

# Expression of Thyroid Hormone Receptors in Villous Trophoblasts and Decidual Tissue at Protein and mRNA Levels Is Downregulated in Spontaneous and Recurrent Miscarriages

Brigitte Ziegelmüller, Aurelia Vattai, Bernd Kost, Christina Kuhn, Simone Hofmann, Birgit Bayer, Bettina Toth<sup>1</sup>, Udo Jeschke<sup>1</sup>, and Nina Ditsch<sup>1</sup>

Department of Obstetrics and Gynaecology-Innenstadt (BZ, AV, BK, CK, SH, UJ), Department of Urology (BZ), Department of Legal Medicine (BB), and Department of Obstetrics Gynaecology-Großhadern (ND), Ludwig-Maximilians University of Munich, Munich, Germany; Department of Gynaecological Endocrinology and Fertility Disorders, University of Heidelberg, Heidelberg, Germany (BT)

## Summary

Thyroid hormones are essential for the maintenance of pregnancy, and a deficiency in maternal thyroid hormones has been associated with early pregnancy losses. The expression of THR $\alpha$ 1, THR $\beta$ 1 and THR $\alpha$ 2 increases with gestational age. The aim of this study was the investigation of the protein and mRNA-levels of THR isoforms THR $\alpha$ 1, THR $\alpha$ 2, THR $\beta$ 1 and THR $\beta$ 2 in normal, spontaneous and recurrent miscarriages. The identification of THR-expressing cells in the decidua was done with double immunofluorescence. The nuclear expression of THR $\alpha$ 1, THR $\alpha$ 2, THR $\beta$ 1 and THR $\beta$ 2 is downregulated at protein level in spontaneous and recurrent miscarriages in villous trophoblast tissue. In decidual tissue, we found a significant downregulation only for THR $\alpha$ 1 in spontaneous miscarriages. For recurrent miscarriages, THR $\alpha$ 1 and THR $\beta$ 1 were both significantly downregulated in decidual tissue. By applying HLA-G as a trophoblast marker, we found a significant co-expression only for THR $\beta$ 2. The results of our study show that thyroid hormone receptors THR $\alpha$ 1, THR $\alpha$ 2, THR $\beta$ 1 and THR $\beta$ 2 are downregulated in spontaneous and recurrent miscarriages. The majority of cells expressing the thyroid hormone receptors in the decidua are decidual stromal cells. (J Histochem Cytochem 63:511–523, 2015)

## Keywords

immunohistochemistry, miscarriage, placenta, TaqMan-PCR, thyroid hormone receptors (THR)

## Introduction

Thyroid hormones are essential for the maintenance of pregnancy, and a deficiency in maternal thyroid hormone expression has been associated with early pregnancy losses (Allan et al. 2000). The ligands of thyroid hormone receptors (THR) play a major role in trophoblast differentiation and in fetal neurodevelopment (Ohara et al. 2004). During the development of the fetal central nervous system, thyroid hormones are involved in the migration and growth of the neuronal cells, the establishment of synapses, myelination and corticogenesis (Lopez-Espinosa et al. 2009). After the 10<sup>th</sup> week of

gestation, the presence of high-affinity nuclear binding sites for the thyroid hormone triiodothyronine (T<sub>3</sub>) can be found in the fetal brain (Bernal and Pekonen 1984). During the 10<sup>th</sup>

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<sup>1</sup>These authors contributed equally to this work

### Corresponding Author:

Udo Jeschke, Department of Obstetrics and Gynaecology, Ludwig-Maximilians-University of Munich, Maistrasse 11, Munich, D-80337, Germany.  
Email: udo.jeschke@med.uni-muenchen.de

and 30<sup>th</sup> weeks of pregnancy, a two- to three-fold increase in the concentration of thyroxine binding globulin (TBG) and a 30% to 100% increase in total T<sub>3</sub> and thyroxine (T<sub>4</sub>) can occur (Kilby et al. 1998; Fantz et al. 1999). T<sub>3</sub> is mostly synthesized from the pro-hormone T<sub>4</sub>, which originates from the thyroid gland and is converted into T<sub>3</sub> through the iodothyronine deiodinase enzymes D1 and D2 (Bianco et al. 2002). Thyroid hormones are involved in gluconeogenesis, lipogenesis, cell proliferation, apoptosis and cell immunity.

Thyroid hormones bind to specific nuclear receptors (THR). Two genes, THRA (NR1A1) and THRB (NR1A2), encode the isoforms THR $\alpha$  and THR $\beta$ , which code for the four ligand-binding thyroid receptors, THR $\alpha$ 1, THR $\beta$ 1, THR $\beta$ 2 and THR $\beta$ 3, and the four non-ligand binding receptors THR $\alpha$ 2, THR $\Delta$ 1, THR $\Delta$ 2 and THR $\Delta$ 3 (Robinson-Rechavi et al. 2001; Williams 2000). Whereas the isoforms THR $\alpha$ 1, THR $\alpha$ 2 and THR $\beta$ 3 are widely expressed, the expression of THR $\beta$ 2 is rather restricted to the hypothalamic/pituitary gland (Bassett et al. 2003). The hormone T<sub>3</sub> is the high-affinity ligand of THR and thereby regulates gene transcription (Velasco et al. 2007). After it has bound to the ligand binding site, THR switches into its active form and recruits specific co-activators such as SRC1-3, PGC-1, TRAP and TRBP (Lin et al. 1997). A systematic review on THR function in reproductive biology was published recently (Pestka et al. 2013).

The expression of THR $\alpha$ 1, THR $\beta$ 1 and the non-ligand binding THR $\alpha$ 2 in the nuclei of syncytiotrophoblasts (ST), cytotrophoblasts, and stromal cells increases with gestational age (Kilby et al. 1998). Maruo et al. (1991) demonstrated that T<sub>3</sub> enhances the production of the epidermal growth factor (EGF). This potent growth factor increases the invasiveness of cytotrophoblast cells (Bass et al. 1994) and stimulates the synthesis of human chorionic gonadotropin (hCG) and human placental lactogen by trophoblast cells (Barber et al. 2005). T<sub>3</sub> and EGF act synergistically in regulating trophoblast differentiation and proliferation (Barber et al. 2005). It has been further shown that T<sub>3</sub> is able to reduce apoptosis of extravillous trophoblasts (EVTs) (Laoag-Fernandez et al. 2004). Hence, due to the inhibition of apoptosis in EVT, T<sub>3</sub> may be able to promote EVT invasion to the decidua (Bass et al. 1994).

The hypothalamic–pituitary–thyroid axis (HPT) and the hypothalamic–pituitary–ovarian axis (HPO) seem to be physiologically related. It is suggested that thyroid hormone receptors located in the ovaries regulate reproductive function, and that estrogens influence the HPT axis as well. Thyroid disorders can lead to bleeding irregularities. Hyperthyroidism can lead to oligomenorrhea or even to anovulatory cycles. Hypothyroidism can result in polymenorrhea. Even if thyroid hormone production is compensated, ovarian function is still affected (Doufas and Mastorakos 2000).

A two-fold increase in miscarriage and stillbirth rates can result from untreated hypothyroidism (Barber et al. 2005).

