The expression of thyroid hormone receptors (THR) is regulated by the progesterone receptor system in first trimester placental tissue and in BeWo cells *in vitro*

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Introduction

Thyroid hormones together with its receptor system are key metabolic regulators coordinating short-term and long-term energy needs [1]. Low thyroid hormone levels can result in early miscarriages or even prevent women from being able to conceive [2]. A 2-fold increase of miscarriages and stillbirth rates can result from untreated hypothyroidism [3]. Hence, the maintenance of a euthyroid state is crucial for a healthy pregnancy [3].

Thyroxine (T_4) is converted into the biological active form T_3 through 5-O-deiodinases [4]. T_3 binds to the thyroid hormone receptor and thereby promotes trophoblast differentiation [5]. Thyroid hormones are also responsible for the growth of neuronal cells, myelinisation and corticogenesis [6]. After the 10th week of

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gestation, high affinity nuclear binding sites for T_3 can be found in the fetal brain [7].

Two genes, THRA (NR1A1) and THRB (NR1A2), encode the isoforms THRα and THRβ which form the four ligand-binding thyroid receptors THRα1, THRβ1, THRβ2 and THRβ3 and the four non-ligand binding receptors THRα2, THRΔα1, THRΔβ2 and THRΔβ3 [8,9]. THRα1 is expressed in the heart, bone and brain, whereas THRβ1 is mostly expressed by the liver and kidney [10]. The expression of THRα1, THRβ1 and the non-ligand-binding-THRα2 increased with gestational age [11].

The functional THR complex consists of a heterodimer with the retinoid X receptor (RXR) that binds to a thyroid hormone response element (TRE) to modulate gene expression [12]. The hormone T_3 regulates gene transcription through binding to THR [13]. The binding of T_3 induces the conformational changes of THRs and results in dissociation of co-repressors and binding of co-activators with THRs [10]. After the hormone T_3 has bound to the ligand binding site, specific co-activators like PGC-1, SRC1-3, TRAP and TRBP are induced [14]. Coactivators only bind to THR in the presence of T_3 and subsequently recruit mediators that further initiate transcription [15].

The progesterone receptor (PR) as a steroid receptor belongs like THR to the nuclear receptor superfamily [16]. The oestrogen receptor (ER), androgen receptor (AR), mineral corticoid receptor (MR) and glucocorticoid receptor (GR) together with PR form the superfamily III of the nuclear receptors [17]. Progesterone plays a major role in the female reproductive system [18].

The anti-progestin Mifepristone induces the withdrawal of progesterone [19]. Mifepristone acts as an emergency contraceptive by blocking or delaying ovulation or as an abortifacient by transforming the endometrium [20–22]. In women, a single dose of mifepristone (200 mg) in the secretory phase of the cycle rapidly renders the endometrium unreceptive, and has been shown to alter gene expression in the uterus within 6 h of oral administration [23,24].

Catalano et al. (2007) could demonstrate that the molecular components necessary to synthesise and metabolise thyroid hormones are expressed in the endometrium, together with THRs and the associated receptor modifiers [19]. Transcripts involved in thyroid hormone metabolism and signalling such as type II iodothyronine deiodinase and thyroid receptors were found to be highly regulated by progesterone antagonism in the endometrium [19].

In a former study we could evaluate a number of nuclear receptors and its role in first trimester pregnancy and miscarriages [16]. Recently, we identified enhanced expression of retinoid X receptor alpha (RXRα) in placentas of miscarriages [25]. RXR as part of multi-protein complexes also include the THR system and transcriptional co-regulators and co-repressors [26]. The receptors bind to DNA response elements and act as ligand-dependent transcription factors [27]. In the absence of ligands, the receptors bind the co-repressors SMRT and NCoR and repress gene expression [28].

As a systematic analysis of the expression of THRs in first trimester placentas is still missing, the aim of this study was to investigate the expression of the THR isoforms THRα1, THRα2, THRβ1 and THRβ2 on protein and on mRNA-level in first trimester placentas and to further analyse the interaction of RU486 with PR as well as THR.

Material and methods

Tissue sample

Samples from elective terminations of pregnancy (ETP, 20 cases) and samples from elective terminations of pregnancy induced by mifepristone (RU 486, 13 cases) were also used. The mean age of women in case of ETP was 29.7 ± 6.0 years and in case of RU 486 induced termination was 26.4 ± 7.6 years ($p > 0.05$). The mean gestational age for the group of ETP was 8.4 ± 1.9 weeks and in case of RU 486 was 9.8 ± 2.2 weeks ($p > 0.05$). Hypothyroidism as well as hyperthyroidism has been excluded in all patients participated in our study. In all samples karyotyping analysis excluded chromosomal abnormalities [29]. This study was approved by the Ethical Committee of the Medical School, Ludwig-Maximilian-University of Munich. Informed consent was obtained from the patients.

