

Article

Receptors for thyroid-stimulating hormone and thyroid hormones in human ovarian tissue



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Abstract

Dysfunction in thyroid regulation can cause menstrual and ovulatory disturbances, the mechanism of which is not clear. The distribution and activity of the thyroid-stimulating hormone (TSHR), and the thyroid hormone receptors (TR) $\alpha 1$, $\alpha 2$ and $\beta 1$ in human ovarian tissue and in granulosa cells was studied using immunohistochemistry, reverse-transcriptase polymerase chain reaction (RT-PCR), quantitative PCR and immunoassays. Strong immunostaining of TSHR, TR $\alpha 1$ and TR $\beta 1$ was observed in ovarian surface epithelium and in oocytes of primordial, primary and secondary follicles, with minimal staining in granulosa cells of secondary follicles. Granulosa cells of antral follicles expressed TSHR, TR $\alpha 1$ and TR $\beta 1$ proteins. Messenger RNA for all receptors was present in ovarian tissue. Mature human granulosa cells expressed transcripts for 5' deiodinases types 2 and 3, but not type 1, indicating the possibility of conversion of peripheral thyroid hormone thyroxine (T_4). Granulosa cells stimulated with TSH showed a significant increase in cAMP concentrations after 2 h of culture ($P = 0.047$), indicating activation through TSHR. Stimulation with T_4 resulted in increased extracellular signal-regulated kinase 1 and 2 activation after 10, 30, 60 min and 24 h. These data demonstrate that TSH and thyroid hormone receptors may participate in the regulation of ovarian function.

Keywords: cAMP, ERK1/2, granulosa cells, human ovary, thyroid hormone receptor, thyroid stimulating hormone receptor

Introduction

Thyroid disease is more common in women than in men and it can cause disturbances in menstruation and ovulation (Thomas and Reid, 1987; Doufas and Mastorakos, 2000). In hyperthyroidism, the manifestation is often oligomenorrhoea, while in hypothyroidism, it is menorrhagia or oligomenorrhoea, pregnancy loss or infertility. However, the mechanisms behind these reproductive abnormalities are not well known.

The principal hormones produced by the thyroid gland are thyroxine (T_4) and tri-iodothyronine (T_3). Thyroxine is the main secretory product of the thyroid gland, and it is deiodinated to

peripherally active T_3 . Thyroid hormone action in humans is receptor mediated. Nuclear binding sites for thyroid hormones have been identified in many tissues and organs of the human body, such as the heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas (Shahrara *et al.*, 1999).

The time of genomic responses to thyroid hormone stimulation varies from hours to days (Aranda and Pascual, 2001). Some thyroid hormone effects occur within a few minutes (so-called non-genomic responses) and are associated with secondary messenger signalling pathways, such as the mitogen-activated

protein kinase (MAPK) pathway (Losel and Wehling, 2003; Tang *et al.*, 2004). Thyroxine promotes these effects by binding to the cell surface and activating MAPK complexes, in particular extracellular signal-regulated kinase 1/2 (ERK1/2) complexes, with thyroid hormone receptor $\beta 1$ (TR $\beta 1$; Davis *et al.*, 2000). The T_4 effect on phosphorylation of MAPK and subsequent activation of TR $\beta 1$ was apparent within 10 min and reached its upper limit by 30–40 min (Davis *et al.*, 2000). The ERK1/2 pathway has been shown to be important for maturation of the oocyte–cumulus complex in mice, under the influence of FSH (Su *et al.*, 2003). ERK1/2 involvement in oocyte maturation was also reported in pig and bovine oocytes (Tomek *et al.*, 2002; Ellederova *et al.*, 2008).

Thyroid-stimulating hormone (TSH), produced in the anterior pituitary gland, is regulated by binding of the thyroid hormone-activated receptor to the *TSH* gene. TSH receptor mRNA has been found to be significantly up-regulated after TSH treatment (Huber *et al.*, 1991; Huber *et al.*, 1992). TSH binding to TSH receptor activates cAMP production, which mediates most TSH effects, especially its short-term actions (Weiss *et al.*, 1984; Davies *et al.*, 2002; Dremier *et al.*, 2002). Mizukami *et al.* (1994) described the distribution of TSH receptors in normal human thyroid tissues, in neoplastic thyroid tissue, and in the thyroid during Grave's disease. TSH has been found to be present in human follicular fluid at concentrations similar to those in serum (De Silva, 1994).

Ovarian cyst formation may be greatly intensified in women with primary hypothyroidism and in experimentally hypothyroid animals exposed to stimulation with gonadotrophins (Lindsay *et al.*, 1983; Fitko *et al.*, 1996). Using ultrasound scans before and after treatment of hyperthyroidism, the appearance of the ovaries was found to be abnormal before treatment in most patients. The ovaries contained follicles of abnormal size, form and number. The ovarian abnormality corresponded more closely to circulating thyroid hormone concentrations than with those of FSH, LH or androgen concentrations (Skjoldebrand Sparre *et al.*, 2002). Earlier, van Voorhis and co-authors reported the development of multicystic ovaries during profound hypothyroidism (Van Voorhis *et al.*, 1994). These findings are in accordance with earlier suggestions that thyroid hormone may exert a direct influence on the granulosa cells of the porcine ovary (Maruo *et al.*, 1992).

In humans, thyroid hormone receptor mRNA was present in luteinized granulosa cells from healthy young non-stimulated women (Wakim *et al.*, 1994). Thyroid hormones were present in follicular fluid in women undergoing IVF treatment, and luteinized granulosa cells from these subjects contain thyroid hormone receptors (Wakim *et al.*, 1993). Mature (MII) oocytes from women undergoing IVF contain TR $\alpha 1$, TR $\alpha 2$, TR $\beta 1$ and TR $\beta 2$ mRNA, which suggested a possible direct effect of T_3 on the human oocyte (Zhang *et al.*, 1997). Goldman *et al.* (1993) demonstrated that luteinized human granulosa cells contained high-affinity thyroid hormone binding sites.

Recent studies reported the presence of thyroid hormone receptors in human ovarian surface epithelium (Rae *et al.*, 2004, 2007) and showed their participation in the hormonal regulation of the ovarian surface epithelium *in vitro* (Rae *et al.*, 2007). They also demonstrated the expression of deiodinases DIO2 and DIO3, but not DIO1, transcripts in human ovarian

surface epithelium (Rae *et al.*, 2007), indicating the presence of an enzyme that generates T_3 from T_4 and the one that inactivates T_3 to reverse T_3 in thyroid hormone target tissues (Kohrle, 1999).

