Retinoid X receptor $\alpha$ and retinoids are key regulators in apoptosis of trophoblasts of patients with recurrent miscarriages

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Abstract

The retinoid X receptor $\alpha$ (RXR$\alpha$) is a nuclear hormone receptor that is able to bind other nuclear receptors in a heterodimeric complex, thereby activating gene transcription. Recently, we identified enhanced expression of RXR$\alpha$ in extravillous trophoblasts (EVT) and villous trophoblasts (VT) of miscarried placentas. In addition, an increased number of apoptotic EVT was present in miscarried placentas. In this study, on the basis of immunocytochemical analysis, western blots, and quantitative real-time reverse transcription PCR, we could demonstrate a reduced expression of RXR$\alpha$ in choriocarcinoma cell lines and in human VTs after stimulation with the retinoids 9-cis-retinoic acid and all-trans-retinoic acid and the prostaglandin 15-deoxy-$\Delta^{12,14}$-prostaglandin $J_2$. Furthermore, a simultaneous expression of RXR$\alpha$ and the apoptotic marker M30 CytoDEATH in EVT of miscarried placentas from the first trimester was shown. In EVT of control placentas from legal termination of pregnancies, no co-expression of RXR$\alpha$ and the apoptotic marker M30 could be detected. A likely conclusion is that RXR$\alpha$ plays an important role in the induction of apoptosis. Downregulation of RXR$\alpha$, as observed in the tested choriocarcinoma cells and trophoblasts, might serve as a protection against apoptosis and miscarriage. In conclusion, RXR$\alpha$ represents a potential target in the treatment of recurrent miscarriages.

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Introduction

In the past two decades, Pierre Chambon, Ronald M Evans, and Elwood V Jensen investigated the role of the nuclear receptor superfamily and discovered that its members interact with each other. The retinoid X receptor (RXR) represents a key player of the receptor family, due to its ability to form homodimers as well as heterodimers with other nuclear receptors and thereby activating their transcription (Zhang & Pfahl 1993, Kastner et al. 1995, Mangelsdorf & Evans 1995). Once a heterodimeric complex has been formed with RXR, the complex binds to specific response elements in the promoter region of target genes (Guibourdenche et al. 2000). Heterodimeric partners of RXR include the retinoid A receptor (RAR$\alpha$, $\beta$, $\gamma$), vitamin D receptor, peroxisome proliferator-activated receptor (PPAR$\alpha$, $\beta$, $\gamma$), thyroid hormone receptor $\alpha$, $\beta$, and liver X receptor $\alpha$, $\beta$; thus, RXR is involved in various metabolic and endocrinological processes.

RXR consists of three isotypes that are referred to as RXR$\alpha$, RXR$\beta$, and RXR$\gamma$ (Mangelsdorf et al. 1992, Mascreez et al. 2001). RXR$\beta$ is expressed ubiquitously, whereas RXR$\gamma$ is mainly expressed in skeletal and cardiac muscle, in the brain, and in the anterior pituitary. RXR$\alpha$ is especially expressed in the kidney, liver, spleen, epidermis, and placenta (Ahuja et al. 2003).

All three RXR isotypes are involved in cell proliferation, cell differentiation, embryonic patterning, and organogenesis (Szanto et al. 2004). The isotype RXR$\alpha$ plays an essential role during embryogenesis and morphogenesis (Mark et al. 2006). Together with its heterodimeric partner PPAR$\gamma$, RXR$\alpha$ is able to promote trophoblast differentiation by inducing the secretion of gestational hormones like human chorionic gonadotropin, leptin, and lactogen (Tarrade et al. 2001). The heterodimer further regulates the uptake of fatty acids in trophoblasts, which is essential for the development of the fetus and the production of placental steroid hormones (Toth et al. 2007, Duttaroy 2009). Invasion of cytotrophoblasts is indirectly correlated to the concentration of RXR$\gamma$ and PPAR$\gamma$ (Toth et al. 2007). The importance of the role of RXR$\alpha$ during embryonic development is pointed by the fact that homozygous RXR$\alpha$-null mice die between embryonic days (E) E13.5 and E16.5 (Kastner et al. 1994, Sucov et al. 1994). Loss of RXR$\beta$ or RXR$\gamma$ is not as severe because RXR$\alpha$ is able to compensate for the roles of both receptors.
(Ahuja et al. 2003). In vivo experiments with RXRα homozygous knockout mice demonstrate that those mice mainly die because of heart defects and disturbed placental development (Kastner et al. 1994, Sucov et al. 1994, Sapin et al. 1997).

RXR and its heterodimeric partner RAR can be activated by vitamin A derivatives, so-called retinoids (Singh et al. 2006). Retinoids are initially taken with food and via oxidation processes they are converted into retinoic acid, of which different isoforms such as 9-cis-retinoic acid (9-cisRA), 13-cisRA, and all-trans retinoic acid (ATRA) exist (Ross et al. 2000, Lee et al. 2004). Trophoblasts are able to synthesize retinoic acid (Tarrade et al. 2001). The vitamin A deficiency syndrome can lead to cardiovascular, respiratory, urogenital, and skeletal defects of the embryo (Wilson & Warkany 1948, Wilson & Barch 1949, Wilson et al. 1953, Kalter & Warkany 1961, Takahashi et al. 1975). An increased level of vitamin A can also have severe outcomes as it causes teratogenic side effects to the embryo (Grune et al. 2010). These congenital malformations are referred to as retinoic acid embryopathy and are mainly characterized by craniofacial abnormalities (Lammer et al. 1985).