Immunohistochemistry

Formalin-fixed paraffin-embedded sections (3 μm) were deparaffinised, rehydrated and subjected to epitope retrieval. Sections were blocked with 3% H_2O_2 in methanol (20 min) for endogenous peroxidase activity. Non-specific binding of the primary antibodies was blocked. Incubation with the primary antibodies followed. Salient features of the antibodies used are presented in Table 1. Reactivity was detected by using the Vectastain Elite ABC-Kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's protocol. Colon (THRα1), term placenta (THRα2) and breast cancer tissue (THRβ1, THRβ2) were used for positive control staining (Fig. 1). Replacement of the primary antibody with mouse or rabbit IgG, respectively served as negative control (Fig. 1). Per slide, ten fields were examined with the semi-quantitative immunoreactive score (IRS) [30]. The immunoreactive score (IRS) is a semiquantitative method to analyse the intensity and distribution pattern of antigen expression. It is calculated by multiplying the optical staining intensity (graded as 0 = none, 1 = weak, 2 = moderate, 3 = strong staining) with the percentage of positively stained 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) [31]. The staining intensity and the percentage of positively stained cells are estimated optically by

Table 1
Salient features of the primary antibodies used for immunohistochemistry.

Antibody (AB)	AB incubation conditions	Blocking solution	Blocking conditions
THRalpha1 Polyclonal (Rabbit IgG) Abcam	1:200 in PBS 1 h RT	Reagent 1 (Polymer kit)	5 min
THRalpha2 Mouse IgG1 Serotec	1:1000 in PBS 1 h RT	Reagent 1 (Polymer kit)	5 min
THRbeta1 Polyclonal (Rabbit IgG) Novus Biologicals	1:300 in Dako VM 1 h RT	Reagent 1 (Polymer kit)	5 min
THRbeta2 Polyclonal (Rabbit IgG) Upstate (cell signalling solutions)	1:100 in Dako VM 1 h RT	Reagent 1 (Polymer kit)	5 min

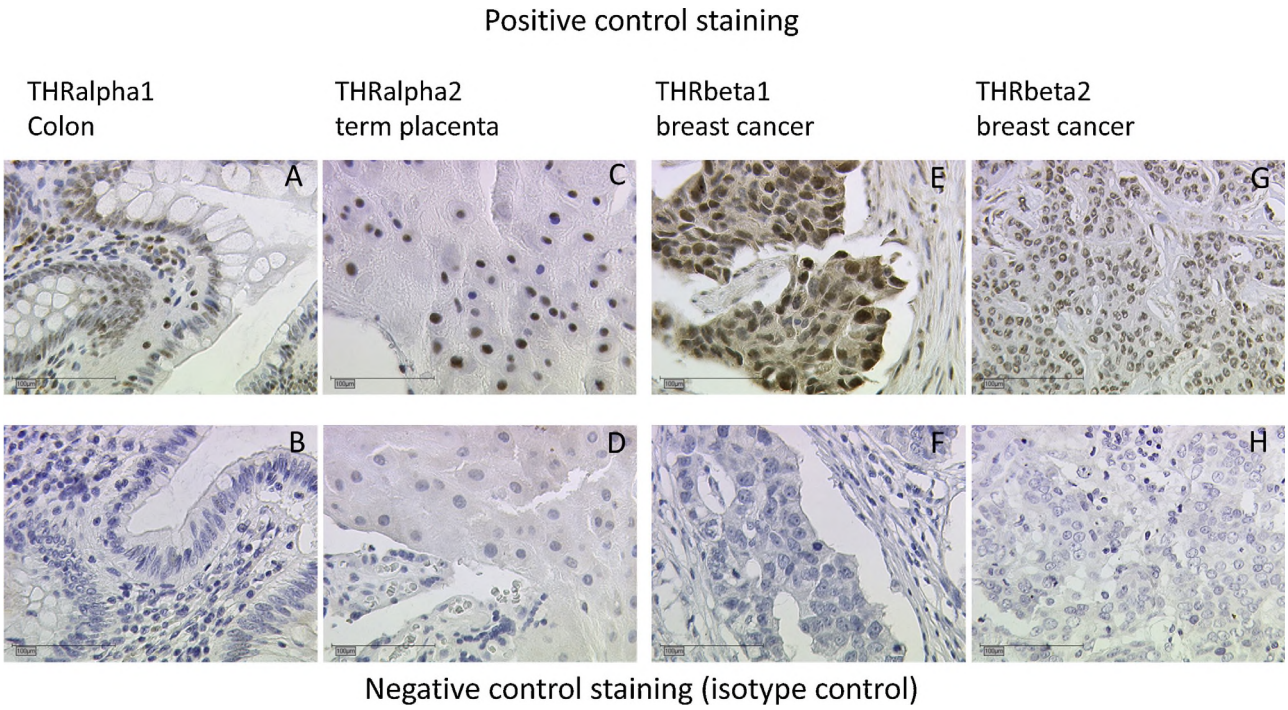

Fig. 1. Colon tissue was used for positive control staining of THRα1 antibodies. THRα1 expression is found specifically in the nucleus of the mucosa (epithelium and pericryptal fibroblast sheath, A). Isotype control staining is shown in B. Term placental tissue was used as for THRα2 positive control staining. THRα2 expression is found in nuclei of villous as well as extravillous trophoblast cells (C). Isotype control staining is shown in D. Breast cancer tissue was used for positive control staining of THRβ1 antibodies. THRβ1 expression is found specifically in the nucleus of the cancer cells with high intensity as well as the surrounding stroma with lower intensity (E). Isotype control staining is shown in (F). Breast cancer tissue was also used for positive control staining of THRβ2 antibodies. THRβ2 expression is found specifically in the nucleus of the cancer cells and not in surrounding stroma (G). Isotype control staining is shown in H.

the observer. Two independent observers evaluated the IRS score for each slide.