Here, the expression, distribution and activation of TSH receptors and the thyroid hormone receptors were studied in healthy human ovaries obtained from fertile women of reproductive age, and in luteinized granulosa cells obtained from women undergoing IVF/intracytoplasmic sperm injection (ICSI) treatment.

Materials and methods

Subjects

Biopsy specimens of ovarian cortical tissue for immunohistochemistry and reverse-transcriptase polymerase chain reaction (RT-PCR) were donated by women undergoing planned Caesarean section ($n = 17$) or laparoscopic tubal sterilization ($n = 10$) at the Karolinska University Hospital, Huddinge. Their mean age was 32.3 years (range 19–41). Granulosa-luteal cells were obtained from 12 women undergoing oocyte retrieval for assisted reproduction treatment in the Fertility Unit, Karolinska University Hospital, Huddinge and at the Centre of Reproduction, Akademiska Sjukhuset, Uppsala. All granulosa cell donors underwent ICSI treatment. Their mean age was 33.7 years (range 28–39). The number of oocytes collected from the granulosa cell donors ranged between seven and 19 (mean 10.5).

Eight women, mean age 33.5 years (range 30–39) donated small ovarian cortical biopsy specimens for tissue culture experiments, when undergoing routine Caesarean sections at Karolinska University Hospital, Huddinge.

The Ethics Committee at the Karolinska Institutet approved this study, and informed consent was obtained from all participating women prior to surgery or oocyte retrieval.

Immunohistochemical analysis

Samples for immunohistochemistry from women undergoing planned Caesarean section ($n = 17$) and from women undergoing laparoscopic sterilization ($n = 10$) were fixed in Bouin's solution for 12 h and then stored in 70% ethanol before embedding. Paraffin-embedded sections were cut (4 μ m), deparaffinized and washed. Endogenous peroxidase was blocked with 3% H_2O_2 in methanol for 10 min. Sections to be stained for TSH receptor were incubated with 10% (blocking) normal horse serum (Vector Laboratories, Burlingame, CA, USA) in phosphate buffered saline (PBS) and sections for TR $\alpha 1$, TR $\alpha 2$ and TR $\beta 1$ analysis were incubated with 10% normal goat serum (Vector Laboratories, Burlingame, CA, USA) for 60 min.

Monoclonal mouse antibody against human TSH receptor (Novocastra Laboratories Ltd, Newcastle, UK) was used at a final concentration of 2.5 μ g/ml at room temperature for 60 min in PBS, and polyclonal rabbit immunoglobulin G (IgG) against human TR $\alpha 1$, mouse anti-TR $\alpha 2$ and rabbit anti-TR $\beta 1$

(Affinity BioReagents, Inc, Golden, CO, USA) were used at 5 µg/ml, 5 µg/ml and 3 µg/ml respectively for 60 min in the same conditions. The specificity of these antibodies for TSH receptor, TRα1, TRα2 and TRβ1 in thyroid tissue has been reported previously (Mizukami *et al.*, 1994; Kilby *et al.*, 2000). Sections of the thyroid gland (donated by the pathology department of Danderyd Hospital) were used as positive controls for TSH receptor and for TRα1, TRα2 and TRβ1 (Wakim *et al.*, 1994).

As negative controls for immunostaining of TSH receptor, TRα1, TRα2 and TRβ1, the primary antibodies were replaced with non-immune IgGs from the same species in which the primary antibodies were raised (SDS, Falkenberg, Sweden). As further negative controls, sections of breast that are known not to express TSH receptor were used (Mizukami *et al.*, 1994). As a non-relevant antibody to validate the results, a specific mouse antibody against human myelin basic protein was used (kindly provided by Dr Nigel Groome, Cambridge, UK), which is not expressed in the human ovary. The slides were then incubated with appropriate biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA, USA) diluted 1:200 for 60 min at room temperature. After rinsing with PBS containing Tween 20 (0.01%), the bound antibodies were visualized by means of avidin–biotin complexed with peroxidase (Vectastain ABC Elite, Vector Laboratories), for 30 min, followed by the application of 3,3-diaminobenzidine in H₂O₂ (DAB-kit, Vector Laboratories). All slides were counterstained with haematoxylin, dehydrated and mounted with Pertex (Histolab, Gothenburg, Sweden). A Leica microscope connected to a video camera and computer was used to assess the stained images. Each experiment was performed in duplicate.

Evaluation of immunohistochemistry

Two observers unaware of the identity of the slides, evaluated the samples. Staining intensity were graded as follows: 0 = no staining, -/+ = a few stained cells, + = faint staining, ++ = moderate staining, +++ = strong staining, and – = no oocytes in antral follicles found.

RT-PCR

Preparation of RNA and cDNA

The ovarian tissue obtained from women undergoing Caesarean section (three samples) was immediately frozen and stored in liquid nitrogen. The frozen tissue was homogenized in lysis buffer. RNA was prepared according to a protocol from SDS-Promega (Falkenberg, Sweden) and its concentration and purity were assessed spectrophotometrically. The synthesis of first-strand cDNA was performed using a Superscript pre-amplification system (Invitrogen, Carlsbad, CA, USA). For each reaction, 2 µg of total RNA were reverse transcribed using oligo(dT) as a primer.

Cultured granulosa cells from four patients were prepared for real-time RT-PCR analysis of 5' deiodinases types 1, 2 and 3 (DIO1, DIO2, DIO3). RNA was prepared by using RNeasy Mini-kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. All samples were analysed by spectrophotometer to determine RNA concentration, yield and purity. Synthesis of granulosa cell cDNA for quantitative

PCR analysis was performed using the Superscript III First Strand Synthesis System for RT-PCR (Invitrogen, Stockholm, Sweden). The reverse transcription was performed according to manufacturers instructions. Each reaction used 200 ng of total mRNA from granulosa cells. Random hexamers were used as primers.

The samples were stored at –20°C until real-time PCR was performed.