Hyperthyroidism and autoimmunity can also have severe effects on pregnancy outcome. Thus, the maintenance of an euthyroid state is crucial during pregnancy and necessary for the prevention of disturbed placentation syndromes such as preeclampsia and intrauterine growth restriction (IUGR) (Barber et al. 2005). IUGR can cause perinatal and neonatal mortality, physical handicaps in low-birth weight babies, and neurodevelopmental delays in toddlers (Gaffney et al. 1994). Kilby et al. (1998) demonstrated that a reduction in the concentration of free T<sub>3</sub> and T<sub>4</sub> occurs in the serum of fetuses affected by IUGR. They compared IUGR placental samples with normal pregnancy samples and found that the immunostaining of THR $\alpha$ 1, THR $\alpha$ 2 and THR $\beta$ 1 was greater in IUGR placental samples. However, RT-PCR results showed no difference in the expression of the messenger RNA of THR between IUGR and normal pregnancy samples. The upregulation of THR proteins in IUGR is possibly a consequence of the reduction in thyroid hormones in order to maintain euthyroidism in the tissue, with the aim of preventing poor long-term fetal outcomes (Kilby et al. 1998).

Based on this data, the aim of the current study was to systematically investigate the protein and mRNA-level THR isoforms THR $\alpha$ 1, THR $\alpha$ 2, THR $\beta$ 1 and THR $\beta$ 2 in normal, spontaneous and recurrent miscarriages. In addition, the identity of THR-expressing cells in the decidua was also analyzed.

## Materials & Methods

### Tissue Samples

This study was approved by the Ethical Committee of the Medical School, Ludwig-Maximilian-University of Munich. Informed consent was obtained from each patient. All data has been encoded.

Samples from elective terminations of pregnancies (ETP, 22 cases) were provided from private practice clinics in Munich, Germany. Maternal and gestational age-matched tissues samples from spontaneous miscarriages (SM, 13 cases) and recurrent miscarriage (RM, 10 cases) were additionally collected from the archives of the Department of Obstetrics and Gynaecology-Innenstadt, Munich, Germany. Recurrent miscarriage is defined as  $\geq 3$  miscarriages. The mean ages of the women for each group were as follows: SM,  $33.8 \pm 4.4$  years; RM,  $34.8 \pm 5.8$  years; and ETP,  $29.7 \pm 6.0$  years ( $p > 0.05$ ). The mean gestational age for the SM group was  $9.6 \pm 1.5$  weeks; for RM group,  $9.6 \pm 2.2$  weeks, and for the ETP group,  $8.3 \pm 1.8$  weeks ( $p > 0.05$ ). A complete list of placentas obtained from different weeks is presented in Table 1. Samples were obtained by dilatation and evacuation without any prior pharmaceutical induction. In cases of spontaneous and recurrent miscarriages, evacuation was performed within the first 24 hr after diagnosis. All women included in the study had no medical or family history. Hypothyroidism as

**Table 1.** Number of Placentas from Different Entities and Weeks of Gestation Used for the Study.

Weeks of Gestation	Control	Spontaneous Miscarriage	Recurrent Miscarriage
5	1	0	0
6	3	0	0
7	2	0	2
8	8	4	2
9	3	4	1
10	0	2	1
11	5	1	2
12	0	2	1
13	0	0	1
	<b>n=22</b>	<b>n=13</b>	<b>n=10</b>

well as hyperthyroidism were excluded in all women. History taking was systematic, aiming to exclude—apart from common disorders—the possible implication of clotting disorders and autoimmune diseases, already known as aggravating factors for increased risk for miscarriages. In all samples, analysis of chromosomal abnormalities was performed. This study was performed using PowerPlex 16HS multiple PCR system, which detects 16 STR loci, with the limitation of this method in picking up chromosomal abnormalities at base pair level. Analysis was performed for all samples as well as controls. DNA extraction and profiling was performed via the following steps: From placental tissue, DNA was isolated with the help of Bio Robot EZ1 (Qiagen, Valencia, CA) as described by Anslinger et al. (2005). The extracted DNA was eluted in 50  $\mu$ l double-distilled water and quantified twice with the Quantifiler Human DNA Quantification Kit (Thermo Fisher Scientific; Waltham, MA) according to the manufacturer's protocol. The arithmetic mean values were calculated. For DNA profiling the PowerPlex® 16HS multiplex PCR system (Promega; Fitchburg, WI) was used, with a total reaction volume of 25  $\mu$ l, applying a 32-cycle program. For each step, 300 pg DNA was added. The PCR products were analyzed via the ABI PRISM 3130XL capillary electrophoresis system (Thermo Fisher) and the results were generated with the GeneScan® Analysis Software and the ABI Prism® GenoTyper Software version 3.7 (Thermo Fischer Scientific) (Anslinger et al. 2005). Additionally, in all samples, microbiology analysis excluded possible intra-uterine infection. All women had a normal first trimester vaginal swab. Following evacuation, specimens from ETP, RM and SM groups were immediately immersed in formalin and embedded in paraffin or snap-frozen and sectioned with a cryostat.

### Immunohistochemistry

Formalin-fixed paraffin-embedded sections (3  $\mu$ m) were deparaffinized in xylol, rehydrated in a descending ethanol gradient, and subjected to epitope retrieval in a pressure

cooker using sodium citrate buffer (pH 6.0). After returning to room temperature, sections were blocked with 3% H<sub>2</sub>O<sub>2</sub> in methanol (20 min) for endogenous peroxidase activity. Non-specific binding of the primary antibodies was blocked using the appropriate blocking solution. Sections were then incubated with primary antibodies; the salient features of the antibodies used are presented in Table 2. Reactivity was detected by using the Vectastain Elite ABC-Kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's protocol. Substrate and chromogen (3,3'-diaminobenzidine DAB; Dako, Glostrup, Denmark) were finally added to the slides, which were then counterstained with Mayer's acidic hematoxylin and covered. Colon (THR $\alpha$ 1), term placenta (THR $\alpha$ 2) and breast cancer tissue (THR $\beta$ 1, THR $\beta$ 2) were used for positive control staining (Fig. 1). Replacement of the primary antibody with mouse or rabbit IgG served as negative controls (Fig. 1).

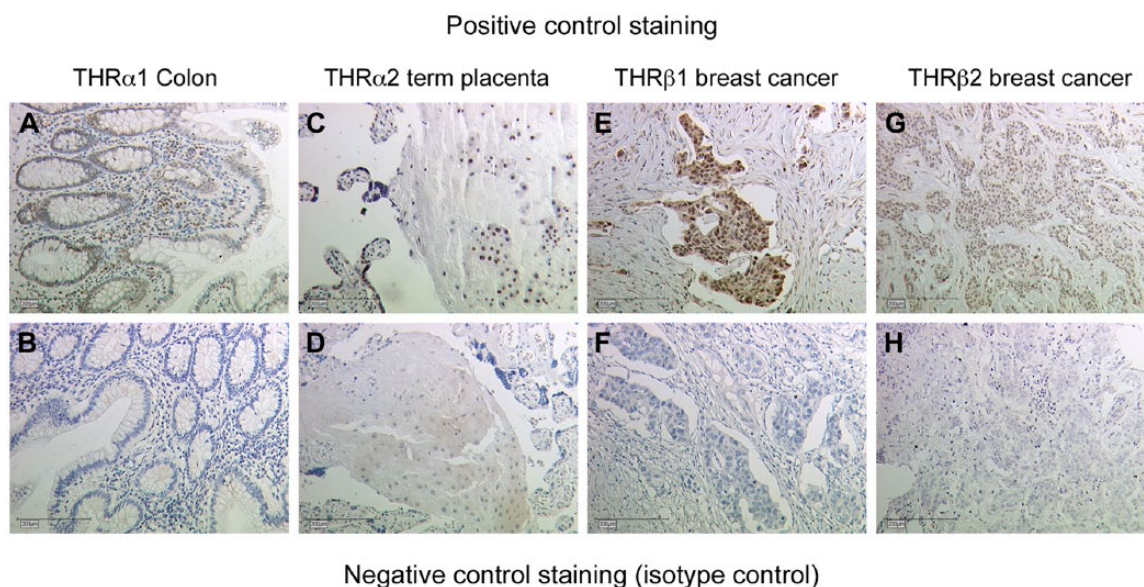
The sections were examined by two independent observers using a Leitz Diaplan microscope (Leitz, Wetzlar, Germany). Per slide, ten fields were examined using a semi-quantitative immunoreactive score (IRS) (Remmele and Stegner 1987). IRS examines the intensity and distribution of antigen expression and is calculated by multiplying the percentage of positively stained cells (0: no staining; 1: <10% of the cells; 2: 11% to 50%; 3: 51% to 80%, 4: >80%) with the intensity of staining of the cells (0: none; 1: weak; 2: moderate; 3: strong).