Stimulation of trophoblast tumour cells BeWo with progesterone

The chorioncarcinoma cell line BeWo was obtained from the European Collection of cell cultures (ECACC, Salisbury, UK). BeWo cells were grown on sterile 24 Multiwell slides at a density of 1 million cells/ml DMEM medium. After 24 h, the cells were stimulated with 0.01 μM or 0.1 μM human progesterone (Sigma-Aldrich, Munich, Germany) for 2 h. Control cells were incubated without stimulants. For the investigation of the total RNA, the NucleoSpin RNAII Kit (Macherey-Nagel, Düren, Germany) was applied according to the manufacturer's protocol.

Evaluation of THRA and THRB expression with real time RT-PCR (TaqMan)

RNA extraction from placental tissue

Total RNA was extracted from placental tissue of 10 women with elective terminations of pregnancy induced by mifepristone and 15 normal controls. Therefore the Rneasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany) was used according to the manufacturer's protocol.

Reverse transcription

The Reverse Transcription (RT) was performed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Weiterstadt, Germany) according to the protocol.

Real-time reverse transcription-PCR

In optical 96-well reaction microtiter plates covered with optical caps the Real-Time Reverse Transcription PCRs were accomplished in a volume of 20 μl, containing 1 μl TaqMan® Gene Expression Assay 20x (Hs00268470_m1 for THRA and Hs00230861_m1 for THRB, both Applied Biosystems), 10 μl TaqMan® Universal PCR Master Mix 2x (Applied Biosystems), 8 μl H_2O (DEPC treated DI water, Sigma, Taufkirchen, Germany) and 1 μl template. Thermal cycling conditions were 20 s at 95 °C, followed by 40 cycles of amplification with 3 s at 95 °C and 30 s at 60 °C. The ABI PRISM 7500 Fast (Applied Biosystems) was used for the PCR assays. Quantification was carried out by the $2^{-\Delta\Delta CT}$ method using GAPDH (Hs99999905_m1) as housekeeping gene.

Statistical evaluation

The SPSS/PC software package, version 20 (SPSS GmbH, Munich, Germany) was used for data collection and processing as well as analysis of statistical data. Values with $p < 0.05$ were considered statistically significant.

Results

Immunohistochemistry

THRalpha1

We identified a strong expression of THRα1 with a median of 7.5 in nuclei of villous trophoblast cells (Fig. 2A). Nuclei of cytotrophoblast cells as well as the syncytiotrophoblast are stained with high intensity and quantity. A similar result was found in the decidua. We identified a strong expression of THRα1 with a median of 6 in nuclei of decidual cells (Fig. 2B). In placental tissue of termination of pregnancy induced by mifepristone (RU 486), a significantly lower expression was found with a median expression of 4 ($p < 0.001$) in the syncytiotrophoblast (Fig. 2C) and a median expression of 3 ($p = 0.04$) in the decidua (Fig. 2D). A