Reverse-transcriptase PCR

The PCR were performed using MasterTaq Kits (Eppendorf AG, Hamburg, Germany) according to the manufacturer's protocol. The primers are shown in **Table 1**. The mixture was heated to 94°C for 3 min and then amplified over 25 cycles (28S rRNA) or 35 cycles (thyroid hormone receptors, TSH receptor): denaturation at 94°C for 1 min for thyroid hormone receptors and 28S rRNA, or 94°C for 20 s for TSH; anneal at 54°C for 1 min for thyroid hormone receptors, 58°C for 1 min for 28S rRNA, or 64°C for 1 min for TSH receptor; extension at 72 °C for 1 min. A control PCR without template cDNA was carried out in each run.

For TSH receptor, nested PCR was performed. The primers are shown in **Table 1**. The same conditions were used for both the first and second PCR.

Amplification products were separated by means of 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. The amounts of mRNA for TRα1, TRα2 and TRβ1 were estimated as equivalence between each target and internal standard (28S rRNA) band intensity using Quantity-One (BioRad Laboratories, Hercules, CA, USA). Each experiment was performed in triplicate.

Real-time RT-PCR

Real-time PCR was performed using an ABI PRISM 7700 sequence detector (Applied Biosystems, Foster City, CA, USA). SYBR green was used to detect the amplification. The reaction mixture consisted of 1 × SYBR green PCR mastermix, 4 µl of the RT-PCR reaction mixture, corresponding to 1 ng cDNA, and primer concentrations of 200 nmol/l for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 1.5 µmol/l for each DIO. The total reaction volume was 25 µl. The cDNA was then heated to 50 °C for 2 min and denatured at 95 °C for 10 min. The template was then amplified over 40 cycles of 15 s melting at 95 °C and 1 min at 62 °C for annealing and extension. Standard curves were run for each primer and were constructed using duplicates of six serial dilutions of cDNA prepared from RNA of known concentration.

Each sample was run in duplicate and the target genes were normalized to GAPDH as endogenous control. Primer sequences and the size of the PCR products for the DIO and the endogenous control gene GAPDH are presented in **Table 1**.

Ovarian tissue culture

The biopsy specimens from eight women at term pregnancy were placed in pre-equilibrated HEPES-buffered oocyte collection

Table 1. List of primers used for reverse transcription-polymerase chain reaction (PCR) amplification and for quantitative PCR.

<i>Gene</i>	<i>Primers (sense)</i>	<i>Primers (antisense)</i>	<i>PCR product (base pairs)</i>
28S	5' GTG CAG ATC TTG GTG GTA GTA GC 3'	5' AGA GCC AAT CCT TAT CCC GAA GTT 3'	552
TR α 1	5' GGT GCT GCA TGG AGA TCA TG 3'	5' GGA ATG TTG TGT TTG CGG TG 3'	325
TR α 2	5' GGT GCT GCA TGG AGA TCA TG 3'	5' TCG ATC TTG TCC ACA CAC AG 3'	259
TR β 1	5' CGG AGG AGA AGA AAT GTA AAG G 3'	5' GCT YCG GTG ACA GTT TTG ATG 3'	421
TSHR	5' AAT CCC TGT GAA TGC TTT TC 3'	5' ACT CAA GGA AAG TGG AAG TT 3'	–
TSHR nested	5' GTG AAT GCT TTT CAG GGA CTA TG 3'	5' GTC CAG GTG TTT CTT GCT ATC AG 3'	272
GAPDH	5' GAA GGT GAA GGT CGG AGT CAA C 3'	5' CAG AGT TAA AAG CAG CCC TGG T 3'	71
Oestrogen receptor β	5' AGA GTC CCT GGT GTG AAG CAA G 3'	5' GAC AGC GCA GAA GTG AGC ATC 3'	143
DIO1	5'GGC TGG GCT TTT AAG AAC AA 3'	5'AAC TGT GGA GCT TTT CCA GAA C 3'	155
DIO2	5'TTG TAC TTA CTC TAA ATT TCC CAA GG 3'	5' CAT TGC CAC TGT TGT CAC CT 3'	159
DIO3	5'GCC AGC ACA TCC TCG ACT AC 3'	5'GAG AAG CCA GAG CAT GAA GG 3'	151

DIO = deiodinase; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; TR = thyroid hormone receptor; TSHR = thyroid-stimulating hormone receptor.

medium (Gamete-100; VitroLife, Kungsbacka, Sweden) and transferred immediately to the culture laboratory. Ovarian tissue samples were cut into small pieces, approximately 1–2 mm³, using a needle and scalpel. The tissue pieces were then either directly fixed for histological analyses (control at 0 days) or placed in inserts and cultured for 3 days, as parallel cultures from the same biopsy specimen.

An established tissue culture method was used, as previously described (Hovatta *et al.*, 1997, 1999; Scott *et al.*, 2004; Carlsson *et al.*, 2006). Cultures were performed in 24-well low-evaporation lid plates (Falcon, Becton Dickinson, Franklin Lakes, NJ, USA) fitted with Millicell CM inserts (12 mm diameter, 0.4 μ m pore size, Millipore, Bedford, MA, USA). The inserts were coated with 100 μ l of growth factor-reduced extracellular matrix (GFR Matrigel, Becton Dickinson, Franklin Lakes, NJ, USA), pre-diluted 1:3 with alpha minimal essential medium with Glutamax (α MEM, Invitrogen, Carlsbad). To test the effects of TSH (Sigma-Aldrich, St. Louis, MO, USA) and T₄ (Sigma-Aldrich) on the ovary, hormones were added to the culture media at concentrations of 0.5 μ g/ml (Wright *et al.*, 1997) and 10 nmol/l (Davis *et al.*, 2000) respectively. Thyroxine was chosen for stimulation, because it has been shown to be capable of binding thyroid hormone receptors, in particular TR β 1, and DIO2 enzyme activating T₄ to T₃ was found in the human ovary (present study; Davis *et al.*, 2000; Rae *et al.*, 2007). Also, newly identified cell-surface receptor for thyroid hormone, α V β 3 integrin, binds preferentially T₄ versus T₃ (Bergh *et al.*, 2005).

Medium without added TSH or T₄ served as a control. The base culture medium used was α MEM supplemented with 10% human serum albumin (HSA; Pharmacia Upjohn, Stockholm, Sweden), 0.5% antibiotic/antimycotic solution (50 IU/ml penicillin, 50 μ g/ml streptomycin sulphate, 0.125 μ g/ml amphotericin B; Invitrogen, Carlsbad) and 1% insulin-transferrin-sodium selenite (Invitrogen, Carlsbad). Culture medium was added to each well; 100 μ l pipetted into the insert and 400 μ l in the well outside the insert. Ovarian tissue samples from eight patients were cultured for 3 days with daily medium replacement (220 μ l). Fresh non-cultured ovarian biopsy material (day 0) and cultured specimens were fixed for histology and follicle counts.