Main natural ligands of PPAR, which is a heterodimeric partner of RXR, include fatty acids, eicosanoids, components of oxidized low-density lipoproteins, and oxidized alkyl phospholipids (Desvergne & Wahli 1999, McIntyre et al. 2003, Schofer et al. 2005). An important group of PPAR agonists are prostaglandins that belong to the eicosanoids (Forman et al. 1995, Kliwer et al. 1997). PPAR agonists can be used in the treatment of type II diabetes mellitus and for patients suffering from polycystic ovary syndrome (Arck et al. 2010).

For both prostaglandins and retinoids (Szanto et al. 2004), a pro-apoptotic role has been described. Retinoids can, therefore, be found in anti-tumor treatments such as in the therapy of acute promyelocytic leukemia (Altucci et al. 2005). During pregnancy, the prostaglandin 15-deoxy-Δ12,14-prostaglandin J2 (15d-J2) promotes apoptosis of trophoblasts and weakens their differentiation (Schaff et al. 2000).

Recently, we detected the expression of RXRα in the nuclei of villous trophoblasts (VT), syncytiotrophoblasts, and interstitial extravillous trophoblasts (EVT) of normal and miscarried placentas (Toth et al. 2008). Enhanced expression of RXRα in comparison to control placentas was found in EVT and VT of miscarried placentas, suggesting a strong regulatory role of RXRα during pregnancy.

In 2007, we could also demonstrate that an increased number of apoptotic EVT is found in placentas of miscarriages in comparison to EVT of placentas from healthy pregnancy terminations (Minas et al. 2007). Trophoblasts react to maternal immunological dysregulation by activation of the intrauterine pro-apoptotic Fas/Fas ligand (FasL) system (Minas et al. 2007). We further observed that an increased expression of FasL is associated with miscarriages (Minas et al. 2007). EVT probably synthesize FasL, which induces apoptosis of activated T-cells, in order to maintain early pregnancy (Runic et al. 1996, Uckan et al. 1997, Kauma et al. 1999, Makrigiannakis et al. 2001, Abrahms et al. 2004).

The aim of our study was to study the (patho-)physiological role of RXRα in the first trimester of human pregnancy with focus on apoptosis and miscarriages.

Materials and methods

Cell culture and cell stimulation

The choriocarcinoma cell lines BeWo (European Collection of Cell Cultures (ECACC), Salisbury, England) and JEG-3 (ATCC, Washington, DC, USA) and human VT (HVT; ScienCell, Carlsbad, CA, USA) were used for the in vitro experiments. HVT are derived from primary cultures, cryopreserved, and then delivered in a frozen state. BeWo, JEG-3, and HVT cells were cultured in DMEM (3·7 g/l NaHCO3, 4·5 g/l d-glucose, 1·028 g/l stable glutamine, and Na-Pyruvate; Biochrom, Berlin, Germany), which was supplemented with 10% heat-inactivated FCS and incubated at 37 ºC and at an atmospheric CO2 concentration of 5%.

BeWo and JEG-3 cells were separately grown to near confluency on sterile 8-well chamber slides (Nunc, Rochester, NY, USA) at a density of 40 000 cells/500 µl DMEM medium and HVT at a density of 150 000 cells/500 µl DMEM medium. In choriocarcinoma cells, medium change was carried out after 24 h, which was followed by treatment of the cells with 1 or 10 nmol/ml 9-cisRA, 15d-J2 (Sigma–Aldrich), ATRA (Sigma–Aldrich), and 13-cisRA (Sigma–Aldrich) for 24 and 48 h. HVT cells were stimulated with 10 nmol/ml 9-cisRA, ATRA, and 15d-J2 for 72 h and no medium change occurred in them before stimulation. As retinoids are sensitive to light, cell stimulation was carried out in a shaded room in order to prevent photodegradation. Untreated cells served as controls. In total, six independent experiments were conducted and evaluated.

Immunocytochemistry

After 24 or 48 h of incubation, the slides were fixed in 1:1 ethanol/methanol solution for 15 min. Otherwise, the experiments were carried out at room temperature. Between each step, the slides were washed with PBS (Biochrom). After incubation with diluted horse serum for 20 min (10 ml PBS and 150 µl horse serum; Vector Laboratories, Burlingame, CA, USA), the slides were incubated with anti-human RXRα mouse monoclonal
IgG2a antibody (Clone No. K8508; PPMX Perseus Proteomics, Tokyo, Japan), diluted 1:200 in PBS, for 17 h at 4°C and then incubated with the secondary antibody from the Vectastain Elite ABC Mouse IgG Kit (Vector Laboratories) for 30 min. Then, the ABC complex (Vector Laboratories) was applied on the slides and incubation lasted for 30 min. Finally, the slides were stained with 3-amino-9-ethylcarbazole plus (AEC plus; Dako, Glostrup, Denmark) for 6 min, counterstained with hemalaun for 30 s, washed in tap water, and coverslipped.