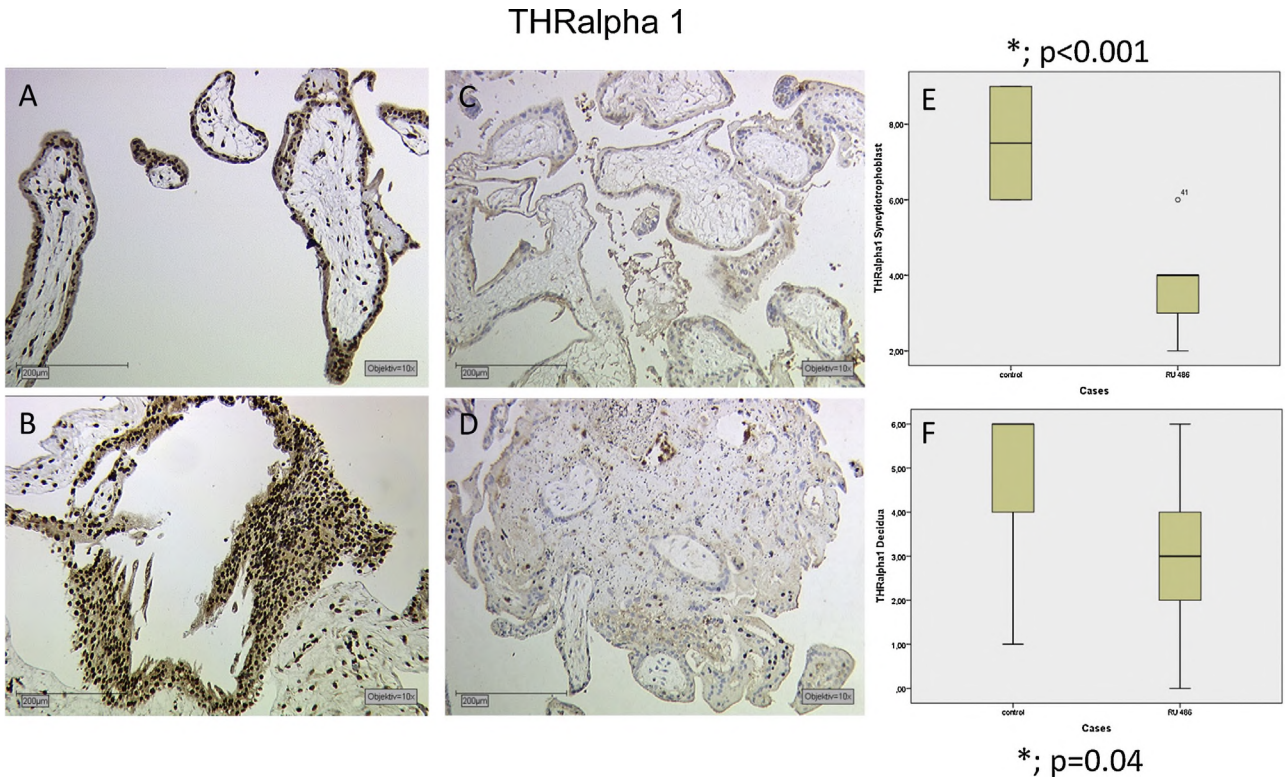

Fig. 2. THRα1 expression is found with high intensity and distribution in normal villous trophoblast cells (A) and in the decidua (B). In placental tissue of women with termination of pregnancy induced by mifepristone (RU 486) we found a significant downregulation of THRα1 in villous trophoblast cells (C) and in the decidua (D). A summary of the staining results is given in Box plot analysis for villous trophoblast tissue (E) and the decidua (F). The boxes represent the range between the 25th and 75th percentiles with a horizontal line at the median. The bars delineate the 5th and 95th percentiles. The circle indicates values more than 1.5 box lengths.

summary of the staining results of villous tissue is presented in Fig. 2E and a summary of staining results in decidual tissue are shown in Fig. 2F.

THRalpha2

We identified a very intense expression of THRα2 with a median of 9 in nuclei of villous trophoblast cells (Fig. 3A). Nuclei of cytotrophoblast cells as well as the syncytiotrophoblast are stained with high intensity and quantity. A median expression was found in the decidua. We identified an expression of THRα2 with a median of 4 in nuclei of decidual cells (Fig. 3B). In placental tissue of patients with termination of pregnancy induced by mifepristone (RU 486), a significantly lower expression was found with a median expression of 4 ($p = 0.004$) in the syncytiotrophoblast (Fig. 3C) and a non-significant change with a median expression of 3.5 ($p = 0.145$) in the decidua (Fig. 3D). A summary of the staining results of villous tissue is presented in Fig. 3E and a summary of staining results in decidual tissue are shown in Fig. 3F.

THRbeta1

We identified an expression of THRβ1 with a median of 4 in nuclei of villous trophoblast cells (Fig. 4A). Nuclei of cytotrophoblast cells as well as the syncytiotrophoblast are both stained. A similar result was found in the decidua. We identified an expression of THRβ1 with a median of 3 in nuclei of decidual cells (Fig. 4B). In placental tissue of termination of pregnancy induced by mifepristone (RU 486), a significantly lower expression was found with a median expression of 3 ($p = 0.023$) in the syncytiotrophoblast (Fig. 4C). A non-significant change with a median expression of 2 ($p = 0.069$) was found in the decidua (Fig. 4D). A summary of the staining results of villous tissue is presented in Fig. 4E and a summary of staining results of decidual tissue are shown in Fig. 4F.

THRbeta2

We identified an intense expression of THRβ2 with a median of 6 in nuclei of villous trophoblast cells (Fig. 5A). Nuclei of cytotrophoblast cells as well as the syncytiotrophoblast are both stained. A similar result was found in the decidua. We identified an expression of THRβ2 with a median of 4 in nuclei of decidual cells (Fig. 5B). In placental tissue of women with termination of pregnancy induced by mifepristone (RU 486), the same median expression was found (IRS = 6, $p = 0.098$) in the syncytiotrophoblast (Fig. 5C) and a non-significant change with a median expression of 3.5 ($p = 0.14$) in the decidua (Fig. 5D). A summary of the staining results of villous tissue is presented in Fig. 5E and a summary of staining results in decidual tissue are shown in Fig. 5F.

Evaluation of THRA and THRB expression with real time RT-PCR (TaqMan)

We identified an expression of THRA and THRB in all cases investigated. The mRNA expression of THRA was significantly down-regulated to a 0.36-fold expression in placental tissue of women with termination of pregnancy induced by mifepristone (RU 486) ($p = 0.022$, Fig. 6A). The mRNA expression of THRB was down-regulated to a 0.51-fold expression in placental tissue of women with termination of pregnancy induced by mifepristone (RU 486) without reaching significance ($p = 0.39$, Fig. 6B).

Stimulation of trophoblast tumour cell line BeWo with progesterone

Stimulation of BeWo cells with 0.01 μM human progesterone led to a 1.3-fold increased expression of THRA after 2 h of cultivation time (Fig. 7, $p < 0.001$). Addition of 0.1 μM progesterone to BeWo cells also led to a significant increased progesterone production of BeWo cells (Fig. 7, 1.15-fold, $p = 0.02$). Addition of