### Identification of THR-expressing Cells in the Decidua with Double Immunofluorescence

Double immunofluorescence staining was performed in order to identify the cells expressing nuclear receptors THR $\alpha$ 1, THR $\alpha$ 2, THR $\beta$ 1 and THR $\beta$ 2 in the decidua. Double immunofluorescence staining was carried out on placentas from healthy pregnancies. The sections were deparaffinized in xylol for 20 min and, after washing in ethanol, were incubated in ethanol/methanol for a further 20 min. Next, the slides were rehydrated in an alcohol gradient and then placed in a pressure cooker with sodium citrate (pH 6.0). The slides were then washed in PBS and blocked with Ultra V blocking solution (Lab Vision, Thermo Scientific) for 15 min. The slides were then incubated with polyclonal anti-THR $\alpha$ / $\beta$  rabbit IgG (Table 2) for 1 hr. After that, the slides were incubated with monoclonal anti-HLA-G antibody. Sections were then incubated with Cy3-labeled goat-anti-rabbit IgG antibody, diluted 1:500, and Cy2-labeled goat-anti-mouse IgG antibody, diluted 1:100 (Dianova; Hamburg, Germany). Next, the slides were embedded in DAPI containing mounting buffer (Vector Laboratories). The slides were then analyzed with a fluorescence Axioskop photomicroscope (Zeiss; Oberkochen, Germany) and pictures were taken with a digital AxioCam camera system (Zeiss).

**Table 2.** Salient Features of the Primary Antibodies Used for Immunohistochemistry and Double Immunofluorescence.

Antibody (AB)	AB Incubation Conditions	Blocking Solution	Blocking Conditions
THRalpha1 Polyclonal (Rabbit IgG) (Abcam; Cambridge, MA)	1:200 in PBS; 1 hr at room temperature	Reagent I (Polymer kit, Zytomed Systems, Berlin, Germany)	5 min
THRalpha1,2 Polyclonal (Rabbit IgG) (Abcam)	1:1000 in PBS; 1 hr at room temperature	Reagent I (Polymer kit)	5 min
THRalpha2 Mouse IgG1 (AbD Serotec, Raleigh, NC)	1:1000 in PBS; 1 hr at room temperature	Reagent I (Polymer kit)	5 min
THRbeta1 Polyclonal (Rabbit IgG) (Novus Biologicals; Littleton, Colorado)	1:300 in Dako VM; 1 hr at room temperature	Reagent I (Polymer kit)	5 min
THRbeta2 Polyclonal (Rabbit IgG) (Upstate, EMD Millipore, Billerica, MA)	1:100 in Dako VM; 1 hr at room temperature	Reagent I (Polymer kit)	5 min
HLA-G mouse IgG1 (Novus Biologicals)	1:100 in Power block; 1-fold dilution	Power block 1× solution.	3 min.



**Figure 1.** Positive and negative control staining. (A) 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). (B) Negative control staining for (A). (C) Term placental tissue was used for THRα2 positive control staining. THRα2 expression is found in the nuclei of villous as well as extravillous trophoblast cells. (D) Negative control staining for (C). (E) Breast cancer tissue was used for positive control staining of THRβ1 antibodies. THRβ1 expression is found specifically in the nucleus of cancer cells, with high intensity staining, and in the surrounding stroma, with lower intensity staining. (F) Negative control staining for (E). (G) 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. (H) Negative control staining for (G). Scale, 200 μm.

### Evaluation of THRA and THRB Expression with Real Time RT-PCR (TaqMan)

**RNA Extraction from Placental Tissue.** Total RNA was extracted from the placental tissues of 13 women with spontaneous

miscarriage, 9 women with recurrent miscarriage and 15 normal controls. The RNA was extracted from both trophoblast (fetal cells) and decidua (maternal cells). The RNeasy Lipid Tissue Mini Kit (Qiagen) was used according to the manufacturer's protocol.

**Reverse Transcription.** Reverse transcription (RT) was performed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Weiterstadt, Germany), according to the protocol using a mastercycler gradient (Eppendorf, Hamburg, Germany). RT conditions were 10 min at 25°C, 2 hr at 37°C, and 5 sec at 85°C, followed by a hold step at 4°C.

**Real-Time Reverse Transcription-PCR.** Optical, 96-well, microtiter reaction plates were covered with optical caps and Real-Time Reverse Transcription PCRs were accomplished in a volume of 20  $\mu$ l containing 1  $\mu$ l 20 $\times$  TaqMan® Gene Expression Assay (Hs00268470\_m1 for THRA and Hs00230861\_m1 for THRB, both Applied Biosystems), 10  $\mu$ l 2 $\times$ TaqMan® Universal PCR Master Mix (Applied Biosystems), 8  $\mu$ l H<sub>2</sub>O (DEPC-treated DI water; Sigma-Aldrich, Taufkirchen, Germany) and 1  $\mu$ l template. Thermal cycling conditions were 95°C for 20 sec, followed by 40 cycles of amplification with at 95°C for 3 sec and 60°C for 30 sec. The ABI PRISM 7500 Fast (Applied Biosystems) was used for PCR assays. Quantification was carried out by the  $2^{-\Delta\Delta CT}$  method using GAPDH (Hs99999905\_m1) as a 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. Mann-Whitney U test was used for the evaluation of two independent groups. Data were analyzed using a one-way analysis of variance with two independent variables.