The cells were examined with a Leitz Diaplan light microscope (Leitz, Wetzlar, Germany) and a digital camera system (JVC, Victor Company of Japan, Yokohama, Japan) was used for attaining images. Totally, eight microscopic fields were analyzed and pictures were taken at random places of the slides. The slides were examined by two independent observers.

The cells' intensity of staining was examined semiquantitatively, including the possibilities of no staining (negative), weak (onefold), moderate (twofold), and strong (threefold) staining and expressed as percentage (%). In order to achieve a more accurate comparison, the average of moderately (twofold) and strongly (threefold) stained BeWo and JEG-3 cells was compared. HVT were compared by the average of their positive staining (%).

MCF7 breast cancer cells (ATCC) served as positive controls. Negative controls were performed by omission of anti-RXRz antibody and incubation of MCF7 cells with mouse IgG preimmune serum (Dako).

**Immunohistochemistry: detection of RXRz and M30 CytoDEATH**

Tissue samples from miscarriages (n = 10) and legal termination of healthy pregnancies (n = 10), of which the latter served as controls, were used for immunohistochemical detection of RXRz and M30 CytoDEATH. The gestational age of both tissue sample groups ranged from gestational week 7 to 12. Tissue samples from miscarriages and legal pregnancy terminations were obtained within 24 h of diagnosis through uterine abrasion and without previous hormonal treatment. Directly after tissue extraction, the samples were fixed in formalin and then embedded in paraffin wax. For immunohistochemistry, the paraffin sections were first deparaffinized in xylol, then washed in absolute ethanol, and afterwards incubated with methanol/H2O2 for 30 min in order to inhibit endogenous peroxidase activity. Then, the tissue samples were rehydrated in an alcohol gradient to distilled H2O and then placed in a pressure cooker containing sodium citrate (pH 6). After washing the sections in PBS for 10 min, they were incubated with diluted horse serum for 20 min (10 ml PBS and 150 μl horse serum; Vector Laboratories). The sections incubated with M30 CytoDEATH were also treated with ultra V blocking solution (Labvision, Fremont, CA, USA) for 45 min. The primary antibodies, anti-human RXRz mouse monoclonal IgG2a antibody (clone No. K8508; PPMX Perseus Proteomics; 1:200 dilution in PBS) or M30 CytoDEATH (M30), a monoclonal mouse IgG2b (Clone M30; Alexis, San Diego, CA, USA; 1:400 dilution in PBS), were then applied on the sections for 16 h at 4°C. This was followed by incubation of the sections with the secondary antibody from the Vectastain Elite ABC Mouse IgG Kit (Vector Laboratories) for 30 min. After washing the sections with PBS, they were incubated with the ABC complex (Vector Laboratories) for 30 min. The sections that had been incubated with anti-RXRz were stained with 3,3-diaminobenzidine substrate solution (Carl Roth, Karlsruhe, Germany) for 2–5 min and the sections that had been incubated with M30 were stained with AEC plus (Dako) for 8 min. All sections were counterstained in hemalaun for 2 min and washed in tap water for 5 min. Finally, the sections were dehydrated in an ascending alcohol series, washed in xylol, and coverslipped with Eukitt quick-hardening mounting medium (Sigma–Aldrich). The sections were examined with a Leitz Diaplan light microscope (Leitz) as described in the immunocytochemistry section. Two independent observers examined ten microscopic fields per section. The semiquantitative immunoreactive score (IRS) was used to examine the intensity and distribution of antigen expression (Remmele & Stegner 1987). The IRS score is calculated by multiplying the cells' staining intensity (0, none; 1, weak; 2, moderate; 3, strong) with the percentage of positive staining cells (0, no staining, 1 ≤ 10% of the cells; 2, 11–50% of the cells; 3, 51–80% of the cells; and 4, ≥ 81% of the cells). Placental slides from the third trimester of healthy pregnancies and MCF7 cells (ATCC) served as positive controls. Negative controls were performed by replacement of anti-RXRz antibody or M30 with mouse IgG preimmune serum (Dako).

**Real-time reverse transcriptase-PCR (TaqMan-PCR)**

**RNA extraction**

BeWo and JEG-3 cells were separately grown on sterile 24-well Multiwell slides (Becton Dickinson, Franklin Lakes, NJ, USA) at a density of 80 000 cells/ml DMEM medium. After 24 h, the cells were stimulated with 10 nmol/ml 9-cisRA and 15d-J2 and incubated for 2, 3, 4, 6, and 8 h. Control cells were incubated in the absence of the stimulants. The NucleoSpin RNAII Kit (Macherey-Nagel, Düren, Germany) was applied for the investigation of the total RNA according to the manufacturer’s protocol. Quantification and evaluation of the purity of the purified RNA was carried out with a
NanoPhotometer (Implen, Munich, Germany). For each cell line, six independent experiments were performed.