Histology and follicle counts

Fresh non-cultured ovarian biopsy material (day 0) and cultured specimens were fixed in Bouin's solution (Sigma-Aldrich) for 4–5 h at room temperature, dehydrated in 70% ethanol, embedded in paraffin and serially sectioned at 4 μ m thickness. To prevent double counting of follicles, eight sections were omitted between those sections mounted on the slide. Double counting of larger follicles was avoided by following the follicle through the sections (Hreinsson *et al.*, 2002; Scott *et al.*, 2004). Following staining with haematoxylin and eosin, the viability of the follicles was analysed using a method previously described (Hovatta *et al.*, 1997, 1999). The presence of pyknotic granulosa cells, eosinophilia of the ooplasm and clumping of the chromatin

material were regarded as signs of atretic follicles (Gougeon, 1986). The follicles were counted and their developmental stages recorded according to the classification of Gougeon (1986). Follicles containing a single layer of flattened granulosa cells were regarded as primordial, those having cuboidal granulosa cells in a single layer were classified as primary, and follicles having two or more layers of cuboidal granulosa cells were identified as secondary.

A digital-imaging analysis system (Easy Image Mätning; Tekno Optik, Huddinge, Sweden) was used to measure the area of the tissue pieces, from which the volume was calculated by multiplying the area by the known section thickness of 4 μm . The density of the follicles was then determined as the total number of follicles per cubic millimetre of ovarian tissue. Also, follicle and oocyte diameters were measured at the largest points.

Collection, isolation and culture of human granulosa cells

Follicular fluid from individual subjects undergoing IVF treatment was collected in separate tubes. Follicular fluid (with granulosa cells) from multiple follicles within the same patient was pooled. The tubes were centrifuged and the cell suspension was incubated with hyaluronidase (Sigma-Aldrich) for 30 min at 37°C. Following this, the granulosa cells were separated over a gradient of Ficoll solution (Amersham Biosciences, Uppsala, Sweden) by centrifugation at 300 g for 30 min at room temperature. The interphase cells were collected and washed twice in PBS. The cell pellet was re-suspended in culture medium. Viability was determined using the trypan blue (0.2% v/v) dye exclusion method. Cell counts were also performed with trypan blue.

Cultured granulosa cells from six subjects were used to analyse the expression of cAMP and ERK1/2, as a response to activation of the corresponding receptors, when stimulated with TSH or T_4 . Cells were seeded overnight in 4-well plates at a density of 0.4×10^6 cells per well in 500 μl of TCM-199 medium, supplemented with 2.5% charcoal-stripped fetal calf serum (Sigma-Aldrich) and 1% penicillin-streptomycin. On the next day, the medium was changed to serum-free medium. Cells were treated with 0.5 μg TSH/ml (Sigma-Aldrich) (Wright *et al.*, 1997) for 0, 30, 60 min, and 2 and 24 h (Bidey *et al.*, 1980, 1981), or with 10 nmol/l T_4 (Sigma-Aldrich) (for dose reference see Davis *et al.*, 2000) for 0, 10, 30, 60 min and 24 h (Tang *et al.*, 2004), with the untreated controls at the same time points. Culture medium from cultures with and without TSH stimulation at different time points was collected for cAMP measurements. 1 mmol/l 3-isobutyl-1-methylxanthine (IBMX, Sigma-Aldrich) was added to cultures 30 min prior to sampling of the medium. Supernatants were then processed for cAMP enzyme-linked immunosorbent assay (ELISA). Cell lysates from cultures with and without T_4 stimulation at different time points were collected and stored at -20°C until proceeding with ERK1/2 ELISA.

Enzyme-linked immunosorbent assay

To evaluate the functional activity of TSH receptor, granulosa cells were stimulated with TSH. Production of

cAMP was measured using an ELISA for cAMP according to the manufacturer's protocol (R and D Systems, Oxford, UK). Stimulation with T_4 was performed to study the functional activity of thyroid hormone receptors. Cell lysates were prepared according to a standard procedure, using RIPA (Radio-Immunoprecipitation Assay) lysis buffer (Assay Designs, Inc, Ann Arbor, MI, USA) with protease and phosphatase inhibitors (Sigma-Aldrich). Assay of phosphorylated ERK1/2 was conducted using a commercial kit (Assay Designs, Inc), according to the manufacturer's protocol. Ninety-six-well flat-bottomed immunoassay microtitre plates with high binding were used for the ELISA. Optical densities were measured using a plate reader and concentrations were calculated using standard curves that were set up in each ELISA plate in order to minimize inter-assay variability. The standard curves lay between 0.039 and 20 pmol/ml for camp, and between 31.25 and 2000 pg/ml for ERK1/2.

Statistical analyses

Differences in developmental stages, density of follicles, and proportions of viable follicles were analysed by using chi-squared and two-tailed *t*-tests. Data are presented as mean \pm SEM. The diameters of the follicles and oocytes were compared using two-tailed type-three *t*-tests. Concentrations of cAMP and ERK1/2 in cells from different subjects and at different time points were compared using Mann-Whitney *U*-test. Significance was accepted at the 0.05 level.

Results

TSH receptor protein expression and distribution in the human ovary

The TSH receptor was highly expressed in the ovarian surface epithelium, showing strong apical and cytoplasmic immunostaining, whereas cell nuclei were negative in both pregnant and non-pregnant women (**Figure 1A**). Primordial follicles showed positive immunoreaction in the oocytes (**Table 2**), while granulosa cells were negative for TSH receptor (**Figure 1B**). A few granulosa cells in primary follicles were immunopositive and there was also staining in the oocytes (**Figure 1C**). The granulosa cells in secondary follicles had faint staining, while oocytes had moderate staining (**Figure 1D**). Stromal cells also exhibited weak TSH receptor immunoreactivity. An antral follicle presented faint staining in granulosa cells. In particular, staining was present in a few granulosa cells lying next to the basal lamina (**Figure 1E**). However, the oocyte was not found in the follicle sections analysed (nine consecutive sections each 4 μm thickness were analysed).

