Journal of Endocrinological Investigation
https://doi.org/10.1007/s40618-019-01169-5

ORIGINAL ARTICLE

Thyroid hormones T3 and T4 regulate human luteinized granulosa cells, counteracting apoptosis and promoting cell survival

V. Di Paolo[1,4] · C. Mangialardo[2] · C. Zacà[3] · M. Barberi[3] · E. Sereni[3] · A. Borini[3] · M. Centanni[2] · G. Coticchio[3] · C. Verga-Falzacappa[2] · R. Canipari[1]

Received: 19 August 2019 / Accepted: 19 December 2019

Abstract

Purpose Fine and balanced regulation of cell proliferation and apoptosis are key to achieve ovarian follicle development from the primordial to the preovulatory stage and therefore assure female reproductive function. While gonadotropins are the major and most recognized regulators of follicle cell growth and function, other factors, both systemic and local, play equally important roles. This work is aimed at evaluating the effects of thyroid hormones (THs) on human granulosa luteinized (hGL) viability.

Methods Human GL cells derived from assisted reproduction treatments were exposed to T3 or T4. Cell viability was evaluated by MTT assay. Apoptosis was evaluated by the TUNEL assay and active caspase-3 staining. *StAR, CYP19A1, Caspase-3, P53* and *BAX* mRNA were evaluated by real-time PCR. LC3-I/-II, AKT and pAKT were evaluated by western blot.

Results T3 and T4 promoted cell viability in a dose-dependent modality and modulate *StAR* and *CYP19A1* expression. T3 and to a lesser extent T4 mitigated cell death induced by serum starvation by inhibition of caspase-3 activity and expression of *P53* and *BAX;* and attenuate cell death experimentally induced by C2-ceramide. Cell death derived from starvation appeared to be involved in autophagic processes, as the levels of autophagic markers (LC3-II/LC3-I ratio) decreased when starved cells were exposed to T3 and T4. This effect was associated with an increase in pAkt levels.

Conclusion From the present study, THs emerge as potent anti-apoptotic agents in hGL cells. This effect is achieved by inhibiting the apoptosis signalling pathway of BAX and caspase-3, while maintaining active the PI3K/AKT pathway.

Keywords Thyroid hormones · Apoptosis · Granulosa luteal cells · Human ovary

Introduction

The production of a mature oocyte is a complex process assisted by mutual interaction between germ and somatic cells. During antrum formation, granulosa cells undergo rapid growth supported by FSH [1]. Both deprivation of survival factors and presence of death ligands are responsible for programmed granulosa cell death and subsequent growth arrest of the follicle [2]. Follicular atresia is a degenerative process mediated by cell apoptosis [3], affecting sequentially granulosa cells [4] and theca cells [5]. Hence, follicular development results from a tight balance between apoptosis and survival. In the absence of proper survival factors, endogenous apoptosis prevails, causing atresia [2]. In addition to gonadotropins, many other factors affect follicular development and regulation [6]. 3,5,3′-Triiodothyronine (T3) and tetraiodo-L-thyronine (T4), detected in follicular fluid of human ovarian follicles are among these factors [7].

V. Di Paolo and C. Mangialardo contributed equally to this manuscript.

✉ R. Canipari
rita.canipari@uniroma1.it

1 DAHFMO, Unit of Histology and Medical Embryology, Sapienza, University of Rome, Via A. Scarpa 14, 00161 Rome, Italy

2 Department of Medical-Surgical Sciences and Biotechnologies, Sapienza, University of Rome, Rome, Italy

3 9.Baby, Family and Fertility Center, Via Dante 15, 40125 Bologna, Italy

4 Present Address: Department of Haematology/Oncology, Bambino Gesù Children's Hospital, IRCCS, Rome, Italy

Published online: 01 January 2020

Thyroid hormones regulate a variety of biological processes, including growth, metabolism, development and differentiation [8]. The ovary is considered to be a selective target for thyroid hormone action [9–11].

Euthyroidism ensures normal fertility function in the female [12, 13]. Both hyperthyroidism and hypothyroidism have adverse effects on female reproduction [14, 15]. In women, thyroid hormone disorders are associated with altered folliculogenesis and adverse pregnancy outcomes [12, 16]. However, the molecular processes underlying the influence of thyroid hormones (THs) on ovarian function remain poorly understood. THs can act indirectly on the ovary by dysregulating the hypothalamic–pituitary–ovarian axis, or impairing levels of sex hormone-binding proteins [17]. In addition to upstream effects of T3 on gonadotropins secretion, T3 targets directly ovarian function [9–11]. T3 and T4 are found in human follicular fluid [7]. THRs (thyroid hormone receptors) and the key enzymes deiodinases (DIO) [18], as well as thyroid hormone transporters [19], are found in the ovary of different species, including humans. Consistent with this, treatment with TH alone promotes the proliferation and survival of rat granulosa cells [20], counteracts chemotherapy-induced apoptosis [21] and acts synergistically with FSH [10]. In addition, T3 counteracts apoptosis in human granulosa cancer cells [22].

The aim of the present study was to detect the presence of TH machinery in human luteinized granulosa cell obtained from in vitro fertilization (IVF) patients and test the hypothesis that T3 and T4 influence cell survival.

Materials and methods

Unless otherwise reported, chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Cell cultures

Human granulosa luteal (GL) cells were obtained from follicular aspirates of women undergoing IVF treatment at 9.baby, Bologna, Italy. Informed consent was obtained from patients. Patients underwent an ovarian stimulation regime based on GnRH analog in combination with recombinant FSH, followed by 10,000 IU of hCG administered 36 h before oocyte recovery. After recovery of cumulus–oocyte complexes, GL cells present in follicle aspirates were collected, washed twice with HEPES-buffered medium, layered over Ficoll-Paque (Amersham, Milan, Italy), and centrifuged at 400*g* for 15 min. The GL cell-enriched interface layer was collected and cultured as previously described [23]. In cell growth experiments, cells were cultured in medium with 1% FBS. In apoptosis experiments, cells were seeded in culture medium and shifted to serum-free medium after 24 h. Adherent cells were treated with T3, or tetraiodo-L-thyronine T4 (Sigma-Aldrich; St. Louis, MO) at different doses (0.1–100 nM). At 24 h intervals, fresh aliquots of hormones were added to culture media in all experiments.

Cell viability assay

Human GL cells were seeded in 96-multiwell plates and treated with T3 and T4. Cell viability was assessed by a Cell Proliferation Kit XTT (Applichem St. Louis, Missouri, USA) as previously described [20].

TUNEL assay

Human GL cells were plated at 60% confluence onto multichamber slides (BD Falcon, BioScientifica S.r.l., Rignano Flaminio (RM), Italy). After 48 h, cells were subjected to the TUNEL assay using the in situ cell death detection kit (Roche, Basel, CH) as previously described [20]. TUNEL positivity was visualized with a Zeiss fluorescence microscope (Carl Zeiss SpA, Milano, Italy).

Active caspase-3 immunofluorescence

Freshly isolated granulosa cells were plated at 60% confluence onto multichamber slides (BD, Falcon) and shifted to serum-free medium after 24 h. THs were added to the culture medium every 24 h. Following the 48-h treatment period, cells were washed with PBS, fixed and permeabilized in acetone: methanol 1:1.