**Reverse transcription**

For reverse transcription (RT), the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) was used according to the protocol and RT was carried out in a mastercycler gradient (Eppendorf, Hamburg, Germany). RT conditions of the mastercycler included temperature cycles of 10 min at 25 °C, 2 h at 37 °C, 5 s at 85 °C, and 4 °C on hold.

**Quantitative real-time RT-PCR (TaqMan-PCR)**

Real-time reverse transcription-PCRs (RT-PCRs) were carried out in Optical Fast 96-well reaction microtiter plates (Applied Biosystems) that were covered with an adhesive cover. In each well, a volume of 20 μl was applied, which contained 1 μl TaqMan Gene Expression Assay 20× (order number Hs00172565_m1 for RXRa, Applied Biosystems), 10 μl TaqMan Fast Universal PCR Master Mix 2× (Applied Biosystems), 8 μl H2O, and 1 μl primer (Table 1). The ABI PRISM 7500 Fast (Applied Biosystems) performed the PCR assays under the following thermal conditions: 20 s at 95 °C, 40 cycles of amplification with 3 s at 95 °C and 30 s at 60 °C.

The ΔΔCt method was used for quantification. Glyceraldehyde phosphate dehydrogenase (order number Hs99999905_m1, Applied Biosystems) served as a reference gene (housekeeping gene).

**Immunochemical detection of RXRa in BeWo and Jeg-3 cell lysates on blots**

BeWo and Jeg-3 cells were separately grown on sterile 6-well chamber slides at a density of 80,000 cells/ml DMEM medium. After 24 h, medium was changed and the cells were incubated with 10 nmol/ml 9-cisRA (Sigma–Aldrich) for 48 h. The remaining medium was removed after incubation and the wells were washed with PBS. Then, 1 ml lysis buffer containing protease inhibitor cocktail (Sigma–Aldrich) and RIPA buffer (Sigma–Aldrich), in a dilution of 1:100, was applied on each well and then incubated with RIPA buffer for 15 min on ice. Finally, the lysate was removed from the wells and frozen at −20 °C. Total protein concentration was subsequently determined via Bradford assay. A protein amount of 10,000 ng was used for western blotting. The lysate proteins were first separated by SDS–PAGE and then transferred onto a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). Between each step, the membrane was washed in Casein solution from the Vectastain ABC-AmP mouse-IgG-Kit for western blot detection (Vector Laboratories). Otherwise, the experiments occurred at room temperature. The membrane was first blocked in Casein solution (dilated 1:10 in distilled water) for 1 h. Then, the membrane was incubated with anti-human RXX mouse monoclonal IgG2a antibody (Clone No. K8508; PPMX Perseus Proteomics), diluted 1:200 in PBS (final concentration: 5 μg/ml), for 16 h at 4 °C and then incubated with the biotinylated anti-mouse IgG secondary antibody from the Vectastain ABC-AmP mouse-IgG-Kit (Vector Laboratories), diluted in Casein solution to a final concentration of 1.5 μg/ml, for 45 min. Afterward, the membrane was incubated with the Vectastain ABC-AmP Reagent (Vector Laboratories) for 20 min. The membrane was then treated with 0.1 M Tris buffer with a pH of 9.5 for 5 min. The blots were developed with the BCIP/NBT–chromogen substrate solution. After 3 min of staining, the reaction was stopped with distilled water and the membrane was kept to dry. The experiments were carried out four times.

Western blots were detected with the Bio-Rad Universal Hood II (Bio-Rad Laboratories) and quantified using the Bio-Rad Quantity One Software (Bio-Rad Laboratories).

**Statistical analysis applied for evaluation of immunocytochemistry, immunohistochemistry, real-time RT-PCR, and western blotting**

The SPSS/PC Software package, version 15.01 (SPSS GmbH, Munich, Germany), was used for evaluation of statistical data including data collection, processing, and analysis. It was performed with the non-parametric Wilcoxon’s signed rank test that compares paired samples. P values with P≤0.05 were considered statistically significant.

**Double-immunofluorescence staining**

Double-immunofluorescence staining was performed on miscarried placentas (n=16) and placentas from elective termination of pregnancies (n=16). Both tissue sample groups were derived from gestational weeks 7 until 12. Immediately after curettage, the sections were frozen. Otherwise, the experiments were performed on embryos derived from elective termination of pregnancies (n=16).

