR (c and d), and first-trimester (e and f) placentas. Black arrowheads mark VTs. Black bars define 100 μ m. Primary villous trophoblast cells were isolated from healthy (Ctrl., n = 10), PE/IUGR (n = 3), PE (n = 3), and IUGR (n = 37) placentas. RNA was extracted and quantified by qRT-PCR analysis (g; mean and SEM). Visualized are the fold changes relative to control trophoblast cells and the significance is marked with stars (* $P \leq .05$ and ** $P \leq .005$). FFPE indicates formaldehyde-fixed and paraffin-embedded; IUGR, intrauterine growth restriction; PE, preeclampsia; qRT-PCR, quantitative real-time polymerase chain reaction; *RARRES*, retinoic acid receptor responder; SEM, standard error of the mean; VT, villous trophoblast.

with the observed failure of inducing *RARRES1* expression by RA in Jeg-3 cells. After AZA treatment, *RARRES1* expression was significantly increased in Jeg-3, which further emphasizes our hypothesis of DNA methylation and subsequently reduced capability to induce *RARRES1* expression in these cells. In breast cancer as well as prostate cancer cell lines, it was shown that *RARRES1* can significantly decrease invasion and proliferation.^{18,57} Thus, we hypothesize that *RARRES1* might slow down invasion and migration of EVT in terminal placentas and reduce the proliferation of VTs. A decrease in proliferation is necessary for the differentiation of VTs and fusion into the SCT. Supportive of this idea, we detected a high *RARRES1* protein expression especially in VTs with spatial fusion capacity, that is, in close proximity to the SCT. As *RARRES1* expression was significantly increased in isolated VTs complicated by PE, we assume that high *RARRES1* expression might negatively influence fusion capacity or increase the presence of apoptotic bodies. This hypothesis might go in line with the observed increase of a VT cell count, decreased number of fusions, and decreased expression of the fusogenic Syncytin 1 in PE placentas.^{42,61,62} On the other hand, re-expression of *RARRES1* in the breast cancer cell line SUM 149 could promote apoptosis, which might be indicative of a similar association in PE placentas.¹⁶ It is well-known that syncytial knots representing apoptotic cells are increased in PE placentas and the maternal circulation.^{63,64} This indicates a potential link between *RARRES1* expression and apoptosis of VTs. Similarly

to *RARRES1*, it was shown earlier that RXRA is significantly increased in placentas complicated by PE and IUGR.⁶⁵ This suggests that *RARRES1* expression might be dependent on RAR/RXR regulation. Interestingly, high syncytial *RARRES1* expression could also be detected in first-trimester placental tissue, implicating that increased *RARRES1* in PE placentas might be due to a disturbed development of trophoblasts. Contrary to the finding that increased apoptosis rates are also reported in IUGR without PE, we did not detect an elevated *RARRES1* expression in villous trophoblasts from IUGR placentas. This might be due to a less pronounced villous trophoblast apoptosis in IUGR.⁶⁶ Furthermore, in contrast to IUGR trophoblasts, cultured trophoblasts from PE/IUGR placentas showed a significantly reduced syncytialisation.⁶⁷ So far, we cannot define whether the increased *RARRES1* expression is a cause or a result of PE, but our results indicate it might be associated with villous trophoblast development. Furthermore, it has to be taken into account that our study focused on the analysis of idiopathic IUGR only. Thus, we currently cannot rule out a differential regulation of *RARRES1* secondary to various other etiopathologic causes of IUGR.

Both *RARRES2* and 3 could only be detected in EVT of the human third-trimester placenta. Interestingly, we observed the highest *RARRES3* gene expression in primary trophoblast cells. This might partly be due to the high *RARRES3* expression in placental blood cells, as the Percoll-gradient-based isolation of primary trophoblast cells contains about 2.4% to 4.5%

mononuclear blood cells.^{14,68} *RARRES3* is known to be a tumor suppressor in many cancers and thus influences proliferation, apoptosis, as well as cell adhesion.^{29,30,69,70} Consequently, we assume *RARRES3* might be involved in the suppression of EVT proliferation and induction of apoptosis, thereby limiting their invasion. Additionally, *RARRES3* is known to be involved in the regulation of epithelial-to-mesenchymal transition, where it, for example, induces adhesion via beta-catenin/cadherin,³⁰ leading to EVT differentiation into placental bed giant cells.⁷¹ Such assumptions could support the detected pattern of *RARRES3* expression.

RARRES2 is well described as a circulating maternal serum factor during pregnancy.^{25,72} Increased serum levels of *RARRES2* were shown to be associated with the pregnancy complication PE.^{73,74} Our findings seem to be in contrast to these results, as we could not detect an increase of *RARRES2* in PE-associated placentas. This might either indicate that serum and tissue-specific *RARRES2* concentrations differ, or, that the soluble form of *RARRES2* originates from a source other than the placenta (e.g. adipocytes). For example, upregulation of *RARRES2* expression was shown in postpregnancy mammary glands and thus might be associated with the indicated increased serum levels.⁷⁵

Overall, we showed that trophoblast *RARRES1*, 2, and 3 expression could be induced via RARA, B, or G signaling. Even though *RARB* protein expression was not detected within the nuclei of trophoblast cells, we showed that *RARB* expression in trophoblastic cell lines is dependent on RA induction and thus might also be involved in the regulation of gene expression. This is in line with former publications, which identified an RA responsive element within the *RARB* promoter.^{34,53} In various carcinomas RA resistance was associated with loss of *RARB* expression.⁷⁶ Here, we detected a loss of *RARB* expression in the choriocarcinoma cell lines, which could be significantly reversed by AZA treatment. Thus, we assume that *RARB* expression might also be dependent on promoter methylation in these cells. In contrast, we found high *RARA* and *RXRA* expression in Jeg-3 cells, with *RXRA* being the predominant receptor. This goes in line with an earlier publication showing high protein and RNA levels of both receptors in Jeg-3 cells.⁷⁷ In placental tissues we detected a specific expression of *RARA* and *RXRA* in the villous and extravillous compartment. This was in line with former publications showing placental *RARA* and *RXRA* expressions.⁷⁸⁻⁸⁰ But in contrast to others, we were able to specify *RXRA* protein expression in the nuclei of VTs and not the SCT.

Taken together, placental *RARRES1*, 2, and 3 expression was detected in trophoblast cells of different placental compartments and could be induced in isolated trophoblast cells with RA derivatives via different RARs. These findings argue for a role of *RARRES1*, 2, and 3 for trophoblast function. Overexpression of *RARRES1* in PE placentas might indicate that *RARRES1* has some unfavorable effects contributing to the development of PE. As nutritional intake of vitamin A is a crucial factor during pregnancy, however, the PE-associated increase in placental *RARRES1* expression might suggest a

possible therapeutic implication for dietary vitamin A restriction in women with severe PE.

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Author's Contribution

The authors FB Fahlbusch and M. Ruebner contributed equally to this study.

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Supplemental Material

Supplemental material for this article is available online.

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