## Results

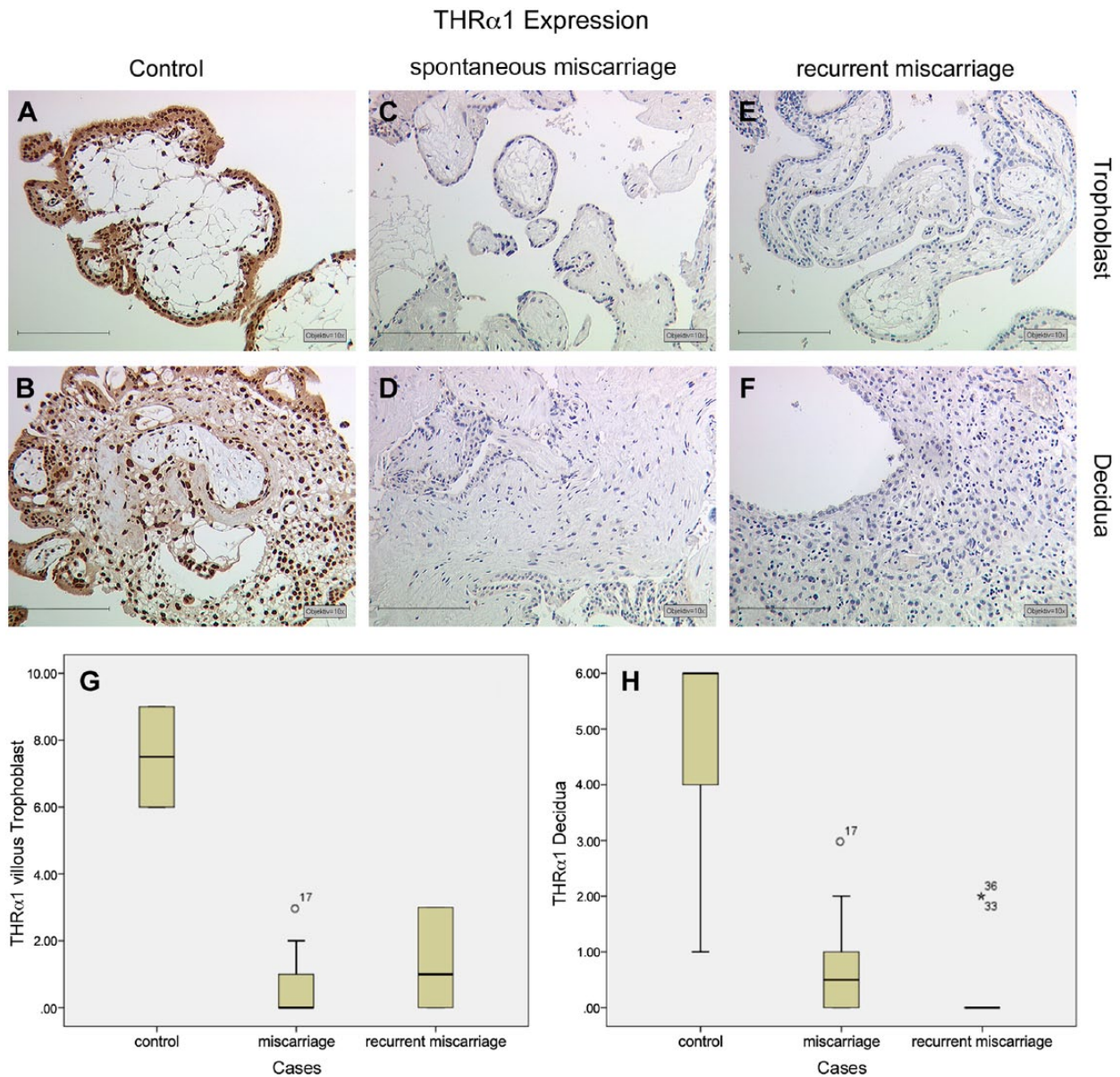
### Immunohistochemistry

**THR $\alpha$ 1.** We identified a strong expression of THR $\alpha$ 1, with a median IRS value of 7.5, in nuclei of villous trophoblasts (Fig. 2A). The nuclei of cytotrophoblasts as well as that of syncytiotrophoblasts stained with high intensity, with an abundance of cells showing this intense staining. A similar result was found for the decidua samples. We identified a strong expression of THR $\alpha$ 1, with a median IRS value of 6, in the nuclei of decidua cells (Fig. 2B). In placental tissue samples from patients in the SM group, we identified a significant downregulation in THR $\alpha$ 1 in villous trophoblasts (Fig. 2C, median = 0;  $p < 0.001$ ) and in decidua cells (Fig. 2D, median = 0.5;  $p < 0.001$ ). In placental tissue samples from RM patients, we also identified a significant downregulation in THR $\alpha$ 1 in villous trophoblasts (Fig. 2E, median = 1;  $p < 0.001$ ) as well as in decidua cells (Fig. 2F, median = 0;  $p < 0.001$ ). A summary of the staining results of villous tissue is presented in Figure 2G and a summary of staining results in decidua tissue is shown in Figure 2H.

**THR $\alpha$ 2.** We identified an intense expression of THR $\alpha$ 2, with a median IRS value of 9, in the nuclei of villous trophoblasts (Fig. 3A). An abundance of high intensity staining was detected for THR $\alpha$ 2 in the nuclei of cytotrophoblasts as well as in syncytiotrophoblasts. THR $\alpha$ 2 showed a median IRS value of 4 in terms of its expression in the nuclei of decidua cells (Fig. 3B). In placental tissue samples from patients in the SM group, we identified a significant downregulation in THR $\alpha$ 2 in villous trophoblasts (Fig. 3C; median = 1;  $p < 0.001$ ) but no significant changes in decidua cells (Fig. 3D, median = 3.5;  $p = 0.182$ ). In placental tissue samples from patients in the RM group, we also identified a significant downregulation in THR $\alpha$ 2 in villous trophoblasts (Fig. 3E; median = 0;  $p < 0.001$ ) but no changes in decidua cells (Fig. 3F; median = 3.5,  $p = 0.167$ ). A summary of the staining results of the villous tissues is presented in Figure 3G, and a summary of staining results in decidua tissues is shown in Figure 3H.

**THR $\beta$ 1.** THR $\beta$ 1 showed a median IRS value of 4 in the nuclei of villous trophoblast cells (Fig. 4A), with staining also observed in the nuclei of cytotrophoblasts and syncytiotrophoblasts. A similar result was found in the decidua. We identified a median IRS value of 3 for THR $\beta$ 1 staining in the nuclei of decidua cells (Fig. 4B). In the placental tissue samples from patients in the SM group, we identified no significant changes in THR $\beta$ 1 expression in villous trophoblasts (Fig. 4C; median = 3.5;  $p = 0.083$ ) or in decidua cells (Fig. 4D, median = 4;  $p = 0.962$ ). In the placental tissue samples from patients in the RM group—in contrast to those in the SM group—we identified a significant downregulation in THR $\beta$ 1 in villous trophoblasts (Fig. 4E; median = 0;  $p < 0.001$ ) and in decidua cells (Fig. 4F, median = 1.5;  $p = 0.004$ ). A summary of the staining results for the villous tissue is presented in Figure 4G and a summary of staining results for decidua tissue is shown in Figure 4H.

**THR $\beta$ 2.** We identified an intense expression of THR $\beta$ 2, with a median IRS value of 6, in the nuclei of villous trophoblast cells (Fig. 5A). Staining was identified in the nuclei of both cytotrophoblasts and syncytiotrophoblasts. A similar result was found in the decidua. We identified THR $\beta$ 2, with a median IRS value of 4, in the nuclei of decidua cells (Fig. 5B). In the placental tissue of patients in the SM group, we identified a significant downregulation in THR $\beta$ 2 in villous trophoblasts (Fig. 5C, median = 3;  $p = 0.008$ ). In decidua cells, no significant changes were observed (Fig. 5D, median = 3;  $p = 0.139$ ). In the placental tissue samples from patients in the RM group, we also identified a significant downregulation in the expression of THR $\beta$ 2 in villous trophoblast (Fig. 5E, median = 0;  $p = 0.001$ ). In decidua cells, again, no significant changes were found (Fig. 5F, median = 3;  $p = 0.094$ ). A summary of the staining results for villous tissue is presented in Figure 5G and a summary of staining results for decidua tissue is shown in Figure 5H.