![Image](https://example.com/image.png)

**Table 1** Primers used for real-time reverse transcriptase-PCR

<table>
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<th>Primer</th>
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<th>Order number</th>
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<td>RXRa</td>
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<td>5–6</td>
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<tr>
<td>GAPDH</td>
<td>Applied Biosystems</td>
<td>Hs99999905_m1</td>
<td>3–3</td>
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<td>β-Actin</td>
<td>Applied Biosystems</td>
<td>Hs99999903_m1</td>
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conducted at room temperature. Between each step, the slides were washed in PBS. The frozen slides were thawed and fixed in acetone for 5 min. After blocking with ultra V blocking solution (Labvision) for 15 min, the sections were incubated with M30 CytoDEATH (M30), a monoclonal mouse IgG2b (Clone M30; Alexis), diluted 1:400 in Dako diluting medium S322 (Dako), and the polyclonal goat anti-RXRα IgG (AbD

Figure 1 Expression of RXRα in BeWo cells. Diagram of immunocytochemical analysis of RXRα protein expression after incubation with 10 nmol/ml 9-cisRA, ATRA, and 15d-J2 for 24 h (*P<0.001) (A) and 48 h (*P<0.001) (C) compared with the control (n=6). Digital images of RXRα expression influenced by 10 nmol/ml 15d-J2, 10× lens (B), and 10 nmol/ml 9-cisRA, 25× lens (D). The staining intensity of the stimulated BeWo cells is weaker than the control. Diagram of immunocytochemical analysis of RXRα protein expression after incubation with 1 nmol/ml 9-cisRA (*P<0.001), ATRA (*P=0.042), and 15d-J2 for 24 h (E) and with 1 nmol/ml 9-cisRA (*P<0.001), ATRA (*P=0.042), and 15d-J2 for 48 h (G) compared with the control (n=6). Digital images of RXRα expression influenced by 1 nmol/ml 9-cisRA, 10× lens (F), and 1 nmol/ml ATRA, 25× lens (H). Quantitative real-time RT-PCR (n=6): significant downregulation of RXRα mRNA expression after incubation with 10 nmol/ml 9-cisRA for 4 h (*P=0.002) (I). Quantitative real-time RT-PCR: significant downregulation of RXRα mRNA expression after incubation with 10 nmol/ml 15d-J2 for 4 h (*P=0.010) (J).
Serotec, Oxford, England), diluted 1:250, for 17 h at 4 °C. Sections were then incubated with the secondary antibodies, Cy2-labeled rabbit-anti-mouse IgG antibody diluted 1:100 (Dianova, Hamburg, Germany) and Cy3-labeled donkey-anti-goat IgG antibody (Dianova), at a concentration of 1:500. The sections were finally embedded in Dapi mounting buffer containing 4',6-diamino-2-phenylindole (Vector Laboratories) and examined with a fluorescent Axioskop photomicroscope (Zeiss, Oberkochen, Germany). A digital Axio-cam camera system (Zeiss) was used to attain images.

Results

**RXRα expression in BeWo cells**

After an incubation time of 24 h as well as 48 h and at a concentration of 10 nmol/ml, 9-cisRA, ATRA, and 15d-J2 significantly (P<0.001) reduced the protein expression of RXRα in BeWo cells in comparison to untreated BeWo cells in immunocytochemistry (Fig. 1A–D). Of all three tested ligands, 10 nmol/ml 9-cisRA had the strongest effect on decreasing RXRα protein expression in BeWo cells with an even more pronounced effect after 48 h of incubation (Fig. 1C and D). At that incubation time, 9-cisRA reduced the average of strongly and moderately stained cells from 34.4±2.3% (Fig. 1C). ATRA (10 nmol/ml) and 15d-J2 also showed a stronger influence on the receptor’s expression after 48 h (Fig. 1C).

At 1 nmol/ml, 9-cisRA induced a significant (P<0.001) reduction of RXRα protein expression in the immunocytochemical analysis after 24 and 48 h (Fig. 1E and G). A weaker staining intensity of BeWo cells stimulated with 1 nmol/ml 9-cisRA in comparison to control BeWo cells can be seen in Fig. 1F. No reduction of RXRα expression could be observed after stimulation of BeWo cells with 1 nmol/ml 15d-J2 for 24 h (Fig. 1E) or 48 h (Fig. 1G). In 1 nmol/ml ATRA-treated cells, RXRα expression was not affected after 24 h; however, after 48 h, ATRA led to a significant (P=0.042) decrease in RXRα protein expression (Fig. 1G and H).

In quantitative real-time RT-PCR, treatment of BeWo cells with 10 nmol/ml 9-cisRA for 4 h significantly (P=0.002) reduced RXRα mRNA expression to over half of its initial concentration (47.2 vs 100% without treatment; Fig. 1I). Incubation with 10 nmol/ml 15d-J2 for 4 h induced a significant (P=0.010) reduction from initially 100 to 67.3% of RXRα mRNA expression (Fig. 1I).

Results of western blotting of RXRα expression in BeWo cell lysates are shown in Fig. 2. RXRα generates a main protein band in the molecular mass range of 60 kDa. The left lane represents control BeWo cell lysates and the right lane represents BeWo cell lysates that have been stimulated with 10 nmol/ml 9-cisRA for 48 h. A stronger staining intensity can be revealed for control BeWo cells in comparison to BeWo cells treated with 10 nmol/ml 9-cisRA (Fig. 2A). Western blotting was quantified with the Bio-Rad Quantity One Software (Bio-Rad Laboratories) (B). The left lane represents control JEG-3 cells and the right lane represents JEG-3 cells that have been stimulated with 10 nmol/ml 9-cisRA for 48 h (P=0.028) (C). Quantification of RXRα protein expression in JEG-3 cell lysates was carried out with Bio-Rad Quantity One Software (D).