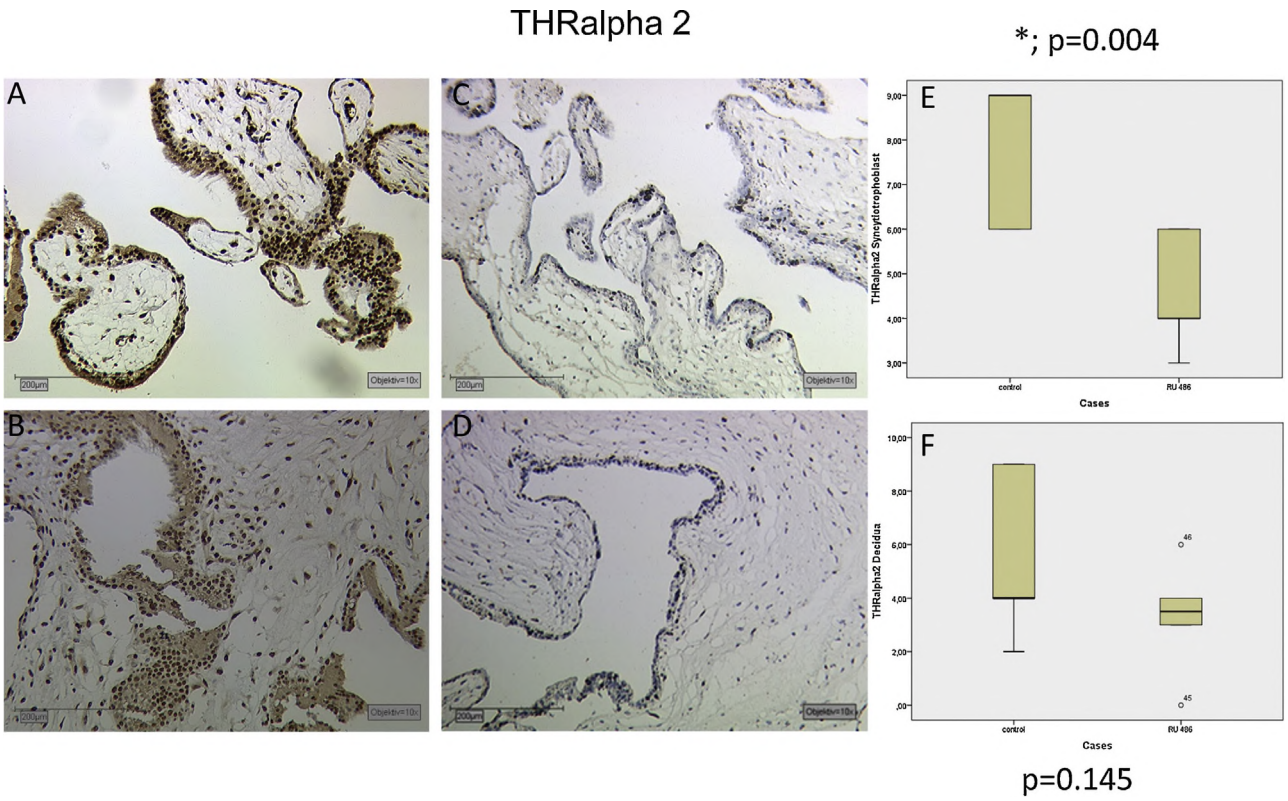

Fig. 3. THRα2 expression is found with high intensity and distribution in normal villous trophoblast cells (A) and in the decidua (B). In placental tissue of women with termination of pregnancy induced by mifepristone (RU 486) we found a significant downregulation of THRα2 in villous trophoblast cells (C) but not in the decidua (D). A summary of the staining results is given in Box plot analysis for villous trophoblast tissue (E) and the decidua (F).