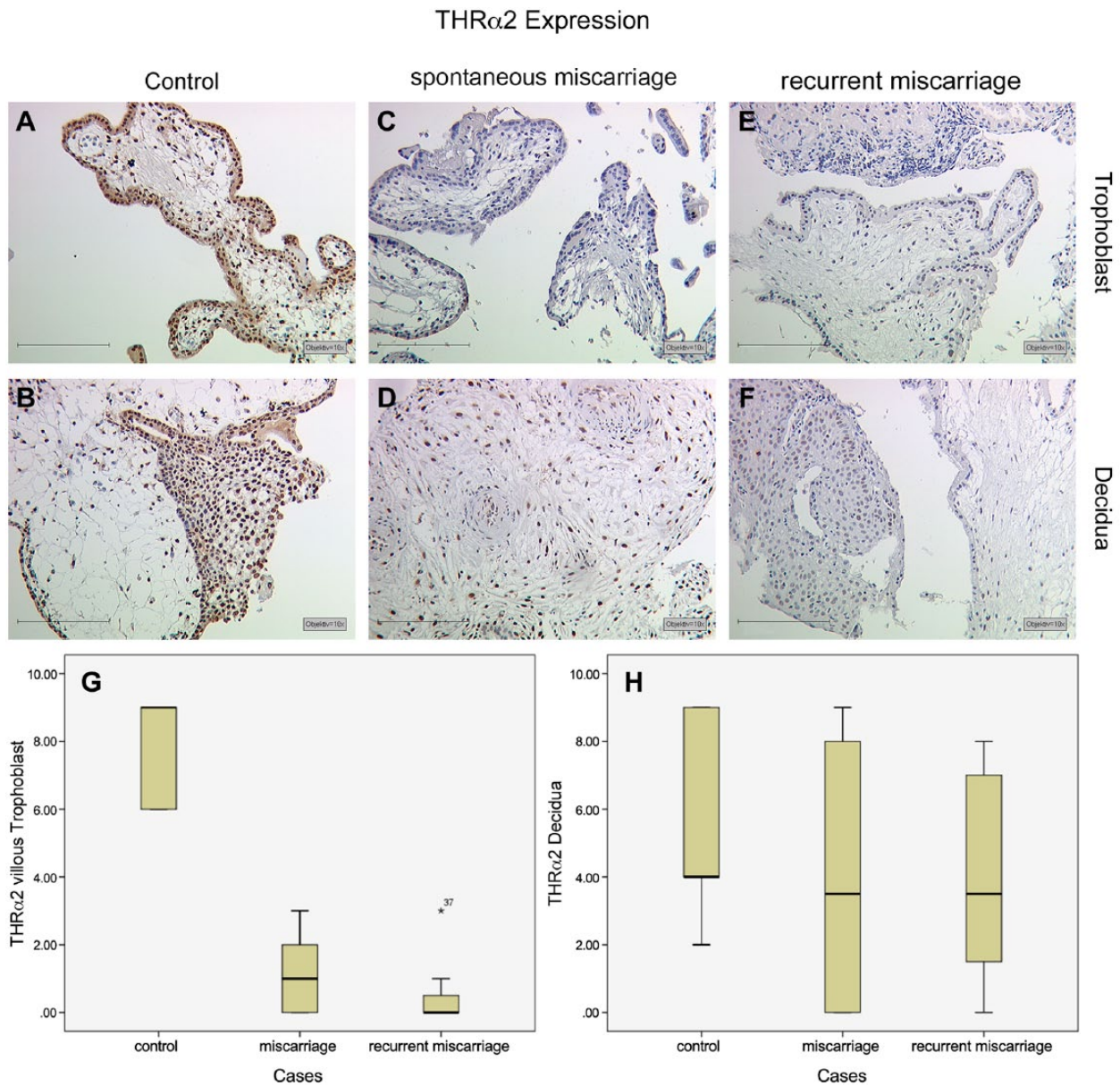
**Figure 2.** THR $\alpha$ 1 expression is found with high intensity and distribution in normal villous trophoblast cells (A) and in the decidua (B) in normal tissues. In samples from patients with spontaneous miscarriage and recurrent miscarriage, we found a significant downregulation in THR $\alpha$ 1 in villous trophoblast cells (C, E) and in the decidua (D, F), respectively. A summary of the staining results is given in Box plot analysis for villous trophoblast tissue (G) and the decidua (H). The boxes represent the range between the 25<sup>th</sup> and 75<sup>th</sup> percentiles with a horizontal line at the median. The bars delineate the 5<sup>th</sup> and 95<sup>th</sup> percentiles. The circle indicates values that are more than 1.5 box-lengths, and the asterisk values more than 3.0 box-lengths from the 75<sup>th</sup> percentile. Scale, 200  $\mu$ m.

### Identification of THR-expressing Cells in the Decidua with Double Immunofluorescence

THR $\alpha$ / $\beta$  + HLA-G double fluorescence staining was used for the identification of EVT cells expressing THR. THR $\alpha$ 1 staining in the nuclei of decidual cells is shown in Figure 6A. Membrane HLA-G staining of EVTs from the same area is shown in Figure 6B. Triple filter excitation showing both THR $\alpha$ 1 and HLA-G is depicted in Figure 6C. The

white arrows mark those cells expressing both antigens. The majority of decidual cells did not stain for HLA-G but were positive for THR $\alpha$ . Therefore, the identity of these cells is decidual stromal cells.

THR $\alpha$ 1/2 staining in the nuclei of decidual cells is shown in Figure 6D. Membrane HLA-G staining of EVTs from the same area is shown in Figure 6E. Triple filter excitation showing both THR $\alpha$ 1/2 and HLA-G is shown in Figure 6F. The white arrows mark the few cells expressing