Samples were stained with anti-cleaved caspase-3 (#9661, CST, Cell Signalling Technology, Danvers, MA. 1:400 in 4% BSA) overnight. After repeated washes in PBS 1x, cells were incubated with a FITC-conjugated secondary antibody (Dako, Denmark A/S, Denmark). Slides were counterstained with Hoechst for nuclear identification. Slides were observed with a Zeiss fluorescence microscope (Carl Zeiss). Apoptotic cells were identified and counted in three or more randomly selected fields, each containing at least 100 cells.

RNA isolation, RT- and real-time PCR analyses

Human GL cells were centrifuged at 250*g* for 5 min and resuspended in lysis buffer for RNA extraction. Total cellular RNA was isolated from cells using Trizol RNA isolation reagent according to the manufacturer's instructions. RNA (1 µg) was subjected to reverse transcription (RT) using a cDNA synthesis kit (High-Capacity cDNA Reverse Transcription Kit, Thermo Fisher Scientific, Waltham, MA USA); cDNA was amplified by random hexamer primers. The presence of specific transcripts was evaluated by RT-PCR or real-time PCR. DNA contamination controls

were performed using gene-specific primers on RNA in the absence of reverse transcriptase treatment.

The PCRs were carried out using Hot Master Taq DNA polymerase (Eppendorf, Milano, Italy) according to manufacturer's instructions. For each primer set (Table 1), the number of cycles for the PCR was chosen in the exponential phase of amplification, using the annealing temperature provided. PCR products were electrophoresed onto a 2% agarose gel containing ethidium bromide (0.5 mg/ml) and visualized under UV light. Beta-actin expression levels were analysed as a control for RNA quality.

SYBR green real-time PCR was performed on an Applied Biosystems7500 Real-Time PCR system as previously described [24]. Each sample was normalized to the *HPRT* content. Threshold cycle (Ct) values were used to calculate the fold-changes in gene expression using the $2^{-\Delta\Delta Ct}$ method. The primers used are shown in Table 2.

All primers were synthesized by MWG Oligo synthesis report (Eurofins MWG Operon, Ebersberg, Germany).

Western blot

Cells were lysed in RIPA buffer supplemented with a protease inhibitor cocktail. Protein content was measured by the Bradford colorimetric assay (Bio-Rad Laboratories, Richmond, CA). Forty micrograms of total protein extracts per sample were loaded onto a 10–12% SDS-polyacrylamide gel, electrophoresed under reducing conditions [25] and transferred onto a nitrocellulose membrane (Bio-Rad) as previously described [20]. Membranes were incubated for 16 h at 4 °C with anti-phospho-protein kinase B (pAKT) (#4060, CST, 1:1000), anti-protein kinase B (AKT) (# 9272, CST, 1:1000), and anti LC3 (#2775, CST, 1:1000) and for 1 h at room temperature with anti-tubulin (T5168, Sigma-Aldrich, 1:500). Membranes were incubated with secondary HRP antibodies (anti-mouse, anti-rabbit, Sigma-Aldrich 1:4000) for 45 min at room temperature. Signals were detected by an ECL immunodetection system (Amersham Corp, Arlington Heights, IL, USA) following the manufacturer's instruction. The relative band intensity was captured by Chemi DocTM XRS 2015 (Bio-Rad Laboratories, Segrate, Milano, Italy), and densitometric analysis was performed using ImageJ software (NIH, 469 Bethesda, MD, USA) and normalized to tubulin.

Table 1 Sequence of oligonucleotides used as RT-PCR primers

Gene	Primers	Product length (bp)
B-ACTIN	Fw 5′-AAAATCTGGCACCACACCTTCTAC-3′ Rv 5′-AGGAGGAGCAATGATCTTGATCTTC-3′	750
THRA1	Fw 5′-CCCTGAAAACCAGCATGTCAG-3′ Rv 5′-TTCTTCTGGATTGTGCGGC-3′	151
THRA2	Fw 5′-GGTGCTGCATGGAGATCATG-3′ Rv 5′-TCGATCTTGTCCACACACAG-3′	259
THRB1	Fw 5′-GGCTGCAAGGGTTTCTTTAG-3′ Rv 5′-CGTTGGTCGCCACATGGGCT-3′	325
DIO1	Fw 5‘-AAGAGGCTCTGGGTGCTCTTGG-3‘ Rv 5‘-GGTTCTGGTGATTTCTGATGTC-3‘	499
DIO2	Fw 5‘-CTCTATGACTCGGTCATTCTGC-3‘ Rv 5‘-TGTCACCTCCTTCTGTACTGG-3‘	211
DIO3	Fw 5‘-CCTGGGACTCTGCTTCTGTAAC-3‘ Rv 5‘-GGGGTGTAAGAAAATGCTGTAGAG-3‘	136
MCT8	Fw 5‘-CAACGCACTTACCGCATCTG-3‘ Rv 5‘-GTAGCCCCAATACACACCAAGAG-3‘	146

Fw forward primer, *Rv* reverse primer

Table 2 Sequence of oligonucleotides used as real-time PCR primers

Gene	Primers	Product length (bp)
HPRT	Fw 5′-TGGAGTCCTATTGACATCGCC-3′ Rv 5′-AACAACAATCCGCCCAAAGGG-3′	197
Caspase-3	Fw 5′-CCATCGCCAAGTAAGAAAGTG-3′ Rv 5′-TCTCCCGTGAAATGTCATACTG-3′	330
BAX	Fw 5′-TGTTTTCTGACGGCAACTTC-3′ Rv 5′-ACAAAGATGGTCACGGTCTG-3′	333
P53	Fw 5′-CCCCTGTCATCTTCTGTCCCTT-3′ Rv 5′-CAGACCATCGTCATCTGAGCAG-3′	291
CYP19A1	Fw 5′-TTGGAAATGCTGAACCCGAT-3′ Rv 5′-TGGATTGTTGTTAAATATGATGCC-3′	408
STAR	Fw 5′-GCTGCTAGCGACATTCAAGC-3′ Rv 5′-CATCACAGCCTGTTGCCTCA-3′	85

Fw forward primer, *Rv* reverse primer

Statistical analysis

SPSS 16.0 for Windows was used for statistical analysis. Data are expressed as the mean ± SD. A comparison of the individual treatments was conducted using one-way ANOVA, followed by Tukey–Kramer post hoc analyses. $p < 0.05$ was considered statistically significant.