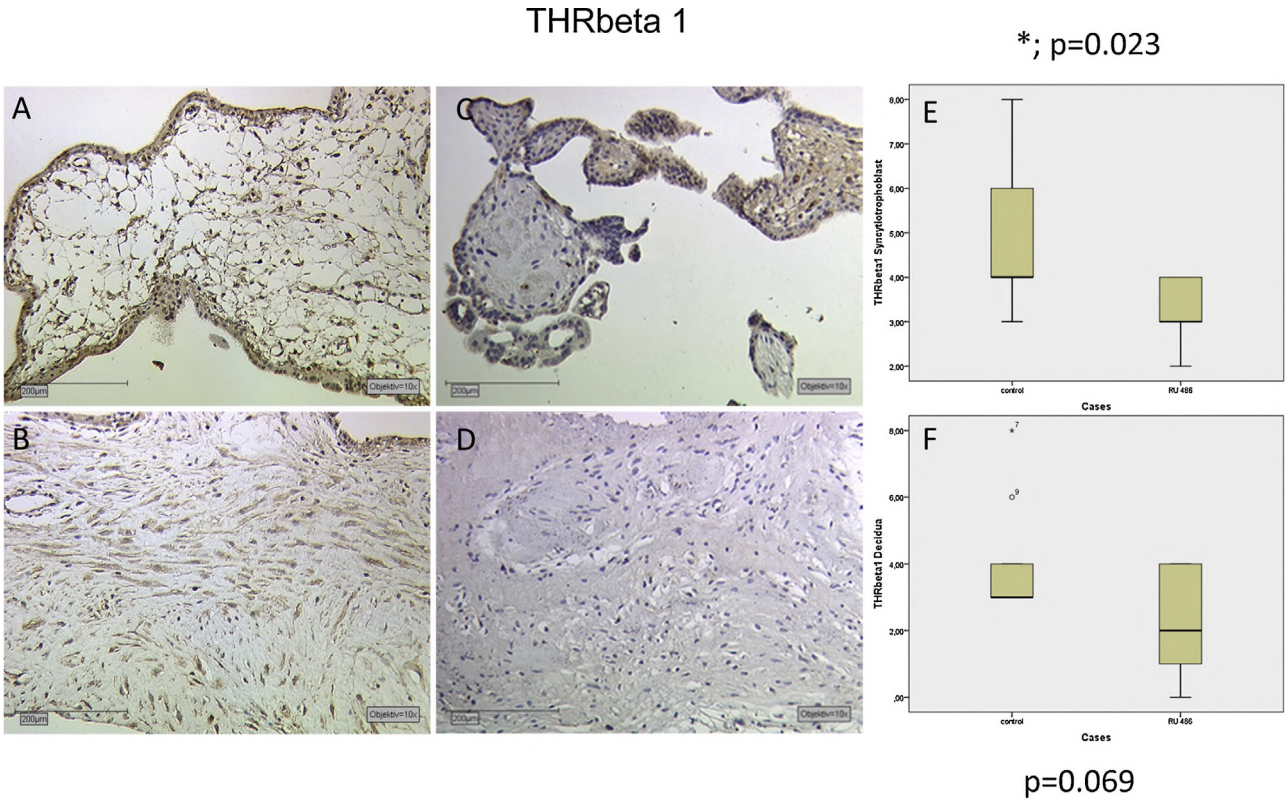

Fig. 4. THRβ1 expression is found with median intensity and distribution in normal villous trophoblast cells (A) and in the decidua (B). In placental tissue of women with termination of pregnancy induced by mifepristone (RU 486) we identified a significant downregulation of THRβ1 in the villous trophoblast (C) but not in the decidua (D). A summary of the staining results is given in Box plot analysis for villous trophoblast tissue (E) and the decidua (F).

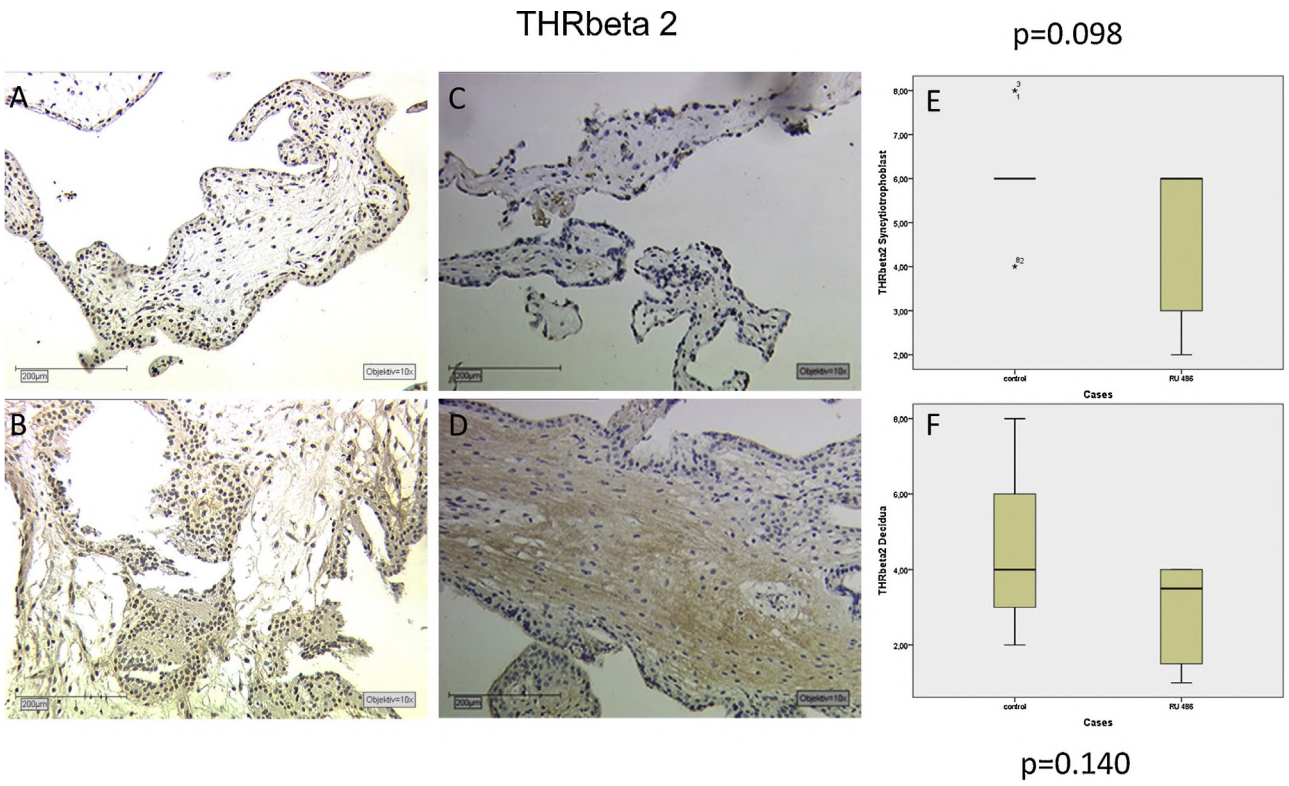

Fig. 5. THRβ2 expression is found with high intensity and distribution in normal villous trophoblast cells (A) and with median expression in the decidua (B). In placental tissue of women with termination of pregnancy induced by mifepristone (RU 486) we found no significant change of THRβ2 in villous trophoblast cells (C) as well as in the decidua (D). A summary of the staining results is given in Box plot analysis for villous trophoblast tissue (E) and the decidua (F).

0.01 μM as well as 0.1 μM human progesterone to BeWo cells *in vitro* induced a 1.1-fold increased expression of THRB after 2 h of cultivation time (Fig. 8), without reaching significance ($p > 0.05$).

Comments

It is already known for decades that steroid hormone receptors like the progesterone receptor and thyroid hormone receptors exhibit striking structural and functional similarity [32]. Early investigation of these two receptor families suggested that nuclear receptors may enhance transcription of target genes by similar mechanisms [33]. Zhang et al. observed transcriptional interference in a ligand-dependent manner between progesterone receptor B (PR-B) and THR α/β in transient transfection experiments [33].