**RXRα expression in JEG-3 cells**

In JEG-3 cells, 10 nmol/ml of 9-cisRA, ATRA, and 15d-J2 led to a significant (P<0.001) downregulation of RXRα protein expression in the immunocytochemical analysis after 24 and 48 h (Fig. 3A–D). The overall effect on RXRα reduction of the three applied substances can be arranged in the following descending order: 9-cisRA> ATRA> 15d-J2. After 48 h, retinoids had a stronger influence on RXRα downregulation (Fig. 3C and D). At a concentration of 1 nmol/ml, both tested retinoids showed a significant reduction in RXRα protein expression (Fig. 3E and G). This result can be observed in Fig. 3F and H, where 1 nmol/ml ATRA and 9-cisRA induced a weaker staining of the stimulated cells in comparison to the control. The effect was greater after 48 h of incubation, where the average of moderately and strongly stained cells was reduced from 29.7 to 0.010 reduction in RXRα mRNA expression (A).

Figure 2 Western blotting of RXRα protein expression in BeWo (n=4) and JEG-3 cell lysates (n=4). The left lane represents control BeWo cells and the right lane represents BeWo cells that have been stimulated with 10 nmol/ml 9-cisRA for 48 h (P=0.043) (A). Western blotting was quantified with the Bio-Rad Quantity One Software (Bio-Rad Laboratories) (B). The left lane represents control JEG-3 cells and the right lane represents JEG-3 cells that have been stimulated with 10 nmol/ml 9-cisRA for 48 h (P=0.028) (C). Quantification of RXRα protein expression in JEG-3 cell lysates was carried out with Bio-Rad Quantity One Software (D).
6.0% by 9-cisRA and to 19.1% by ATRA (Fig. 3G). 15d-J2 (1 nmol/ml) did not have an influence on RXRα protein expression after an incubation time of either 24 or 48 h (Fig. 3E and G).

9-cisRA (10 nmol/ml) significantly \((P=0.023)\) reduced RXRα mRNA expression to 65.6% compared with 100% of the control in quantitative real-time RT-PCR (Fig. 3I). 15d-J2 led to a significant \((P=0.026)\) downregulation of RXRα mRNA expression after incubation with 10 nmol/ml 9-cisRA for 8 h. Significant downregulation of RXRα mRNA expression after incubation with 10 nmol/ml 15d-J2 for 8 h \((P=0.026)\) in quantitative real-time RT-PCR (n=6) (J).
A reduction of 84.0% RXR\textsubscript{a} mRNA in JEG-3 cells after 8 h (Fig. 3J).

Western blot detection of RXR\textsubscript{a} protein expression of JEG-3 cell lysates is shown in Fig. 2. RXR\textsubscript{a} generates a main protein band in the molecular mass range of 60 kDa. The left lane, which represents RXR\textsubscript{a} protein expression of control JEG-3 cells, is stained more intensely than the right lane, representing JEG-3 cells that were incubated with 10 nmol/ml 9-cis\textsubscript{RA} for 48 h (Fig. 2C). Applying Bio-Rad Quantity One Software (Bio-Rad Laboratories), a significant decrease in RXR\textsubscript{a} expression of JEG-3 cells induced by 10 nmol/ml 9-cis\textsubscript{RA} after 48 h could be quantified (Fig. 2D). Staining intensity of RXR\textsubscript{a} expression in unstimulated (control) and stimulated JEG-3 cells in square millimeter volume was normalized to \(\beta\)-actin staining intensity respectively.

**RXR\textsubscript{a} expression in HVT**

In immunocytochemical analysis, the retinoids 9-cis\textsubscript{RA} and ATRA and the PPAR\textgamma ligand 15d-J\textsubscript{2} also led to a significant \((P<0.05)\) downregulation of RXR\textsubscript{a} protein expression in HVT at a concentration of 10 nmol/ml and after incubation for 72 h (Fig. 4A). Retinoids had a stronger effect on reducing the receptor’s protein expression than 15d-J\textsubscript{2} (Fig. 4A). Their effect can be observed (Fig. 4B and C) where the control is more strongly stained than the retinoid-treated cells.

**Expression of RXR\textsubscript{a} in EVT of placentas from miscarriages**

Protein expression of RXR\textsubscript{a} in EVT of placentas from miscarriages (gestational weeks 7–12) was compared with its expression in EVT of placentas from legal termination of healthy pregnancies, which served as a control (gestational weeks 7–12). In EVT of miscarriages, an increased protein expression of RXR\textsubscript{a} could be observed in comparison to the control \((P=0.001; \text{Fig. 5A})\). No significant difference in RXR\textsubscript{a} expression in EVT of miscarriages could be detected for the different gestational weeks (7–12) of the occurrence of miscarriage.