Results

Expression of components of the TH machinery in hGCs

Gene expression levels of the main TH machinery molecules were evaluated by RT-PCR in hGL cells. As shown in Fig. 1, genes encoding for thyroid receptors A (THRA1,

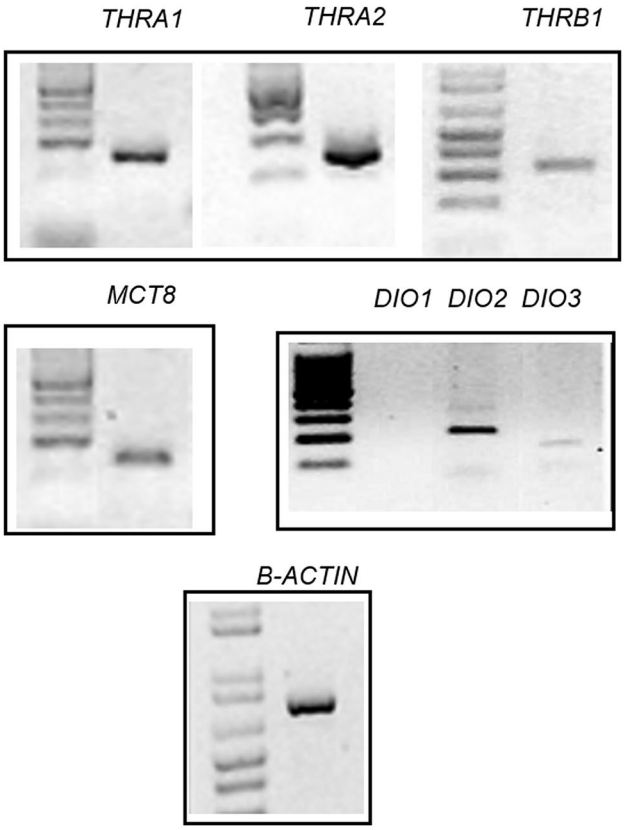

Fig. 1 TH machinery gene expression in hGL cells. Total RNA was prepared from freshly isolated hGL cells and analysed by semiquantitative RT-PCR as described in the "Materials and methods". The primers are indicated in Table 1. An aliquot of each PCR product was electrophoresed onto 2% agarose gel and stained with ethidium bromide

THRA2) and B (THRB1) and thyroid transporter MTC8 were expressed in hGL cells. In accordance with evidence reported by Aghajanova [18], expression of genes encoding for Type 1 deiodinases (DIO1) was not detectable, while both transcripts of deiodinases II and III (DIO2, DIO3) were present (Fig. 1).

Effect of T3 and T4 on GL cell viability

To evaluate the presence of functional receptors in hGL cells, we studied the effect of T3 and T4 on cell viability. Cells were cultured for 48 h in the presence of 1% FBS to prevent nutrient starvation, with or without T3 and T4 at different concentrations (0.1–100 nM). Cells cultured in the presence of 5% FBS were utilized as a positive control (Ctrl).

As shown in Fig. 2, separate addition of T3 and T4 promoted cell viability in a dose-dependent manner, with T3 being more effective.

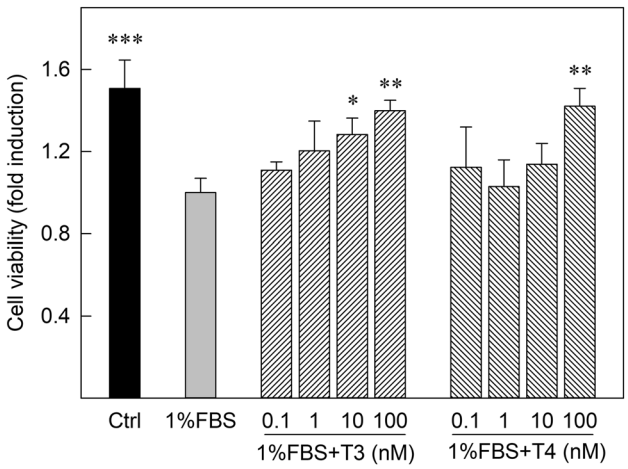

Fig. 2 Granulosa cell viability was assessed by the XTT assay. Freshly isolated hGL cells were exposed to increasing concentrations of T3 and T4 (0.1–100 nM) in complete medium supplemented with 1%FCS. 5% FCS was utilized as positive control (Ctrl). Data are presented as fold induction with 1% FBS value arbitrarily set to 1. All data are presented as the mean ± SD and are the result of at least three individual experiments. *$p < 0.05$, **$p < 0.01$. ***$p < 0.001$ vs. 1%FCS

Effect of T3 and T4 on expression of CYP19A1 (aromatase) and StAR

To evaluate the effect of T3 and T4 on the expression levels of granulosa cell-specific genes implicated in follicle steroidogenesis, such as *CYP19A1* (aromatase) and steroidogenic acute regulatory protein (*StAR)*, the mRNA extracted from hGCs cultured for 48 h in the presence of 1% FBS to prevent nutrient starvation, with or without 100 nM T3 and T4 was analysed by real-time PCR. As shown in Fig. 3, the addition of 100 nM THs significantly reduced the level of expression of *CYP191A,* while the expression of *StAR* was upregulated.

Impact of T3 and T4 on hGL cell death

To test whether thyroid hormones T3 and T4 were able to counteract serum starvation-induced cell death in hGL cells, cells were cultured in the absence of FBS (-FBS) for 48 h and simultaneously exposed to increasing concentrations of hormones. Cells cultured in the presence of 5% FBS were utilized as a positive control (Ctrl).

As shown in Fig. 4, cell death of cultured hGL cells increased significantly in response to serum starvation (-FBS). The addition of T3 or T4 counteracted cell death in a dose-dependent manner.

Since caspase-3 cleavage is a key step in apoptosis, we also analysed whether the pro-survival effect of THs was dependent on a direct regulation of this function.

Serum-deprived cells were cultured in the presence or absence of 100 nM T3 and T4 for 24 h and the presence

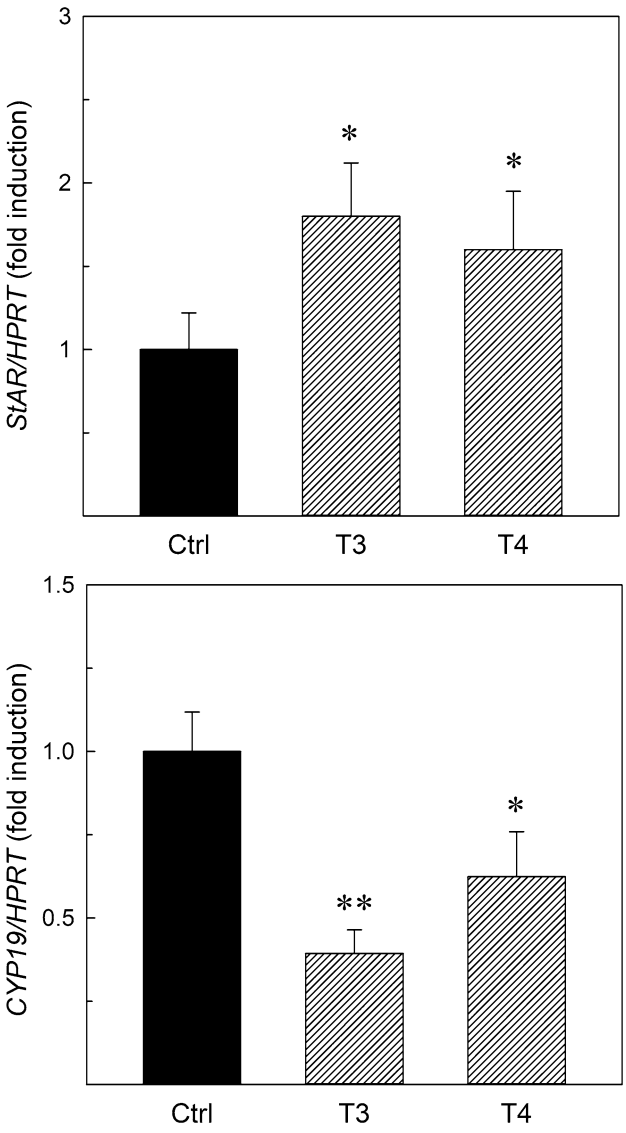

Fig. 3 Effects of T3 and T4 on *CYP-19* and *StAR* gene expression in hGL cells. Cells were exposed to T3 or T4 (100 nM) for 48 h in complete medium supplemented with 1% FCS. Extracted total RNA extracted subject to real-time PCR using specific primers indicated in Table 2. Each sample was normalized against its HPRT content. Results are expressed as fold induction with Ctrl value arbitrarily set to 1. Data are represented as the mean ± SD of three independent cell preparations. *$p < 0.05$, **$p < 0.01$ vs. Ctrl

of active caspase-3 evaluated by IF. As expected, immunofluorescence for active caspase-3 increased in cells cultured for 24 h in the absence of FBS (-FBS) but decreased after treatment with either T3 or T4 (Fig. 5).