There was no difference in TSH receptor expression intensity in ovarian tissue components between pregnant and non-pregnant women.

Immunostaining with anti-human myelin basic protein antibody did not reveal any protein expression (**Figure 1F**). Normal non-immune IgG did not produce any immunoreaction (**Figure 1F** insert). Positive staining was observed in positive

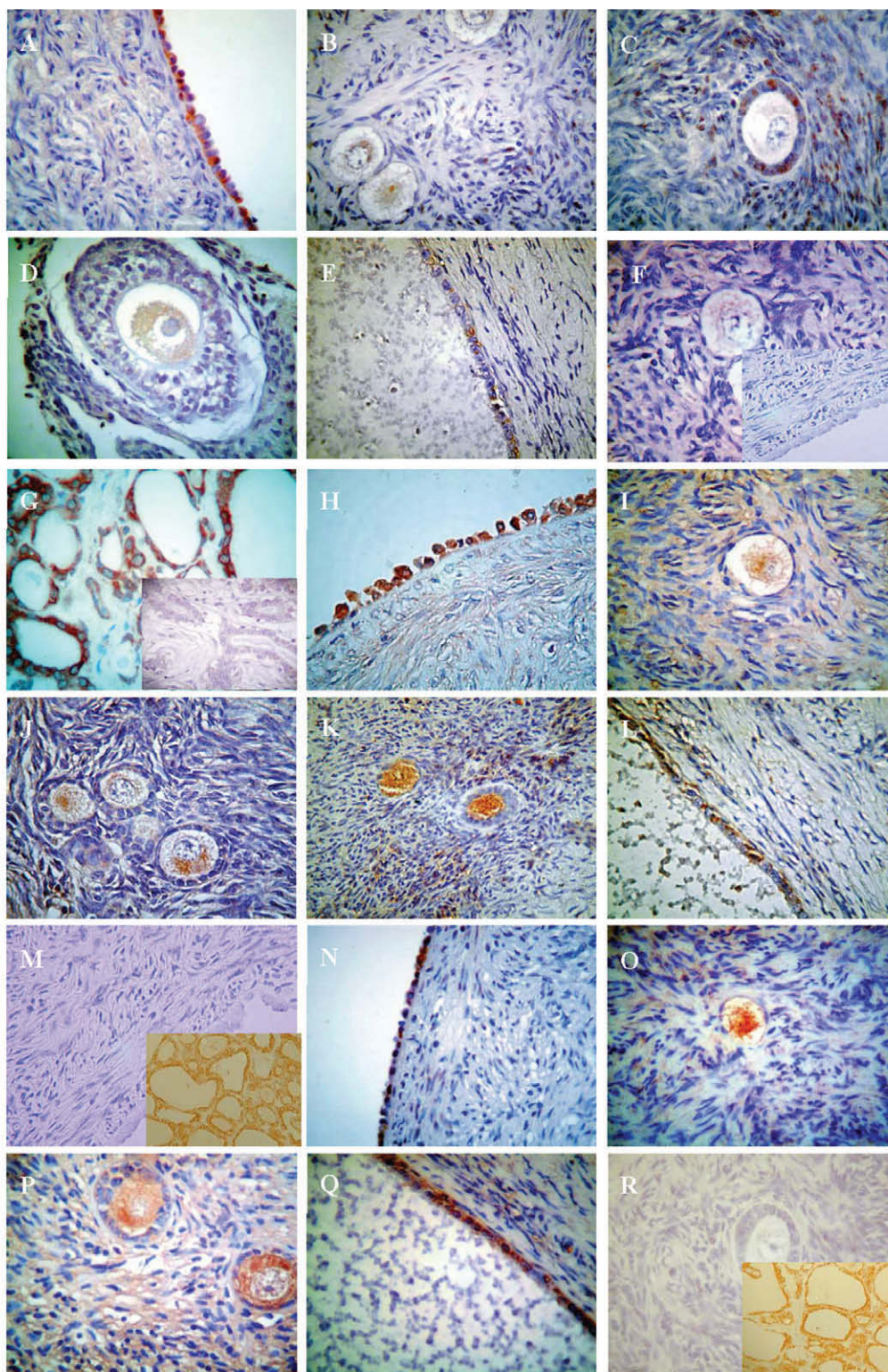


Figure 1. (A–G) Distribution of thyroid-stimulating hormone receptor in the human ovary: (A) immunostaining in surface epithelium; (B) primordial follicles; (C) primary follicle; (D) secondary follicle; (E) antral follicle; (F) human ovarian cortex treated with non-immune immunoglobulin G (insert is ovarian surface epithelium treated with mouse anti-human myelin basic protein); (G) thyroid tissue as positive control (insert is human breast tissue as negative control). (H–M) Distribution of thyroid hormone receptor TR α 1: (H) immunostaining in surface epithelium; (I) primordial follicle; (J) primary follicles; (K) secondary follicle; (L) antral follicle; (M) negative control slide with ovarian surface epithelium treated with non-immune immunoglobulin G (insert is thyroid tissue as positive control). (N–R) Distribution of TR β 1 in human ovary: (N) immunostaining in surface epithelium; (O) primordial follicles; (P) primary and secondary follicles; (Q) antral follicle; (R) negative control treated with non-immune immunoglobulin G (insert is thyroid tissue as positive control). All images are $\times 40$ magnification.

Table 2. Evaluation of immunohistochemical staining in ovarian tissue biopsies.

Ovarian tissue	Protein TSHR	TR α 1	TR α 2	TR β 1
Epithelium	+++	+++	0	+++
Stroma	+	-/+	0	+
Primordial				
Oocyte	+	+	0	++
Granulosa cell	0	0	0	0
Primary				
Oocyte	+	++	0	++
Granulosa cell	+	0	0	0
Secondary				
Oocyte	++	++	0	+
Granulosa cell	-/+	0	0	-/+
Antral				
Oocytes	-	-	-	-
Granulosa cell	-/+	+	0	++

TR = thyroid hormone receptor; TSH = thyroid-stimulating hormone receptor. 0 = no staining; -/+ = a few stained cells; + = faint staining; ++ = moderate staining; +++ = strong staining; - = no oocytes found in antral follicles.

control tissue (thyroid) (**Figure 1G**), and there was no staining in the negative tissue (breast) (**Figure 1G** insert).