**Expression of M30 CytoDEATH in EVT of placentas from miscarriages**

Apoptosis of EVT of placentas from miscarriages and elective terminations of healthy pregnancies (both gestational weeks 7–12) was immunocytochemically detected with M30 CytoDEATH applied as a primary antibody. Less than 2% of interstitial EVT from elective pregnancy terminations were apoptotic. In miscarriages, a significantly increased number of apoptotic EVT could be detected \((P<0.05)\) in comparison to the control (Fig. 5B). No difference in the amount of apoptotic EVT could be found in placentas of miscarriages from different gestational weeks (7–12). Hence, the number of apoptotic EVT was independent.
The IRS score has been used for both immunohistochemical investigations. (A) In EVT of placentas from miscarriages, a significantly ($P<0.05$) increased expression of RXR$\alpha$ can be found in comparison to the control (EVT of placentas from terminations of healthy pregnancies). (B) A significantly (* $P<0.001$) increased expression of the apoptotic marker M30 can be found in EVT of placentas from miscarriages in comparison to the control.

Figure 5 Immunohistochemical detection of RXR$\alpha$ ($n=10$) and M30 ($n=10$) in EVT of placentas from miscarriages. The IRS score has been used for both immunohistochemical investigations. (A) In EVT of placentas from miscarriages, a significantly ($P<0.05$) increased expression of RXR$\alpha$ can be found in comparison to the control (EVT of placentas from terminations of healthy pregnancies). (B) A significantly (* $P<0.001$) increased expression of the apoptotic marker M30 can be found in EVT of placentas from miscarriages in comparison to the control.

to the time of occurrence of miscarriage. The tissue samples used for the investigation were immediately processed after curettage.

**Co-expression of M30 CytoDEATH and RXR$\alpha$**

Incubation of first trimester miscarried placentas in double-immunofluorescence experiments with cytokeratin 7 (CK7) and prolactin were used to identify/exclude EVT. Positive staining of the cells for CK7 and negative staining for prolactin served as markers for EVT. RXR$\alpha$-positive cells were identified as EVT.

The primary antibody M30 CytoDEATH (M30) detects apoptosis in tissue and epithelial cells. In this study, interstitial EVT of miscarried placentas from the first trimester expressed RXR$\alpha$, stained in red (Fig. 6A), and M30 stained in green (Fig. 6B). Triple filter excitation showed expression of RXR$\alpha$ and M30 in the same EVT (Fig. 6C), which can be identified by yellow staining. Thus, a simultaneous expression of RXR$\alpha$ and M30 in EVT of placentas from miscarriages can be shown. EVT of placentas from legal termination of healthy pregnancies expressed RXR$\alpha$, which was stained in red (Fig. 6D). Some cells were stained in green; thus, they express the apoptotic marker M30 (Fig. 6E). No simultaneous expression of RXR$\alpha$ and M30 could be detected in EVT of placentas from legal pregnancy terminations (Fig. 6F). Hence, EVT of placentas from healthy pregnancy terminations that express RXR$\alpha$ are not apoptotic.

**Discussion**

In our study, we analyzed the expression of RXR$\alpha$ in the choriocarcinoma cell lines BeWo and JEG-3 and in HVT influenced by the retinoids 9-cisRA, ATRA, and the PPAR$\gamma$ agonist 15d-J2. Incubation with 10 nmol/ml of each of the stimulants induced a significant ($P<0.05$) downregulation of RXR$\alpha$ expression in all tested cell lines on protein level via immunocytochemistry and western blot and in choriocarcinoma cells also on mRNA level via real-time RT-PCR. Hence, 9-cisRA, ATRA, and 15d-J2 influence the expression of RXR$\alpha$ in choriocarcinoma cells on transcriptional and thus translational levels.

We previously described that upregulation of apoptotic EVT is found in abortive placenta tissue (Minas et al. 2007). In addition, we could show that EVT in placentas of miscarriages show a significantly higher expression of RXR$\alpha$ in comparison to EVT in placentas of elective terminations of healthy pregnancies (Toth et al. 2008). We also speculated that the time span between abortion and formalin fixation or freezing of the tissue samples does not lead to an additional upregulation of apoptotic EVT (Minas et al. 2007). If apoptosis of EVT was due to degradation of the extracted tissue, a higher apoptosis rate would have been observed in the tissue samples of elective terminations, as those samples have been processed exactly the same way as the tissue samples from miscarriages (Minas et al. 2007).

Ligand-dependent downregulation of RXR$\alpha$ in choriocarcinoma cells and HVT might be a response of RXR$\alpha$ to escape repression by retinoids and prostaglandins in vivo. This phenomenon has already been observed in neutrophilic granulocytes where downregulation of RXR$\alpha$ induced by retinoids turned out to be necessary for neutrophilic development (Taschner et al. 2007). It can be hypothesized that a reduced expression of RXR$\alpha$ might serve as a response to apoptosis. As an increased expression of RXR$\alpha$ is found in EVT of miscarriages and those are associated with an increased number of apoptotic EVT, down-regulation of RXR$\alpha$ in HVT might act protectively during early pregnancy. In vivo studies have to be undertaken to further study retinoid signaling and to
clarify whether inhibition of RXRα expression could serve as a protection against miscarriages.