Moreover, caspase-3 mRNA expression also decreased after stimulation with T3 or T4 at the same concentration (Fig. 6).

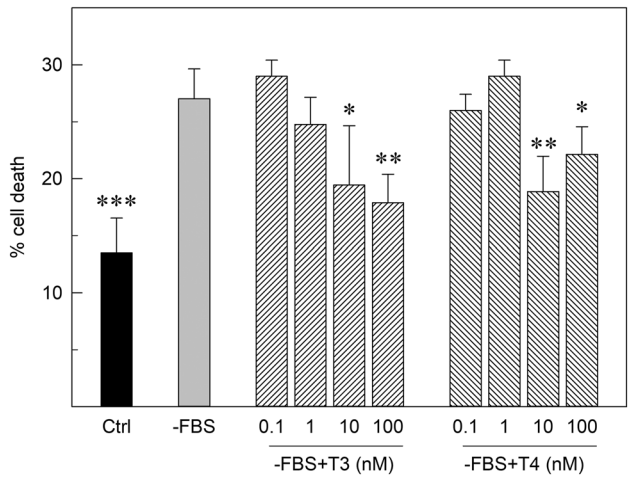

Fig. 4 Percentage of hGL cell death. Cells were cultured in the absence of serum (-FBS) and in the presence of increasing concentrations (0.1–100 nM) of T3 or T4. 5% FCS was utilized as positive control (Ctrl). Apoptotic nuclei were detected as TUNEL positive, and nuclei were counter-stained with Hoechst 33258. At least ten fields per chamber and three independent cultures were examined. The percentage of cell death was calculated as percentage of TUNEL-positive nuclei on Hoechst nuclei. *$p < 0.01$, **$p < 0.005$, ***$p < 0.001$. vs. -FCS

Effect of T3 and T4 on expression of P53 and BAX

The expression of *BAX* and *P53* mRNA was analysed by real-time PCR in cells cultured in the absence of FBS (-FBS) for 24 h and simultaneously exposed to 100 nM T3 or T4. Cells cultured in the presence of 5% FBS were comparatively assessed as a positive control (Ctrl). As shown in Fig. 7, starvation induced an increase in the expression of both genes and the addition of 100 nM THs significantly reduced their level of expression.

Effect of T3 and T4 on starvation-induced autophagy

To evaluate if autophagy could be involved in starvation-induced cell death, hGL cells were cultured in the absence of FBS for 24 h and simultaneously exposed to 100 nM T3 or T4. At the end of culture, the conversion of LC3-I to LC3-II was evaluated by western blot, the ratio between LC3-II/LC3-I being a reliable marker of autophagy. As shown in Fig. 8a, b, in starved cells (-FBS) the expression of LC3 protein significantly decreased, while the ratio LC3-II/LC3-I increased. Treatment with T3 and T4 for 24 h reverted this effect.

T3 and T4 activate AKT in hGL cells

To evaluate the effect of T3 and T4 on AKT activation, hGL cells were cultured in the absence of FBS (-FBS) and treated

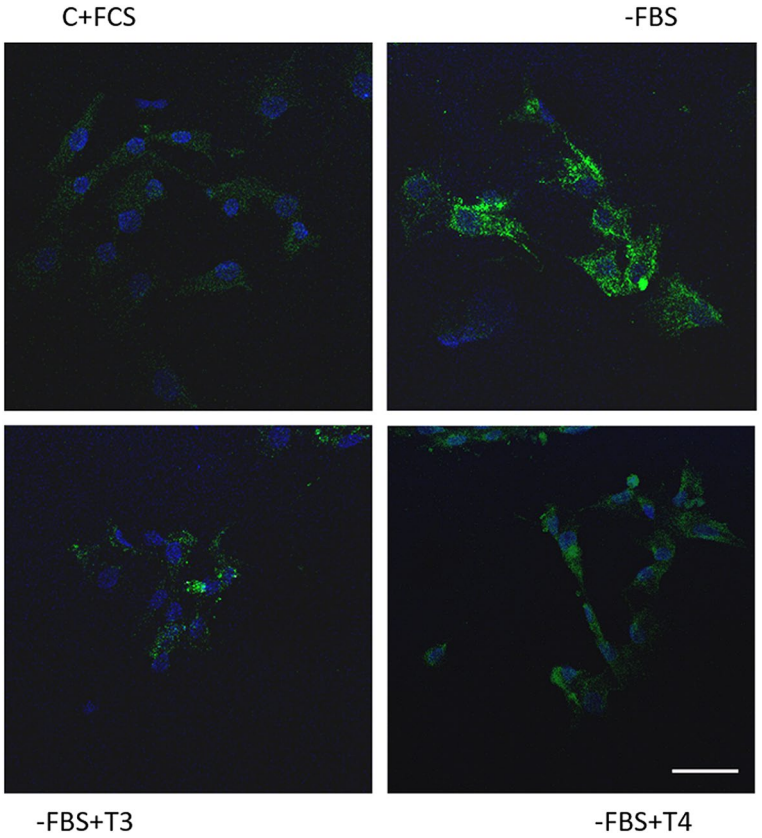

Fig. 5 Detection of cleaved caspase-3. Microphotographs of hGL cells cultured in serum-free medium for 24 h without (-FCS) or with 100 nM T3 (-FCS + T3) or T4 (-FCS + T4) and then stained with active caspase-3 antibody and counter-stained with Hoechst 33258. The photographs were taken on a Zeiss Axioplan fluorescent microscope with multiwavelength filter pairs (UV for Hoechst stain and green light for caspase-3). Note the apoptotic cells showing green staining in the cytoplasm. Scale bar = 50 µm

with 100 nM T3 or T4 for 48 h. AKT phosphorylation was evaluated by western blot. Cells cultured in the presence of 5% FBS were utilized as a positive control (Ctrl). In the absence of serum, a significant reduction of AKT phosphorylation was observed; treatment for 24 h with T3 or T4 reverted this effect (Fig. 9a, b).

Effect of TH on C2-ceramide-induced apoptosis

C2-ceramide induces hGL cell death by increasing cell apoptosis [26]. We investigated the influence of THs on survival of hGL cells after treatment with 100 µM C2-ceramide. Treatment with ceramide induced a significant decrease in cell viability (Fig. 10a) and a corresponding increase in cell death (Fig. 10b) (60%; $p < 0.005$). The addition of 100 nM T3 improved cell viability reducing significantly cell death (Fig. 10). Treatment with 100 nM T4 did not induce a statistically significant effect on cell death.