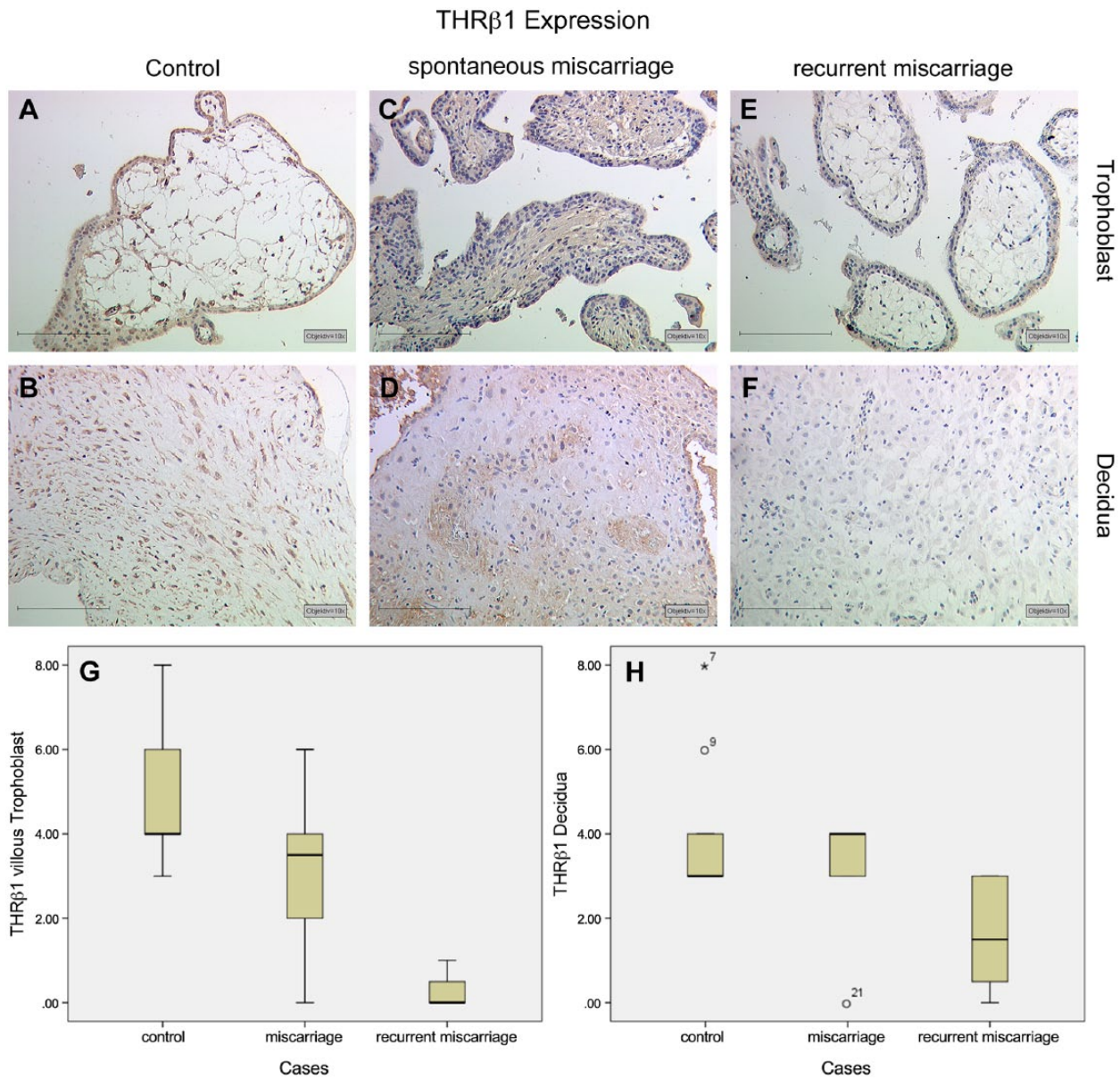


**Figure 3.** High-intensity staining and elevated distribution of THR $\alpha$ 2 expression in normal villous trophoblast cells (A) and in the decidua (B). In spontaneous miscarriage samples as well as in recurrent miscarriage samples, we found a significant downregulation of THR $\alpha$ 2 in villous trophoblast cells (C, E) but not in the decidua (D, F), respectively. A summary of the staining results is given in the Box plot analysis for villous trophoblast tissue (G) and decidua (H). The boxes represent the range between the 25<sup>th</sup> and 75<sup>th</sup> percentiles with a horizontal line at the median. The bars delineate the 5<sup>th</sup> and 95<sup>th</sup> percentiles. The circle indicates values that are more than 1.5 box-lengths from the 75<sup>th</sup> percentile. Scale, 200  $\mu$ m.

both antigens. The majority of THR $\alpha$ 1/2-expressing cells is not stained for HLA-G. The identity of these cells is decidual stromal cells.

THR $\beta$ 1 staining in the nuclei of decidual cells is shown in Figure 7A. Membrane HLA-G staining of EVT from the same area is shown in Figure 7B. Triple filter excitation showing both THR $\beta$ 1 and HLA-G is shown in Figure 7C. THR $\beta$ 1-expressing cells are not stained for HLA-G. Therefore, the identity of these cells is decidual stromal cell.

THR $\beta$ 2 staining in the nuclei of decidual cells is shown in Figure 7D. Membrane HLA-G staining of EVT from the same area is shown in Figure 7E. Triple filter excitation showing both THR $\beta$ 2 and HLA-G is shown in Figure 7F. The white arrows mark cells expressing both antigens. The majority of cells stained for HLA-G are also positive for THR $\beta$ 2. A great number of cells expressing THR $\beta$ 2 do not express HLA-G and therefore are decidual stromal cell.



**Figure 4.** Median intensity and distribution of THRβ1 expression in normal villous trophoblast cells (A) and in the decidua (B). In spontaneous miscarriage samples, we found no significant changes in THRβ1 in villous trophoblast cells (C) or in the decidua (D) as compared with the control. In recurrent miscarriage samples, we identified a significant downregulation in THRβ1 in the villous trophoblasts (E) as well as in the decidua (F). A summary of the staining results is given in Box plot analysis for villous trophoblast tissue (G) and the decidua (H). The boxes represent the range between the 25<sup>th</sup> and 75<sup>th</sup> percentiles with a horizontal line at the median. The bars delineate the 5<sup>th</sup> and 95<sup>th</sup> percentiles. The circle indicates values that are more than 1.5 box-lengths, and the asterisk values more than 3.0 box-lengths from the 75<sup>th</sup> percentile. Scale, 200 μm.

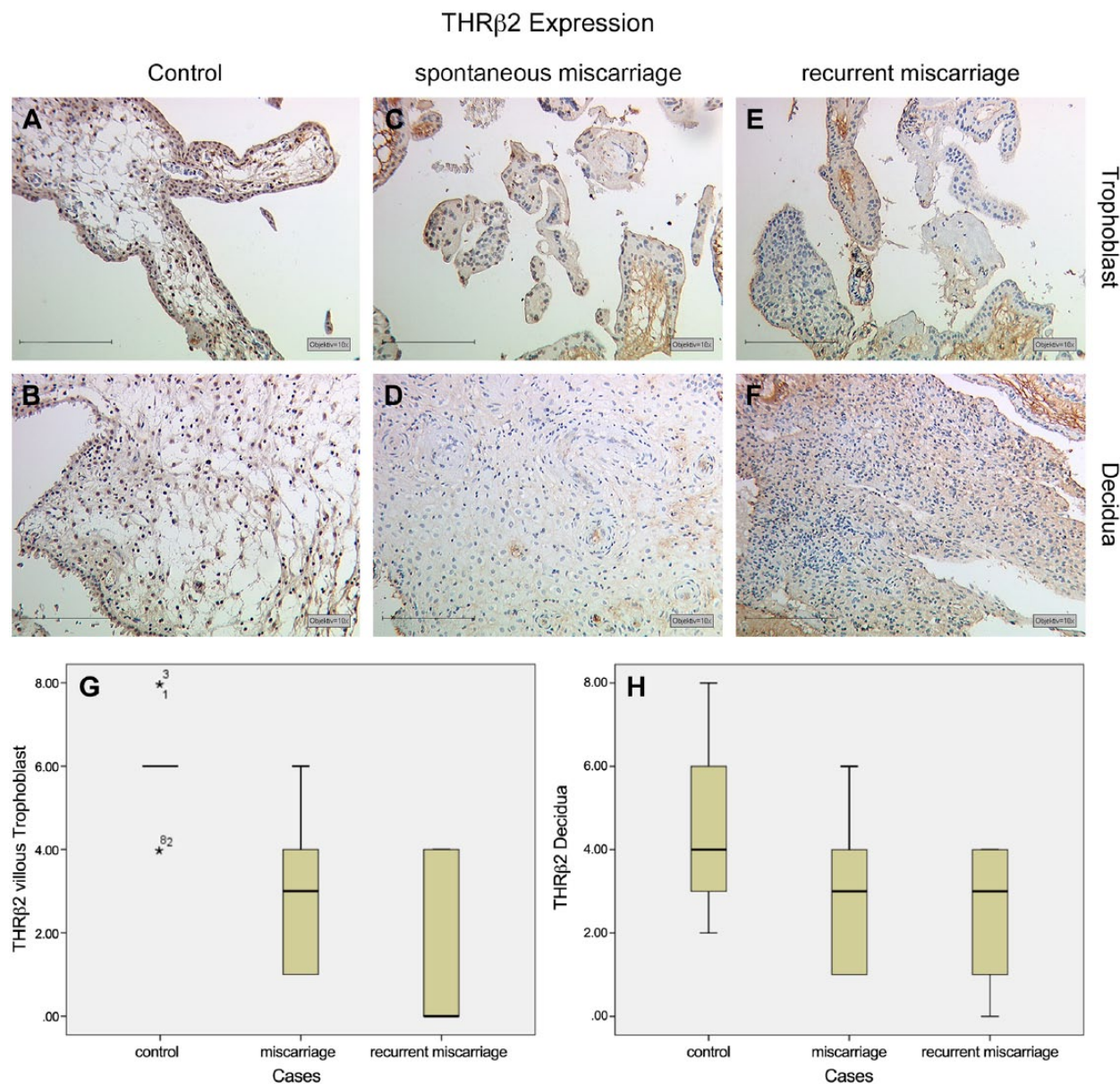
#### Evaluation of THRA and THRB Expression with Real Time RT-PCR (TaqMan)

We identified the expression of THRA and THRB in all cases investigated. The mRNA expression of THRA was downregulated to a 0.14-fold expression in samples from the SM group and to a 0.096-fold expression in samples from the RM group ( $p=0.001$ , Fig. 8A). The mRNA

expression of THRB was downregulated to a 0.28-fold expression in the SM group and to a 0.04-fold expression in the RM group ( $p<0.001$ , Fig. 8B).