Wagner et al. showed that the nuclear receptor co-repressor (NCoR) and the silencing mediator for the retinoid and the thyroid hormone receptor (SMRT) are differentially associated with the progesterone receptor. This differential association depends on the type of ligand bound to the receptor [34]. Not only co-repression but also ligand-dependent transcriptional activation by nuclear receptors is mediated by interactions with co-activators [35]. A consensus interaction motif (LXXLL) has been identified in a number of co-activators such as the steroid receptor coactivator-1

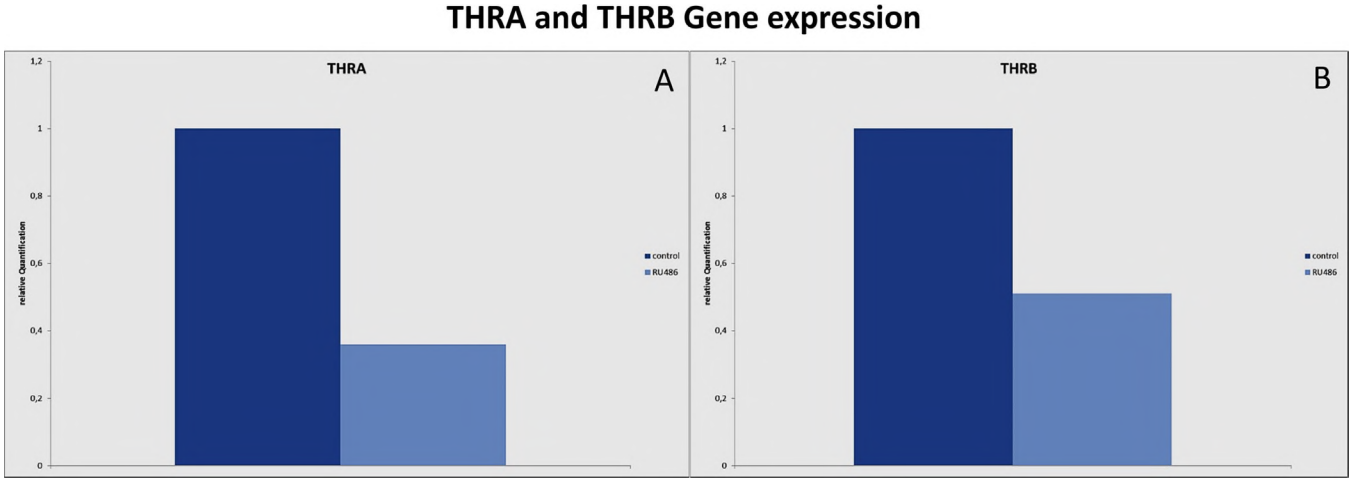

Fig. 6. Results of gene expression analysis with TaqMan RT-PCR. THRA expression is shown in A. We identified a significant downregulation of THRA in placental tissue of women with termination of pregnancy induced by mifepristone (RU 486). In addition, we identified no significant downregulation of THRB in placental tissue of women with mifepristone (RU 486) induced termination of pregnancy (B).

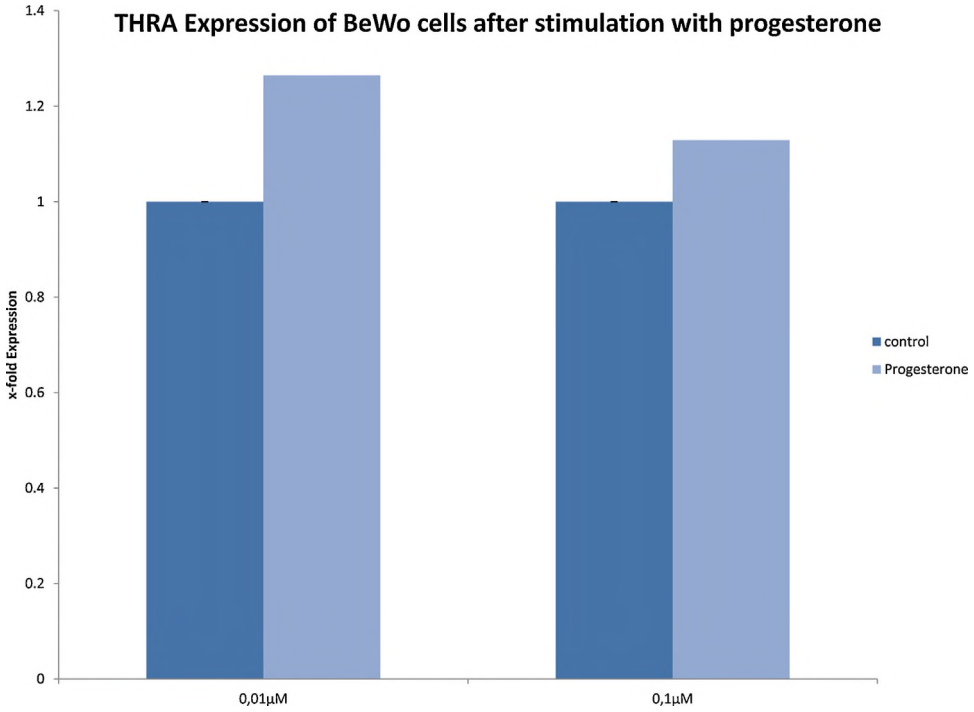

Fig. 7. THRA expression of BeWo cells after stimulation with progesterone. Results of gene expression analysis after stimulation with 0.01 µM progesterone in comparison with unstimulated controls are shown in the two left columns and results of gene expression analysis after stimulation with 0.1 µM progesterone in comparison with unstimulated controls are shown in the two right columns.