Discussion

We previously demonstrated that thyroid hormone T3 can act as a survival factor for human granulosa cells COV434 [22] and rat granulosa cells [20], thus supporting follicle growth. Moreover, we reported that the ability of T3 to protect granulosa cells from apoptosis is PI3K/AKT mediated. In the present study, we confirm the presence of the TH machinery in hGL cells and show that treatment of hGL cells with thyroid hormones induces cell survival and decreases cell death and caspase activation.

We observed that the thyroid hormones T3 and T4 caused a significant increase in the cell growth rate and a concomitant decrease in cell death. The effect of T3 was dose-dependent and statistically significant at 10 and 100 nM, while T4 showed significant action only at 100 nM. Indeed, this difference is consistent with the fact that T3 is at least ten times more effective than T4 [27].

Successful follicle development depends on a delicate balance between apoptosis and survival. In absence of the proper survival factors, endogenous apoptosis pathways intrinsic to the follicle are triggered and atresia occurs. In addition to gonadotropins, a number of other factors have been reported to affect follicular development and act directly or indirectly on follicular cell differentiation, growth or atresia [6]. Thyroid hormones T3 and T4 have been found among these factors.

In the rat ovary, THs play a synergistic effect with FSH to promote proliferation and survival of granulosa cells [28]. It has also been shown that T3 alone significantly downregulates the levels of Bcl-2-associated death promoter (Bad) mRNA, protects granulosa cells from ceramide-induced

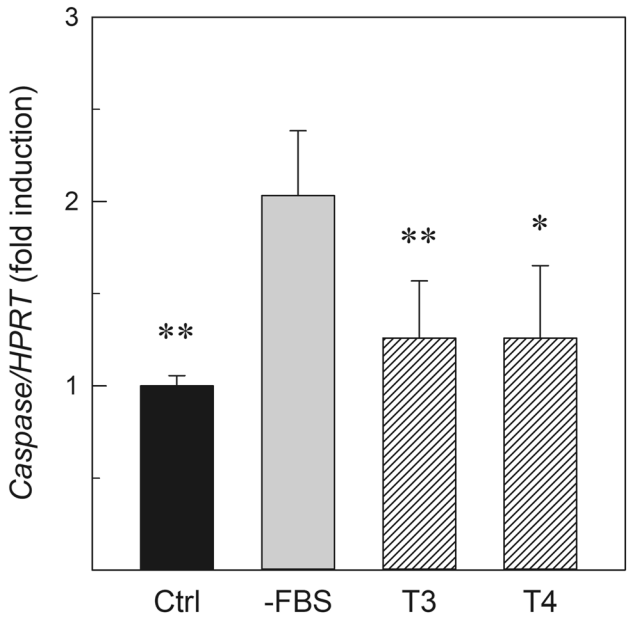

Fig. 6 Effects of T3 and T4 on *Caspase-3* gene expression in hGL cells. Cells were serum deprived (-FBS) and exposed to T3 or T4 (100 nM) for 24 h; hGL cells cultured in the presence of 5%FCS were used as positive control (Ctrl). Total RNA extracted from cells was subject to real-time PCR using specific primers indicated in Table 2. Each sample was normalized to its HPRT content. Results are expressed as fold induction with Ctrl value arbitrarily set to 1. Data are represented as the mean ± SD of three independent cell preparations. *$p < 0.05$, **$p < 0.01$ vs. -FBS

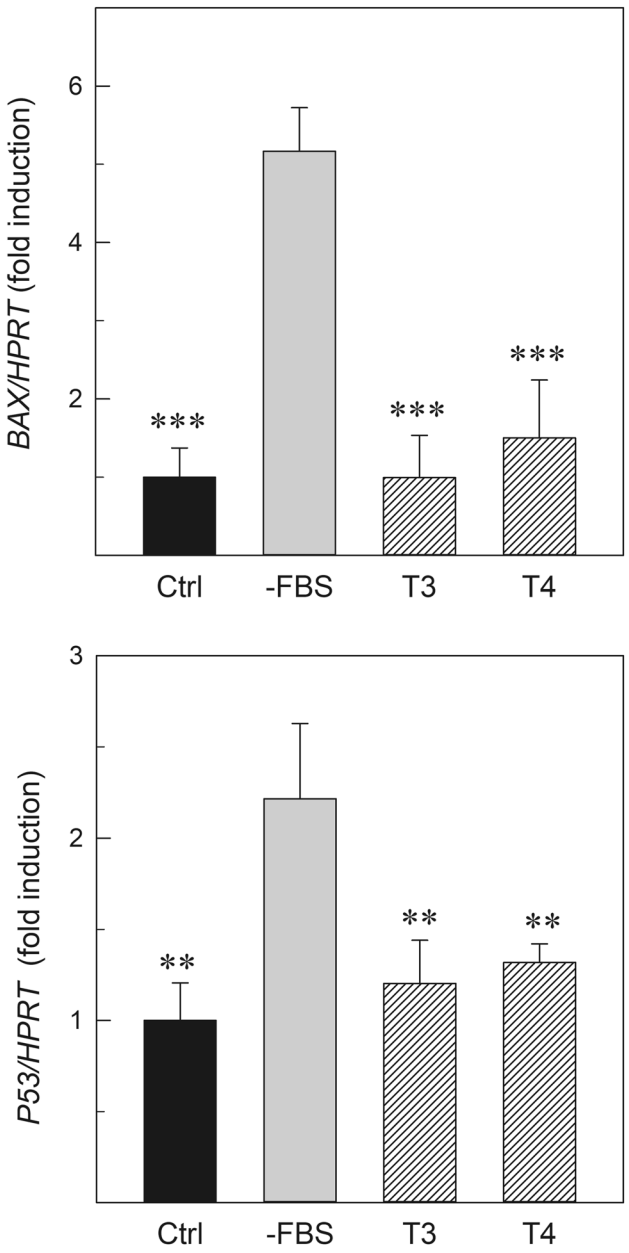

Fig. 7 Effects of T3 and T4 on *BAX* and *P53* gene expression in hGL cells. Cells were treated as described in Fig. 6. Total RNA extracted from cells was subject to real-time PCR using specific primers indicated in Table 2. Each sample was normalized to its HPRT content. Results are expressed as fold induction with 5% FCS value arbitrarily set to 1. Data are represented as the mean ± SD of three independent cell preparations. **$p < 0.01$, ***$p < 0.001$ vs. -FBS

apoptosis [10] and promotes the proliferation and survival of granulosa cells [20].

THs are found in follicular fluid of human ovarian follicles [7] and have been shown to influence ovarian follicles functions [29]. In addition, in a dose-dependent fashion, they modulate the responsiveness to FSH and hCG of human and porcine granulosa cells in terms of steroidogenic activity, cellular morphology, and expression of hCG receptors [30, 31].

Moreover, in human granulosa luteal cells, T4 alone was found to activate ERK1/2 phosphorylation [18] and stimulate TIMP 1 [32]. Moreover, T3 and T4 modulated the expression of enzymes involved in the steroidogenesis. We observed a decrease in the expression of *CYP19A1,* as already observed in the rat [33] and mouse [34] ovary. Conversely, *StAR* expression was upregulated by stimulation with T3 and T4. These data suggest that thyroid hormone action on survival can be mediated by progesterone production [35].