A detailed matched analysis revealed no significant differences in THR expression in the control placentas from the 5<sup>th</sup> to the 11<sup>th</sup> weeks of gestation. We also analyzed fetal gender-related THR expression but could not identify significant differences ( $p>0.05$ ) for fetal gender.



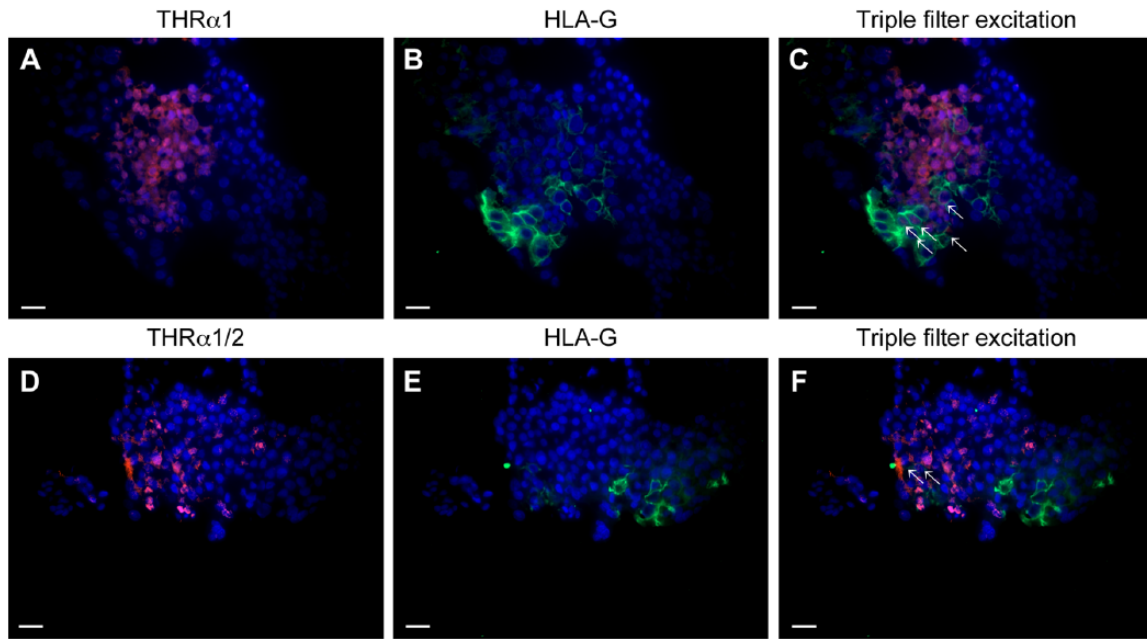


**Figure 5.** THR $\beta$ 2 expression is found with high intensity and distribution in normal villous trophoblast cells (A) and with median expression in the decidua (B). In spontaneous miscarriage as well as in recurrent miscarriage samples, we found a significant downregulation in THR $\beta$ 2 expression in villous trophoblast cells (C, E) but no significant change in the decidua (D, F). A summary of the staining results is given in Box plot analysis for villous trophoblast tissue (G) and the decidua (H). The boxes represent the range between the 25<sup>th</sup> and 75<sup>th</sup> percentiles with a horizontal line at the median. The bars delineate the 5<sup>th</sup> and 95<sup>th</sup> percentiles. The asterisk indicates values that are more than 3.0 box-lengths from the 75<sup>th</sup> percentile. Scale, 200  $\mu$ m.

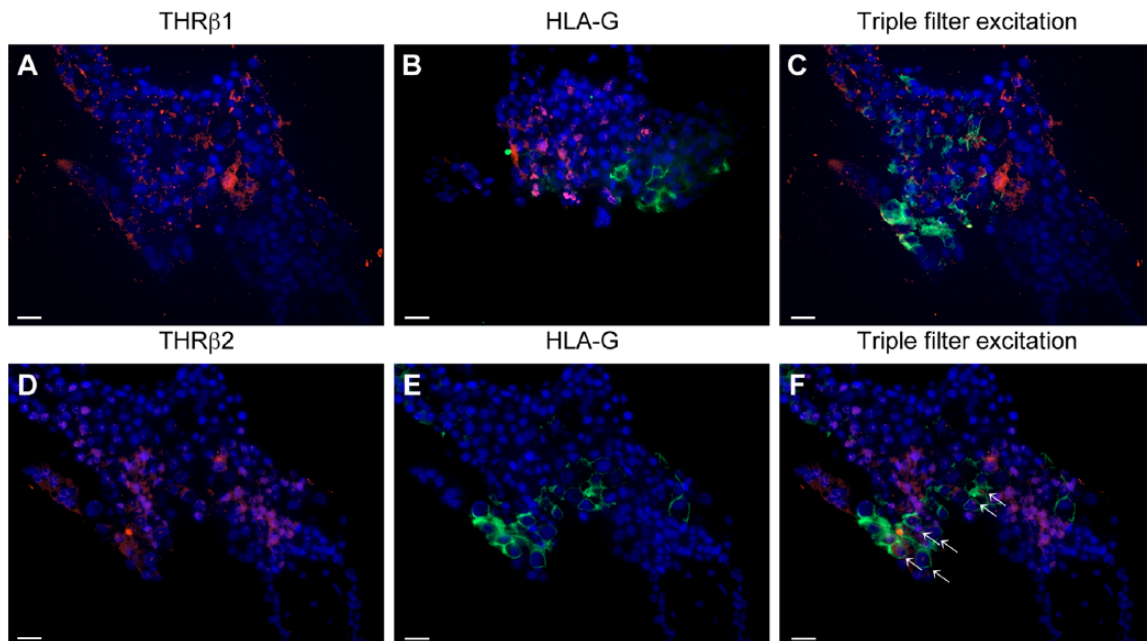
## Discussion

Within this study, we investigated the nuclear expression of THR $\alpha$ 1, THR $\alpha$ 2, THR $\beta$ 1 and THR $\beta$ 2. We found that these receptors are downregulated at protein level in villous trophoblast tissue in patients who suffer from spontaneous and recurrent miscarriages. In decidua tissue, we found a significant downregulation only in THR $\alpha$ 1 in spontaneous miscarriage samples, and in THR $\alpha$ 1 and

THR $\beta$ 1 for recurrent miscarriage samples. Using double immunofluorescence and HLA-G as trophoblast markers (Proll et al. 1999), we found a significant co-expression for THR $\beta$ 2 only with HLA-G. Therefore, decidual stromal cells seem to express the majority of THR $\beta$ 2. Further, we were able to confirm the results found at protein level at mRNA level. THRA and THRB are both downregulated in spontaneous and recurrent miscarriage placental tissue.