Thyroid hormone receptor protein expression and distribution in the human ovary

Ovarian surface epithelial cells showed strong nuclear and perinuclear staining for TR α 1 (**Figure 1H**, **Table 2**). The oocytes in all follicles presented faint to moderate immunostaining, while the granulosa cells were negative at all stages of follicular development observed, with the exception of antral follicles (**Figure 1I–L**), in which few stromal cells stained positive. Positive and negative controls are presented (**Figure 1M**).

TR α 2 immunostaining was not observed in any of the human ovarian samples.

The ovarian surface epithelial cells had strong nuclear and perinuclear anti-TR β 1 staining in all samples (**Figure 1N**, **Table 2**). The oocytes in primordial, primary and secondary follicles stained positively for TR β 1, whereas the granulosa cells showed little or no staining (**Figure 1O, P**). No oocytes were visualized in the antral follicle samples. Ovarian stroma stained weakly for TR β 1.

Some granulosa cells in antral follicles showed positive TR β 1 immunostaining. Positive immunoreactivity was especially observed in granulosa cells lying next to the basal lamina (**Figure 1Q**). Positive and negative controls are presented (**Figure 1R**).

No difference was observed in TR α 1 and TR β 1 immunoexpression in ovaries from pregnant and non-pregnant women.

TSH receptor and thyroid hormone receptor mRNA expression in human ovary

Ovarian tissue showed the presence of mRNA for TR α 1, TR α 2 and TR β 1 (**Figure 2A**). Nested PCR showed the presence of TSH receptor mRNA in the human ovary (**Figure 2B**).

DIO mRNA expression in human granulosa cells

The presence and relative expression of three types of DIO were studied in human luteinized granulosa cells from four patients. There was no expression of DIO1 in granulosa cells from any of the patients, while both DIO2 and DIO3 transcripts were present in the granulosa cells from all four patients (**Figure 3**). These results are similar to a previous report on DIO expression in human ovarian surface epithelium (Rae *et al.*, 2007).

Ovarian tissue culture

The primordial follicles had initiated their growth in all cultured tissue. The proportion of primordial follicles was higher in non-cultured control tissue, while the proportion of primary and secondary follicles increased in culture conditions (**Figure 4A**). This phenomenon has been documented previously in different experimental settings (Wandji *et al.*, 1996; Hovatta *et al.*, 1997, 1999). Cultured tissue in all groups had significantly fewer viable follicles than the non-cultured control tissue ($P \leq 0.03$; **Figure 4B**). There was no significant difference in the densities of the follicles when any one group was compared with any other (data not shown). Finally, the sizes of the follicles and diameters of the oocytes in primordial, primary and secondary follicles were compared in all groups (**Figure 4C, D**). The diameters of non-cultured control primordial follicles ($42.2 \pm 1.2 \mu\text{m}$) and those of cultured control primordial follicles ($38.6 \pm 0.7 \mu\text{m}$) were significantly different ($P = 0.02$). The diameters of the non-cultured control primordial follicles were significantly greater than those of primordial follicles cultured in the presence of TSH ($38.8 \pm 0.8 \mu\text{m}$) ($P = 0.03$) with no significant difference between non-cultured control primordial follicles and cultured primordial follicles after exposure to T_4 (**Figure 4C**). The mean diameter of the non-cultured primary oocytes ($37.2 \pm 1.0 \mu\text{m}$) was significantly greater than that of the corresponding primary oocytes cultured in the presence of T_4 ($33.4 \pm 1.1 \mu\text{m}$) ($P = 0.006$) (**Figure 4D**).

Effects of TSH and T_4 on luteinized granulosa cells

Concentrations of cAMP as a sign of activation of cAMP-signalling pathway varied between the control sample and samples at different time points with and without stimulation with TSH (**Figure 5A**). The increase in cAMP reached significantly different concentrations compared with the corresponding control after 2 h in culture with exogenous TSH ($P = 0.047$). cAMP responses continued to rise and were highest after 24 h of TSH stimulation; however, the difference was not significant compared with the control unstimulated culture (**Figure 5A**).

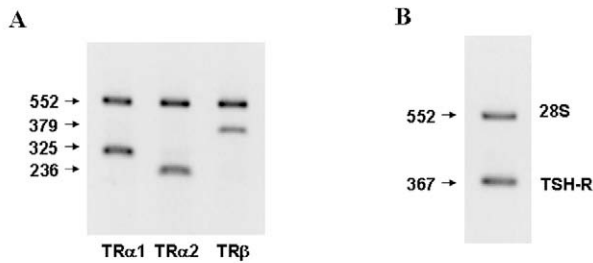


Figure 2. (A) Thyroid hormone receptors TRα1, TRα2 and TRβ1 mRNA expression in human ovarian tissue. (B) thyroid-stimulating hormone receptor (TSH-R) mRNA expression in the ovary. 28S rRNA was used as an internal standard.

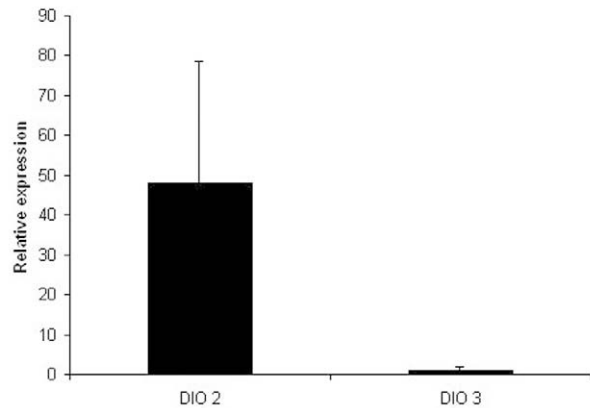


Figure 3. Relative expression of 5' deiodinases DIO2 and DIO3 in luteinized granulosa cells obtained from four patients undergoing IVF/intracytoplasmic sperm injection. DIO1 mRNA was not detected. Values are mean \pm SEM.