During follicle development in mammals, granulosa and luteal cell death is an important selective process based on apoptosis [36] and autophagy [37]. These two pathways are strictly correlated. In fact, autophagy is prominently induced in rat luteal cells during corpus luteum (CL) regression, while autophagosome accumulation induces apoptotic cell death [37].

Serum deprivation, or the absence of growth factors or hormones such as FSH or hCG, can induce cell death by inhibiting the PI3K/AKT pathway [38] and inducing pro-apoptotic molecules.

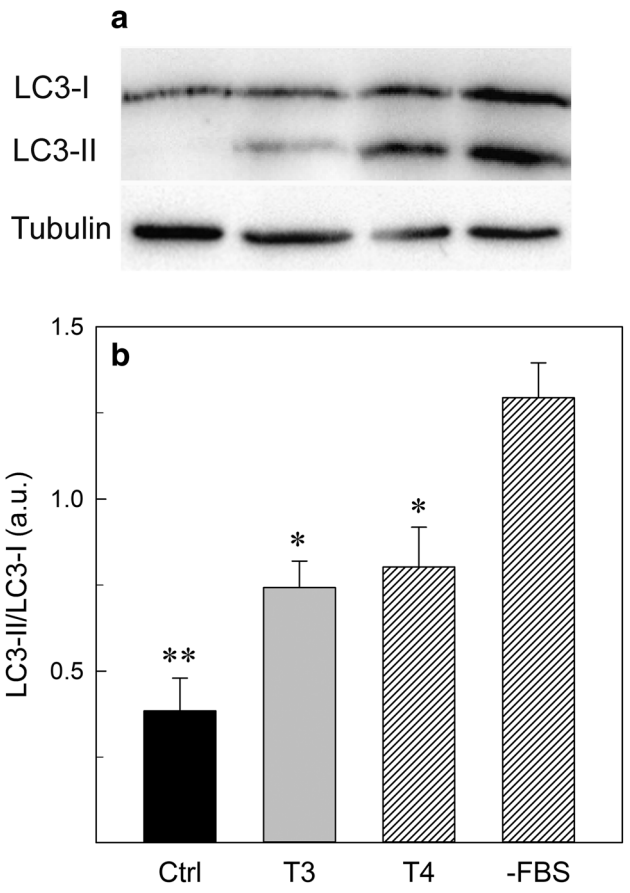

Fig. 8 Western blot analysis for LC3-I and -II. Cells, obtained from pooled follicles of individual patients, were incubated for 24 h in serum-free medium in the presence of 100 nM T3 or T4. Western blot analyses were performed as described in the "Materials and methods". **a** Representative Western blot showing two bands corresponding to the LC3 I and II. In the absence of serum (Ctrl), the band corresponding to the proenzyme significantly decreased, whereas the addition of T3 and T4 maintained higher levels of inactive enzyme. **b** Ratio values normalized by their respective tubulin values. Each bar represents the mean ± SD of three separate experiments and is expressed as arbitrary units (a.u.). *$p < 0.01$, **$p < 0.001$ vs. -FBS

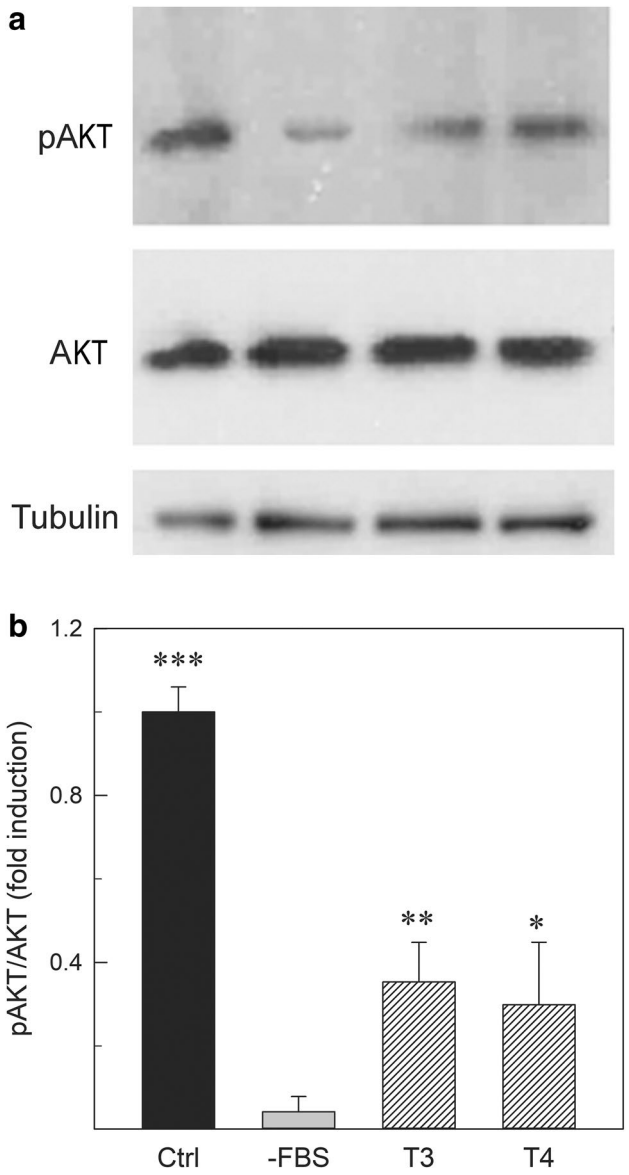

Fig. 9 Western Blot analysis for pAKT/AKT. Western blot analyses were performed as described in the Materials and Methods, and a specific band corresponding to the phosphorylated AKT (Ser 473) was detected. Cells were serum deprived (Ctrl) and exposed or not to T3 and T4. Tubulin was analysed as a control for gel loading. **a** Representative Western blot. **b** Densitometric absorbance values from three separate experiments were averaged (± SD) after they had been normalized to that of Tubulin for equal loading. Data are presented in the histogram as fold induction with Ctrl value arbitrarily set to 1. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. -FBS

Caspase-3 is the most characterized effector caspase required for granulosa cell apoptosis, since granulosa cells from caspase-3 null ovaries do not undergo apoptosis in response to serum starvation [39]. Caspase-3 is the downstream regulator of many other effectors, including BCL-2 family proteins. Among these, BAX, a protein that acts as an antagonist of BCL-2, is expressed both in the oocyte and granulosa cells, and increases its expression in granulosa cells of atretic follicles compared to healthy follicles [2]. Moreover, in ovaries of young adult female $Bax^{-/-}$ mice, more primordial follicles are found compared with those of their wild-type sisters, with a maintenance of ovarian reserve in advanced chronological age [40].