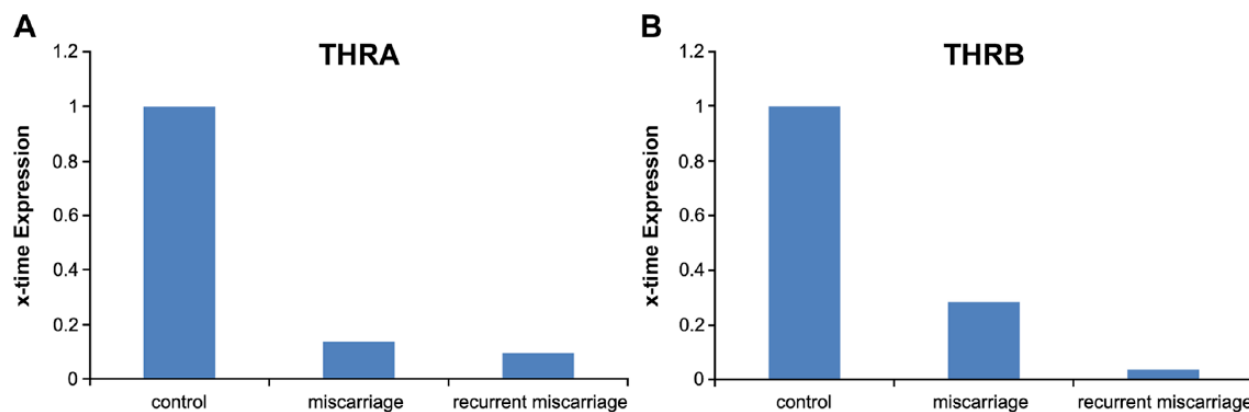
**Figure 6.** (A)  $\text{THR}\alpha 1$  expression (Cy3; red) shows a high distribution in normal decidua. (B) Extravillous trophoblast cells (EVTs) (HLA-G positive) appear green. (C) Triple filter excitation shows cells expressing both markers (as indicated by the white arrows). The majority of  $\text{THR}\alpha 1$ -expressing cells are not HLA-G positive. (D)  $\text{THR}\alpha 1/2$  expression (Cy3; red) is found in the normal decidua. (E) EVT (HLA-G positive) appear green. Triple filter excitation (F) shows cells expressing both marker (marked with white arrows). Only very few  $\text{THR}\alpha 2$  expressing cells are HLA-G positive. Scale, 20  $\mu\text{m}$ .



**Figure 7.** (A)  $\text{THR}\beta 1$  expression (Cy3; red) is found in normal decidua. (B) Extravillous trophoblasts (EVTs) (HLA-G positive) appear green. (C) Triple filter excitation shows that there are no cells expressing both markers. (D)  $\text{THR}\beta 2$  expression (Cy3; red) is found in the normal decidua. (E) EVT (HLA-G positive) appear green. (F) Triple filter excitation shows cells expressing both markers (marked with white arrows). The majority of HLA-G-expressing cells are also  $\text{THR}\beta 2$ -positive. Scale, 20  $\mu\text{m}$ .

The scientists Pierre Chambon, Ronald M. Evans and Elwood V. Jensen investigated the nuclear receptor

superfamily in the past two decades and discovered that its members interact with each other. The retinoid-X-receptor



**Figure 8.** Results of gene expression analysis with TaqMan RT-PCR. (A) THRA expression. We identified a significant downregulation in THRA in samples from patients with spontaneous miscarriage as well those with recurrent miscarriage. (B) We identified a significant downregulation in THRB in samples from patients with spontaneous miscarriage and recurrent miscarriage.

(RXR) acts as a key player of the receptor family through forming heterodimers with other nuclear receptors. The formation of heterodimers further activates transcription (Kastner et al. 1995; Mangelsdorf and Evans 1995; Zhang and Pfahl 1993). The thyroid hormone receptor family,  $THR\alpha$  and  $THR\beta$ , are heterodimeric partners of RXR. We previously described that upregulation of apoptotic EVT is found in spontaneous and recurrent miscarriage placental tissue (Minas et al. 2007). In addition, we have shown that EVTs in the placentas of miscarriages show a significantly higher expression of  $RXR\alpha$  in comparison to EVTs in placentas of elective terminations of healthy pregnancies (Pestka et al. 2011; Toth et al. 2008). Therefore, the key heterodimeric partner receptor of THRs is rather upregulated in contrast to the THRs. A similar trend in terms of the downregulation of the nuclear receptor LXR has recently been described by our group. Expression of the liver X receptor (LXR) is decreased in both villous trophoblasts and decidual tissues of spontaneous and recurrent miscarriages (Knabl et al. 2013). Peroxisome proliferator-activated receptor gamma ( $PPAR\gamma$ ) expression has further been investigated and was shown to be increased in miscarriages (Pestka et al. 2011; Toth et al. 2009; Toth et al. 2007). Interestingly, a recent study showed that activation of  $PPAR\gamma$  signaling via rosiglitazone induced a strong downregulation in both THRA and THRB in brown adipose tissue and in rats in vivo (Festuccia et al. 2012). Based on these results, we may speculate that, in miscarriages, downregulation of THRs is mediated by activated  $PPAR\gamma$  as well as RXR. Therefore, we consider that THR downregulation is a cause mediated by a number of different factors.

On the other hand, an impairment of the maternal thyroid function, such as in hypothyroidism or thyroid autoimmune diseases, can also have detrimental consequences to embryonic and fetal development and result in an increased risk of miscarriage. In fact, thyroid autoantibodies are associated with a higher rate of both spontaneous and

recurrent miscarriages (Budenhofer et al. 2013). Thyroid autoimmunity leads to an increase in miscarriage rate (Abalovich et al. 2007; Anselmo et al. 2004; De Geyter et al. 2009; Poppe et al. 2008; Smallridge et al. 2005; Todorova et al. 2008), especially in the first trimester (De Geyter et al. 2009; Poppe et al. 2008). A significant correlation between the presence of thyroid antibodies and spontaneous pregnancy loss has been confirmed by several studies: A two-fold increase in the miscarriage rate was seen in women who were thyroid antibody-positive in the first trimester as compared with antibody-negative women (Stagnaro-Green et al. 1990). A four-fold increase in the miscarriage rate was present in women affected by autoimmune thyroid disease as compared to the healthy controls (Glinioer et al. 1991). In addition, it has been shown that miscarriage in women affected by thyroid autoimmunity mainly occurs during the first trimester (Lejeune et al. 1993) and patients with recurrent miscarriage (RM) showed rather high incidences of thyroid antibodies (Bussen and Steck 1995) (Reznikoff-Etievant et al. 1999). In accordance with the Endocrine Society Guidelines, hypothyroidism in pregnant women is indicated to be treated with levothyroxine, even if subclinical (Gartner and Reincke 2008; Verga et al. 2009). The benefit of levothyroxine replacement in pregnant women has been demonstrated by several studies. Levothyroxine administration reduced rates of miscarriage and premature delivery in thyroid antibody-positive women to values similar to those of controls with euthyroid function (Negro et al. 2006). The incidence of obstetric complications is decreased when euthyroidism can be achieved and maintained by levothyroxine treatment in pregnant hypothyroid women (Glinioer 1998; Verga et al. 2009). However, an improvement in fertility by levothyroxine administration has not yet been proven (Gartner and Reincke 2008). Maternal thyroid dysfunction, especially hypothyroidism and thyroid autoimmunity, does have a negative influence on fetal development

and obstetric outcome, and thyroid autoantibodies are associated with a significant increase in miscarriage rates. However, a consistent screening routine for thyroid (dys-) function in pregnant women does not yet exist (Vaidya et al. 2012).

In summary, thyroid hormones  $T_3$  and  $T_4$  are essential for the maintenance of pregnancy and a deficiency in maternal thyroid hormones is associated with miscarriages. The results of our study show that the expression of thyroid hormone receptors  $THR\alpha_1$ ,  $THR\alpha_2$ ,  $THR\beta_1$ , and  $THR\beta_2$  is downregulated in villous trophoblasts from spontaneous and recurrent miscarriages. The majority of cells expressing the  $THR$ s in the decidua are decidual stromal cells. Only  $THR\beta_2$  is also expressed in EVT<sub>s</sub>.

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The authors declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: Mayr, Doris prof LMU Munich, Pathology may have a conflict of interest.

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