Furthermore, we were able to demonstrate a simultaneous expression of the apoptotic marker M30 CytoDEATH and RXRα in EVT of abortion placentas from the first trimester, indicating that apoptotic EVT express the nuclear receptor RXRα. As apoptotic cells feature an increased expression of RXRα and in non-apoptotic cells, the expression of RXRα is reduced, the assumption can be made that RXRα plays an essential role in the induction of apoptosis. A reduced RXRα expression is, therefore, desirable to prevent apoptosis of EVT as found in abortive tissue. In future investigations, natural ligands that are able to enhance the expression of RXRα and thereby induce apoptosis should be identified. This knowledge will further improve the understanding of RXRα regulation in apoptosis.

In line with our results, the research group of Nomura et al. (1999) was able to demonstrate that 1 μM 9-cisRA significantly reduced protein expression of RXRα in JEG-3 cells after an incubation time of 24 h. They argued that 9-cisRA might accelerate RXRα degradation through enzymes such as lysosomal cathepsin 1-type protease. Furthermore, Nomura et al. (1999) explained the decrease in RXRα expression by the ability of 9-cisRA to promote a shift of RXRα from the nucleus to the cytoplasm. However, we were unable to detect RXRα in the cytoplasm of the investigated cells. We speculate that during proliferation, RXRα stays attached to the DNA via response elements and its concentration remains unchanged. As the amount of DNA is doubled at the same time, the ratio of RXRα bound to DNA consequently decreases and therefore a reduced RXRα expression can be observed in immuno-cytochemical staining.

Boudjelal et al. (2002) investigated that 1 μM ATRA as well as 1 μM 9-cisRA led to a significant decrease in RXRα protein expression of immortalized human keratinocytes (HaCaT cells). In their investigation, the transcript levels of RXRα mRNA expression were not affected by retinoids in comparison to our results. Boudjelal et al. (2002) were able to verify the assumption of Nomura et al. (1999) that 9-cisRA accelerates the breakdown of RXRα protein. They argued that a ligand-induced decrease in RXRα expression is mediated by a proteasome degradation pathway. Ligand binding to RXRα thus activates the receptor as well as negatively regulates its expression. Ligand-induced receptor degradation via the proteasome pathway has also been described for other members of the nuclear receptor superfamily: Nawaz et al. (1999) showed that estrogen induces a reduction of the estrogen receptor via proteasome-mediated degradation and thereby decreases the transcriptional activity of the estrogen receptor.

The effect of retinoids and PPARγ agonists on the expression of RXRα is also dependent on the examined tissue. In 3T3-L1 mouse adipocytes, the research group of Hollung et al. (2004) discovered

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**Figure 6** Double-immunofluorescence staining of extravillous trophoblasts (EVT). RXRα is marked with the Cy-3 labeled secondary antibody and stained in red. It is expressed in EVT of placentas from miscarriages (A) and legal terminations of healthy pregnancies (D). Green staining represents the apoptotic marker M30 CytoDEATH (M30) that is marked with the Cy-2 labeled secondary antibody. It is expressed in EVT of miscarried placentas (B) and in some cells of placentas from legal pregnancy terminations, shown in the upper left corner of the image (E). Triple filter excitation shows a simultaneous expression of RXRα and M30 in EVT of miscarried placentas (C). No co-expression of RXRα and M30 can be detected in EVT of the control (healthy pregnancy terminations). The blue staining of the nucleus results from the Dapi mounting buffer. All images: 40× lens, co-expression of M30 and RXRα.
a significant increase of RXRα mRNA expression after incubation with 1 or 10 µM ATRA. Krskova-Tybitanclova et al. (2008) also showed an upregulation of RXRz in rat adipose tissue induced by 13-cisRA. The deviating results of RXRz expression in adipose tissue in comparison to its expression in choriocarcinoma cells and trophoblasts can be explained by the varying functions of retinoids in different tissues. 13-CisRA may be able to increase the expression of RXRz in adipose tissue, as synthetic RXR ligands are able to promote adipogenesis and RXR expression is increased during the differentiation of adipocytes (Villarroya et al. 1999). Hence, the impact of retinoids on the expression of nuclear receptors depends on its concentration, incubation time, tissue, and species (Hollung et al. 2004).

The cytotoxic effect of dimethyl sulfoxide (DMSO) and ethanol on cell lines such as BeWo is described in the former literature (Rebourcet et al. 2004). After dissolving 9-cisRA and ATRA in ethanol or DMSO, while keeping a limit of 3% for the substances in order to prevent cell death, we observed a change in the cells’ morphology and staining ability. As an altered expression of the non-steroidal nuclear receptors induced by DMSO and ethanol could be observed, we alternatively dissolved the retinoids in DMEM medium.

In conclusion, we assume that a ligand-dependent reduced expression of RXRz in trophoblasts might occur as a protection against apoptosis, respectively, abortion. RXRz possibly represents a ‘potential therapeutic candidate’ (Desvergne 2007) in the treatment of recurrent miscarriages. In this study, vitamin A derivatives have been identified to influence RXRz expression. Owing to the pleiotropic functions of RXRz, future studies could focus on identifying ligands that are able to influence RXR and its heterodimeric partners in order to treat multifactorial diseases. Examples include patients who suffer from hypothyroidism or diabetes mellitus in addition to recurrent miscarriages.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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