Conclusions

In our study, we have demonstrated that THs counteract human ovarian granulosa cell apoptosis induced by nutrient deprivation. This effect is achieved by inhibiting the apoptosis signalling pathway of BAX and caspase-3, while maintaining active the PI3K/AKT pathway. In fact, the increase

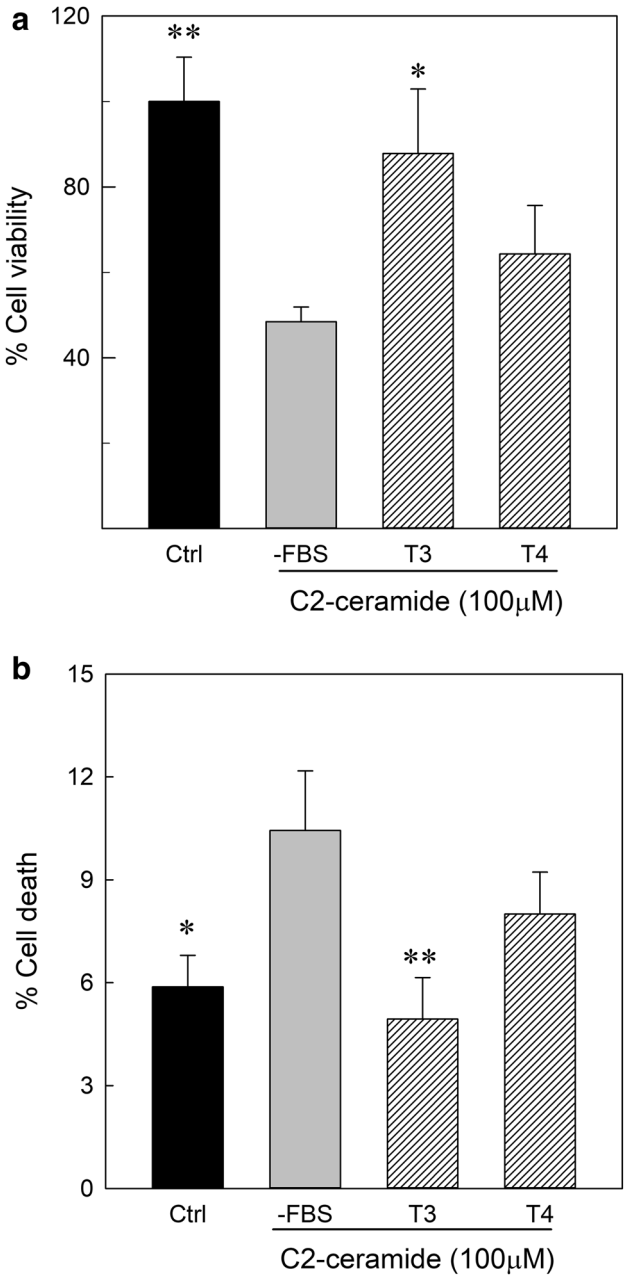

Fig. 10 **a** Granulosa cell viability was assessed by the XTT assay. Cells were exposed to C2-ceramide in complete medium without FCS in the absence (-FCS) or in the presence of 100 nM T3 or T4. 5% FCS was utilized as positive control (Ctrl). Data are presented as percentage of the OD (570 and 630 nm) with Ctrl value arbitrarily set to 100. All data are presented as the mean ± SD and are the result of at least three individual experiments. *$p < 0.05$, **$p < 0.01$ vs. -FCS. **b** Percentage of hGL cell death. Cells were cultured as described above. Apoptotic nuclei were detected as TUNEL positive, and nuclei were counter-stained with Hoechst. At least ten fields per chamber and three independent cultures were examined. The percentage of cell death was calculated as percentage of TUNEL-positive nuclei on Hoechst nuclei. *$p < 0.01$, ** $p < 0.005$, vs. -FCS

of cleaved caspase-3 and BAX observed in the absence of FCS is downregulated by THs treatment. In agreement with data presented by Herr et al. [41], our data suggest that the inhibition of apoptosis in hGL cells is accompanied by a decreased expression of P53. Moreover, the induction of autophagy indicated by the conversion of the LC3-I into its active form LC3-II caused by serum deprivation appears partially reverted by THs.

Overall, these results are consistent with the notion that T3 acts as an anti-apoptotic factor, as previously demonstrated in our previous work and in various cell systems [20]. Moreover, supported by the analysis of pAKT levels, our experiments suggest a positive action of T3 on granulosa cells through activation of PI3K, a widely recognized target of this hormone in many tissues [42].

Funding This study was supported by grants from Sapienza University of Rome, Ateneo Federato 2017–2018 (to R. C.) (Grand No.: Finanziamenti Ateneo per la ricerca 2018).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The research was carried out according to the principles of the Declaration of Helsinki and was approved by the local institutional review board.

Informed consent Informed consent was obtained from all patients.

References

1. McGee EA, Hsueh AJ (2000) Initial and cyclic recruitment of ovarian follicles. Endocr Rev 21(2):200–214. https://doi.org/10.1210/edrv.21.2.0394
2. Matsuda F, Inoue N, Manabe N et al (2012) Follicular growth and atresia in mammalian ovaries: regulation by survival and death of granulosa cells. J Reprod Dev 58(1):44–50. https://doi.org/10.1262/jrd.2011-012
3. Jiang JY, Cheung CK, Wang Y et al (2003) Regulation of cell death and cell survival gene expression during ovarian follicular development and atresia. Front Biosci 8:d222–d237. https://doi.org/10.2741/949
4. Palumbo A, Yeh J (1995) Apoptosis as a basic mechanism in the ovarian cycle: follicular atresia and luteal regression. J Soc Gynecol Investig 2(3):565–573. https://doi.org/10.1177/107155769500200310
5. Tilly JL, Kowalski KI, Johnson AL et al (1991) Involvement of apoptosis in ovarian follicular atresia and postovulatory regression. Endocrinology 129(5):2799–2801. https://doi.org/10.1210/endo-129-5-2799
6. Canipari R (2000) Oocyte–granulosa cell interactions. Hum Reprod Update 6(3):279–289. https://doi.org/10.1093/humupd/6.3.279
7. Wakim AN, Polizotto SL, Buffo MJ et al (1993) Thyroid hormones in human follicular fluid and thyroid hormone receptors in