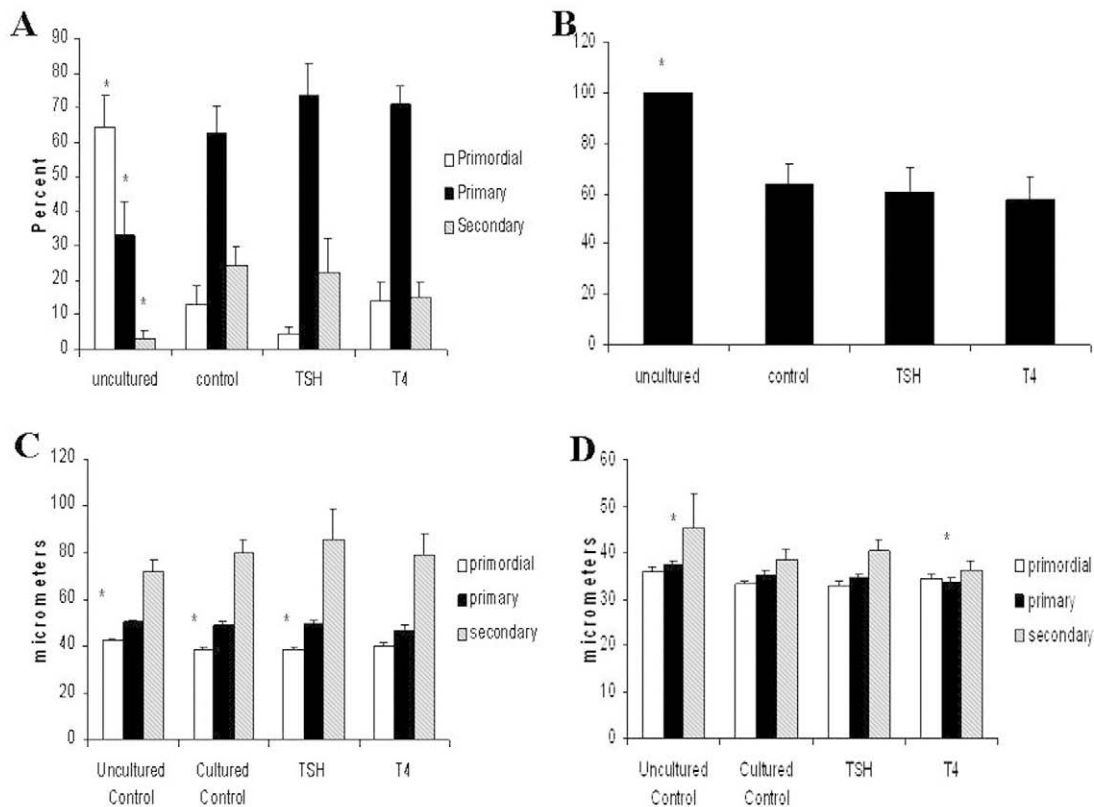
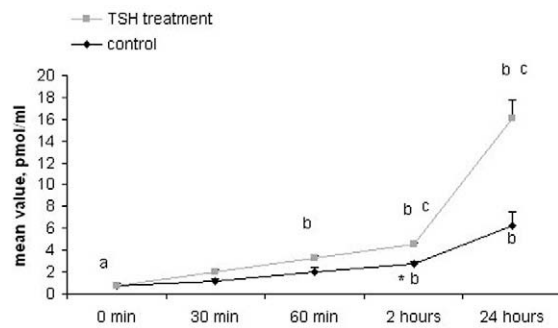


Figure 4. Culture of normal human ovarian tissue ($n = 8$) under control conditions and treatment with thyroid-stimulating hormone (TSH) and thyroxine (T_4). (A) Follicle development, * = significant difference between uncultured control tissue and cultured tissue under different conditions at all follicle developmental stages, $P \leq 0.03$. (B) Changes in follicle viability, * = significant difference between uncultured control tissue and cultured tissue under different conditions, $P \leq 0.03$. (C) Changes in follicle diameter, * = significant difference between uncultured and cultured primordial follicles, $P \leq 0.03$. (D) Changes in oocyte diameter, * = significant difference between uncultured primary oocytes and primary oocytes cultured with T_4 , $P \leq 0.03$. Values are mean \pm SEM.

A



B

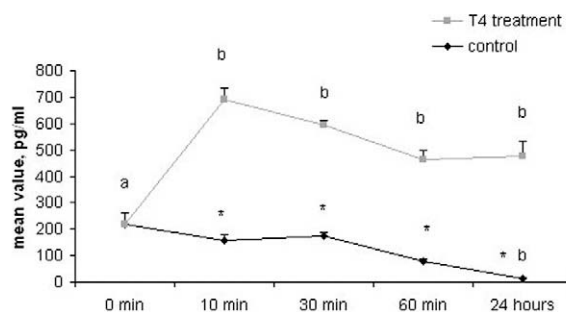


Figure 5. (A) Time-dependent production of cAMP by cultured human granulosa cells ($n = 6$) after thyroid-stimulating hormone treatment. Significant difference between a and b , $P \leq 0.02$. Significant difference between c and c , $P = 0.005$. * = significant difference between corresponding control and test sample at 2 h time point, $P = 0.047$. Values are mean \pm SEM. (B) Time-dependent extracellular signal-regulated kinase 1/2 activation in cultured human granulosa cells ($n = 6$) after thyroxine treatment. Significant difference between a and b , $P \leq 0.02$. * = significant difference between corresponding control and test sample at each time point, $P = 0.02$. Values are mean \pm SEM.

ERK1/2 phosphorylation was measured in granulosa cells cultured with and without T_4 for 10, 30, 60 min and 24 h. Phosphorylation of ERK1/2 was similar in control cells at 10, 30, 60 min time points, with a significant decrease after 24 h of culture ($P \leq 0.02$). Concentrations of ERK1/2 activity differed between control samples and T_4 -stimulated cells at 10, 30, 60 min and 24 h time points ($P = 0.02$) (Figure 5B).

Discussion

As far as is known, this study is the first to show expression of TSH receptor mRNA and protein in the human ovary. Oocytes, granulosa cells, surface epithelial cells and stromal cells of healthy human ovaries expressed TSH receptor. The expression was particularly strong in the surface epithelium.

The presence and distribution of TSH receptors in different organs of humans (not including human ovary) and other mammals have been presented (Davies *et al.*, 2002). TSH receptor signals via cAMP protein kinase A pathway (Davies *et al.*, 2002; Dremier *et al.*, 2002). Here, increased production of cAMP is demonstrated after stimulation of cultures with recombinant TSH, which was significant after 2 h of stimulation.