(SRC-1) [36]. SRC-1 contains three LXXLL motifs in the centre (nuclear receptor binding domain-1, NBD-1) [37]. Moreover, an isoform of SRC-1, SRC-1E, which lacks the LXXLL motif in NBD-2, exhibited enhanced ligand-stimulated transactivation of both THR and PR on their response elements [38].

Despite the new finding within our study that progesterone regulates THRA in the trophoblast, former investigations showed that the two thyroid hormone receptor genes (THRA and THRB) have opposite effects on oestrogen-stimulated sex behaviour [39]. Thyroid hormones can interfere with oestrogen stimulation *via* the oxytocin receptor promoter [39].

Additional studies confirmed that agonist-bound PR interacts with steroid receptor co-activators such as SRC-1. RU486 bound to PR, on the other hand, activates both co-activator SRC-1 but also

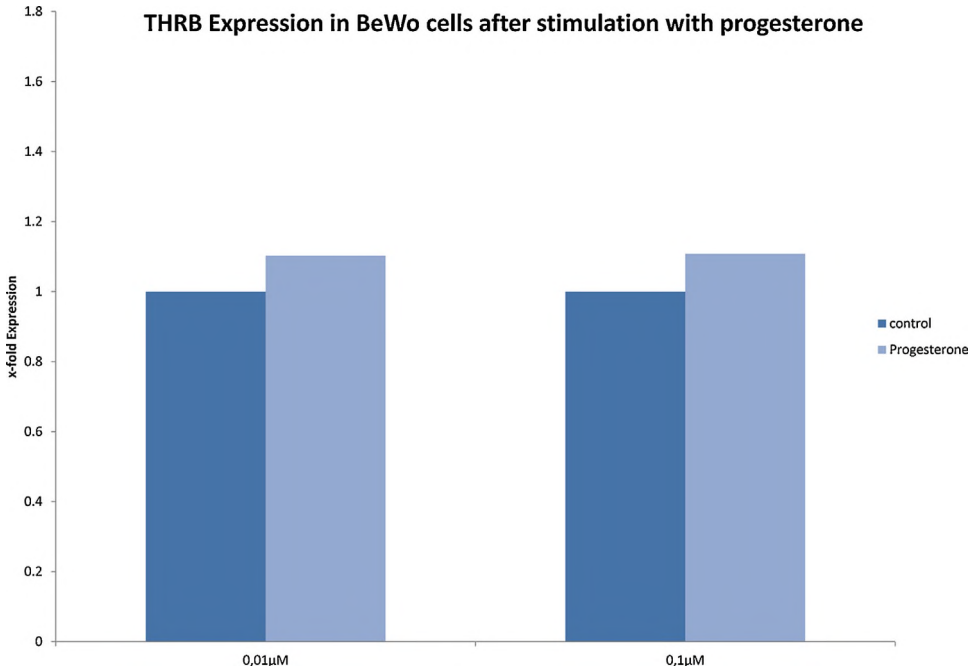

Fig. 8. THRB expression of BeWo cells after stimulation with progesterone. Results of gene expression analysis after stimulation with 0.01 µM progesterone in comparison with unstimulated controls are shown in the two left columns and results of gene expression analysis after stimulation with 0.1 µM progesterone in comparison with unstimulated controls are shown in the two right columns.

SMRT *in vitro*. SMRT is the silencing mediator for RXR and THR [40]. Peterson et al. found that SMRT plays a positive role in regulating ERα transcriptional activity [41]. Karmakar and co-workers found that the SRC-3 co-activator and the SMRT co-repressor directly interact with each other and form a trimeric complex with ERα [42]. It also is possible that SMRT can interact with the PR and cyclin D1 genes *via* interactions with the ligand-independent AF1 domain of ERα [42]. Based on the described results we could also identify a positive correlation between THRα1/β1 expression and the ER/PR status of breast cancer patients [43,44].

Mifepristone (RU486), the first PR antagonist which has been used in clinical practice is also a potent antagonist of the glucocorticoid receptor (GR) and the androgen receptor (AR) [45–47]. Progesterone dependent phosphorylation of PR serine 294 has been shown to be mandatory for PR transcriptional activation [48,49]. RU 486 triggers this by activating phosphorylation and inhibits interaction with an unknown partner that is mandatory for PR degradation [50]. This mechanism may be responsible for the stabilisation of the PR-RU 486 complex in the nucleus that could be responsible for partial agonist effects of RU 486 [51].

A very recent publication on THR expression in the corpus luteum of rats during gestation showed that the expression of THRα1 and THRβ1 throughout gestation showed a pattern similar to the profile of circulating progesterone [52]. In parallel to the decline of progesterone later on in pregnancy, the expression of THRα1 and THRβ1 also declines [52].

Results obtained in this study show that the nuclear expression of THRα1, THRα2 and THRβ1 on protein level is decreased in mifepristone (RU 486) treated villous trophoblast tissue. On mRNA level, we also found a significantly reduced expression of THRA in the placenta. Interaction of RU 486 with the progesterone receptor may be responsible for that down regulation.

Acknowledgements

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