human granulosa cells. Fertil Steril 59(6):1187–1190. https://doi.org/10.1016/S0015-0282(16)55974-3
8. Pascual A, Aranda A (2013) Thyroid hormone receptors, cell growth and differentiation. Biochim Biophys Acta 1830(7):3908–3916. https://doi.org/10.1016/j.bbagen.2012.03.012
9. Zhang C, Wang X, Wang Z et al (2013) Effect of different culture systems and 3, 5, 3′-triiodothyronine/follicle-stimulating hormone on preantral follicle development in mice. PLoS One 8(4):e61947. https://doi.org/10.1371/journal.pone.0061947
10. Zhang C, Guo L, Zhu B et al (2013) Effects of 3, 5, 3′-triiodothyronine (t3) and follicle stimulating hormone on apoptosis and proliferation of rat ovarian granulosa cells. Chin J Physiol 56(5):298–305. https://doi.org/10.4077/CJP.2013.BAB186
11. Fedail JS, Zheng K, Wei Q et al (2014) Roles of thyroid hormones in follicular development in the ovary of neonatal and immature rats. Endocrine 46(3):594–604. https://doi.org/10.1007/s12020-013-0092-y
12. Poppe K, Velkeniers B, Glinoer D (2007) Thyroid disease and female reproduction. Clin Endocrinol (Oxf) 66(3):309–321. https://doi.org/10.1111/j.1365-2265.2007.02752.x
13. Dittrich R, Beckmann MW, Oppelt PG et al (2011) Thyroid hormone receptors and reproduction. J Reprod Immunol 90(1):58–66. https://doi.org/10.1016/j.jri.2011.02.009
14. Mintziori G, Anagnostis P, Toulis KA et al (2012) Thyroid diseases and female reproduction. Minerva Med 103(1):47–62
15. Cho MK (2015) Thyroid dysfunction and subfertility. Clin Exp Reprod Med 42(4):131–135. https://doi.org/10.5653/cerm.2015.42.4.131
16. Vissenberg R, Manders VD, Mastenbroek S et al (2015) Pathophysiological aspects of thyroid hormone disorders/thyroid peroxidase autoantibodies and reproduction. Hum Reprod Update 21(3):378–387. https://doi.org/10.1093/humupd/dmv004
17. Duarte-Guterman P, Navarro-Martin L, Trudeau VL (2014) Mechanisms of crosstalk between endocrine systems: regulation of sex steroid hormone synthesis and action by thyroid hormones. Gen Comp Endocrinol 203:69–85. https://doi.org/10.1016/j.ygcen.2014.03.015
18. Aghajanova L, Lindeberg M, Carlsson IB et al (2009) Receptors for thyroid-stimulating hormone and thyroid hormones in human ovarian tissue. Reprod Biomed Online 18(3):337–347. https://doi.org/10.1016/S1472-6483(10)60091-0
19. Kinne A, Schulein R, Krause G (2011) Primary and secondary thyroid hormone transporters. Thyroid Res 4(Suppl 1):S7. https://doi.org/10.1186/1756-6614-4-S1-S7
20. Canipari R, Mangialardo C, Di Paolo V et al (2018) Thyroid hormones act as mitogenic and pro survival factors in rat ovarian follicles. J Endocrinol Invest 42(3):271–282. https://doi.org/10.1007/s40618-018-0912-2
21. Verga-Falzacappa C, Timperi E, Bucci B et al (2012) T(3) preserves ovarian granulosa cells from chemotherapy-induced apoptosis. J Endocrinol 215(2):281–289. https://doi.org/10.1530/JOE-12-0153
22. Verga-Falzacappa C, Mangialardo C, Patriarca V et al (2009) Thyroid hormones induce cell proliferation and survival in ovarian granulosa cells COV434. J Cell Physiol 221(1):242–253. https://doi.org/10.1002/jcp.21849
23. Morelli MB, Barberi M, Gambardella A et al (2008) Characterization, expression, and functional activity of pituitary adenylate cyclase-activating polypeptide and its receptors in human granulosa-luteal cells. J Clin Endocrinol Metab 93(12):4924–4932. https://doi.org/10.1210/jc.2007-2621
24. Innocenti F, Cerquetti L, Pezzilli S et al (2017) Effect of mitotane on mouse ovarian follicle development and fertility. J Endocrinol 234(1):29–39. https://doi.org/10.1530/JOE-17-0203
25. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685
26. Perks CM, Newcomb PV, Grohmann M et al (2003) Prolactin acts as a potent survival factor against C2-ceramide-induced apoptosis in human granulosa cells. Hum Reprod 18(12):2672–2677. https://doi.org/10.1093/humrep/deg496
27. Samuels HH, Stanley F, Casanova J (1979) Relationship of receptor affinity to the modulation of thyroid hormone nuclear receptor levels and growth hormone synthesis by L-triiodothyronine and iodothyronine analogues in cultured GH1 cells. J Clin Invest 63(6):1229–1240. https://doi.org/10.1172/JCI109418
28. Kobayashi N, Orisaka M, Cao M et al (2009) Growth differentiation factor-9 mediates follicle-stimulating hormone-thyroid hormone interaction in the regulation of rat preantral follicular development. Endocrinology 150(12):5566–5574. https://doi.org/10.1210/en.2009-0262
29. Fitko R, Kucharski J, Szlezyngier B et al (1996) The concentration of GnRH in hypothalamus, LH and FSH in pituitary, LH, PRL and sex steroids in peripheral and ovarian venous plasma of hypo- and hyperthyroid, cysts-bearing gilts. Anim Reprod Sci 45(1–2):123–138. https://doi.org/10.1016/s0378-4320(96)01568-0
30. Maruo T, Hayashi M, Matsuo H et al (1987) The role of thyroid hormone as a biological amplifier of the actions of follicle-stimulating hormone in the functional differentiation of cultured porcine granulosa cells. Endocrinology 121(4):1233–1241. https://doi.org/10.1210/endo-121-4-1233
31. Goldman S, Dirnfeld M, Abramovici H et al (1993) Triiodothyronine (T3) modulates hCG-regulated progesterone secretion, cAMP accumulation and DNA content in cultured human luteinized granulosa cells. Mol Cell Endocrinol 96(1–2):125–131. https://doi.org/10.1210/jcem.82.6.3997
32. Goldman S, Dirnfeld M, Abramovici H et al (1997) Triiodothyronine and follicle-stimulating hormone, alone and additively together, stimulate production of the tissue inhibitor of metalloproteinases-1 in cultured human luteinized granulosa cells. J Clin Endocrinol Metab 82(6):1869–1873. https://doi.org/10.1210/jcem.82.6.3997
33. Hatsuta M, Tamura K, Shimizu Y et al (2004) Effect of thyroid hormone on CYP19 expression in ovarian granulosa cells from gonadotropin-treated immature rats. J Pharmacol Sci 94(4):420–425. https://doi.org/10.1254/jphs.94.420
34. Cecconi S, Rucci N, Scaldaferri ML et al (1999) Thyroid hormone effects on mouse oocyte maturation and granulosa cell aromatase activity. Endocrinology 140(4):1783–1788. https://doi.org/10.1210/endo.140.4.6635
35. Hirata R, Hojo T, Sano M et al (2015) Potential role of hCG in apoptosis of human luteinized granulosa cells. J Reprod Dev 61(1):67–73. https://doi.org/10.1262/jrd.2014-115
36. Kim WG, Zhu X, Kim DW et al (2013) Reactivation of the silenced thyroid hormone receptor beta gene expression delays thyroid tumor progression. Endocrinology 154(1):25–35. https://doi.org/10.1210/en.2012
37. Choi J, Jo M, Lee E et al (2011) Induction of apoptotic cell death via accumulation of autophagosomes in rat granulosa cells. Fertil Steril 95(4):1482–1486. https://doi.org/10.1016/j.fertnstert.2010.06.006
38. Choi J, Jo M, Lee E et al (2014) AKT is involved in granulosa cell autophagy regulation via mTOR signaling during rat follicular development and atresia. Reproduction 147(1):73–80. https://doi.org/10.1530/REP-13-0386
39. Matikainen T, Perez GI, Zheng TS et al (2001) Caspase-3 gene knockout defines cell lineage specificity for programmed cell death signaling in the ovary. Endocrinology 142(6):2468–2480. https://doi.org/10.1210/endo.142.6.8078
40. Perez GI, Robles R, Knudson CM et al (1999) Prolongation of ovarian lifespan into advanced chronological age by Bax-deficiency. Nat Genet 21(2):200–203. https://doi.org/10.1038/5985

41. Herr D, Keck C, Tempfer C et al (2004) Chorionic gonadotropin regulates the transcript level of VHL, p53, and HIF-2alpha in human granulosa lutein cells. Mol Reprod Dev 69(4):397–401. https://doi.org/10.1002/mrd.20137
42. Davis PJ, Leonard JL, Davis FB (2008) Mechanisms of non-genomic actions of thyroid hormone. Front Neuroendocrinol 29(2):211–218. https://doi.org/10.1016/j.yfrne.2007.09.003