This study also showed the presence and cellular distribution of thyroid hormone receptors in non-stimulated ovaries from healthy euthyroid women at term pregnancy undergoing Caesarean section, and in ovaries from healthy fertile women undergoing tubal sterilization as a result of fulfilled family planning. Most studies regarding thyroid receptors in the human ovary have been carried out in connection with IVF treatment. Wakim *et al.* (1994) reported the presence of $TR\alpha$ and $TR\beta$ mRNA in human granulosa and stromal cells of pre-ovulatory antral follicles in non-stimulated ovaries, using *in situ* hybridization. In the present study, ovaries from healthy women were used for immunohistochemistry and RT-PCR experiments.

The presence of $TR\alpha$ and $TR\beta$ mRNA in human granulosa cells from stimulated ovaries has been previously described, and in particular, the preponderance of $TR\alpha 1$ (Wakim *et al.*, 1993). However, these studies were performed in non-physiological conditions with rapidly rising concentrations of oestrogen, which can influence the concentration of the most important binder of thyroid hormone, thyroxine-binding globulin (Skjöldebrand *et al.*, 1982). The presence of T_3 and T_4 in follicular fluid from women stimulated with human menopausal gonadotrophin in connection with oocyte retrieval has been described. The concentrations of thyroid hormone in both serum and follicular fluid were similar (Wakim *et al.*, 1993; Zhang *et al.*, 1997).

The presence of mRNA of all four studied receptors (TSH receptor, $TR\alpha 1$, $TR\alpha 2$ and $TR\beta 1$) has been found in the human ovary. The results of the immunohistochemical analyses suggested that granulosa cells at early stages of follicular development do not actively participate in thyroid hormone binding. $TR\alpha 1$, $TR\alpha 2$ and $TR\beta 1$ proteins were not expressed in granulosa cells of primordial and primary follicles. The granulosa cells of secondary follicles expressed $TR\beta 1$, and the granulosa cells of antral follicles expressed a low amount of $TR\alpha 1$ and a moderate amount of $TR\beta 1$, especially in the cells lying next to the lamina basalis. These results are similar to earlier findings reported by Wakim *et al.* (1994), who showed thyroid hormone receptor mRNA expression in granulosa cells of pre-ovulatory follicles.

Positive faint-to-moderate immunostaining was found for $TR\alpha 1$ and $TR\beta 1$ proteins in human oocytes in primordial, primary and secondary follicles. However, since no oocytes were observed in antral follicles in tissue sections, it is difficult to draw any conclusions about the expression of receptor protein in premature versus mature oocytes. Nevertheless, in the extensive gene expression study on human oocytes (with an Affymetrix microarray) expression of the $TR\alpha$ gene was found in germinal vesicle-stage, and metaphase I and II oocytes (Zhang *et al.*, 2007). This relates to previous results (Zhang *et al.*, 1997) demonstrating the presence of thyroid hormone

receptor isoforms in mature human oocytes from stimulated follicles, suggesting that T_3 can act either directly on the oocyte or through the thyroid receptors in cumulus or granulosa cells.

TR β 1 is generally regarded as being localized in the nucleus, but it has been reported to be present in cytosol (Zhu *et al.*, 1998). This study found cytosolic immunostaining of TR β 1, although the role of cytosolic TR β 1 is not clear (Davis *et al.*, 2000). mRNA was detected, but not protein corresponding to TR α 2. TR α 2 does not bind T_3 . TR α 2 acts as a negative dominant transcriptional factor that binds to a specific thyroid hormone-response element in thyroid hormone-regulated genes, but without generating transcriptional activity (Sylvén *et al.*, 1996).

It is known that serum concentrations of thyroid hormone increase during pregnancy, although the concentrations of free (non-bound and active) hormones do not change (Glinioer *et al.*, 1990). This is in line with the observation in this study that there was no difference regarding the intensity of expression of TSH and thyroid hormone receptors between pregnant and non-pregnant women.

Relative expression of DIO2 and DIO3 transcripts in luteinized human granulosa cells was also shown, as well as absence of DIO1 expression in those cells. These data reveal the ability of granulosa cells to regulate the conversion of T_4 to either T_3 or reverse T_3 (to a lesser extent, due to low expression of DIO3 transcript), thereby locally controlling the hormone activity. This notion is also supported by activation of p-ERK1/2 after stimulation with T_4 , which may be evidence for the conversion of T_4 to T_3 and activation of the TR in the granulosa cell cultures. Further studies are planned including the quantification of deiodinase activity according to Schomburg *et al.* (1997) in ovarian tissue.

The effects of TSH and T_4 treatment of cultured human ovarian tissue on the development and viability of follicles and oocytes was analysed. Cecconi *et al.* (1999) reported that, in mice, T_3 did not have any effect on the process of cumulus expansion and meiotic maturation of the oocyte. This study did not find any pronounced differences in the development of follicles and oocytes in stimulated and non-stimulated cultures. It can be concluded that TSH and thyroid hormone do not have acute (short-term) effects on the development of follicles and oocytes in fresh human ovarian tissue from healthy fertile women. Longer term in-vitro studies in the future may help us to understand the possible role of TSH and thyroid hormones in human folliculogenesis.

These results confirmed the previous reports on the expression of thyroid hormone receptor mRNA in human ovarian surface epithelium (Rae *et al.*, 2004, 2007). Together with strong TSH receptor expression in surface epithelium, this may mean a special role for thyroid hormones, which may have a certain effect during folliculogenesis and ovulation in the healthy state. Thyroid hormone influence and expression of thyroid hormone receptors in human ovarian surface epithelium was also suggested to be involved in the pathogenesis of pathological conditions such as ovarian cancer and probably ovarian endometriosis (Rae *et al.*, 2007).

This study analysed the effect of TSH and T_4 stimulation on

the relative expression of oestrogen receptor β , a predominant receptor in human granulosa cells, in human ovarian tissue and luteinized granulosa cells after 24, 48 and 72 h of treatment (data not shown). However, no significant difference in the treated groups compared with the controls was observed. Rae *et al.* (2007) demonstrated stimulation of mRNA expression for oestrogen receptor α (the oestrogen receptor isoform strongly associated with ovarian cancer) by T_3 in human ovarian surface epithelium without any effect on oestrogen receptor β mRNA concentrations. These combined data suggest that oestrogen receptor β may not be directly affected by thyroid hormone in the human ovary.

In conclusion, it is possible that TSH and thyroid hormones act directly on the human ovary through binding to their specific receptors, but their contribution to physiological and pathological processes in the ovary remains to be elucidated.

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