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REPRODUCTIVE PHYSIOLOGY AND DISEASE

Co-culture of human cryopreserved fragmented ovarian tissue with theca progenitor cells derived from theca stem cells

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Abstract

Purpose Despite the significant advances in the in vitro development of human primordial follicles, it is still a challenging approach with great potential for improvements. Therefore, the present study aimed to investigate the effect of a feeder layer of human theca progenitor cells (hTPCs) on the development of primordial follicles embedded in human ovarian tissue.

Methods Fragments of frozen-thawed ovarian tissue were activated using the vanadate-derivative dipotassium bisperoxo (5-hydroxy-pyridine-2-carboxylic) oxovanadate (V) and kit ligand for 24 h. Then, the specimens were divided into the co-culture and mono-culture groups and were cultured with and without a hTPC feeder layer for 6 days, respectively. Afterward, the follicles were counted and classified, and the hormone levels and expression levels of apoptosis- and folliculogenesis-related genes were assessed.

Results Both culture groups showed significant follicle growth ($P < 0.05$). However, the co-culture group had a significantly higher number of growing follicles compared to the other group ($P < 0.05$). Moreover, the expression levels of *ZP1*, *ZP2*, *ZP3*, *BMP-7*, *AMH*, and *GDF9* were significantly higher in the co-culture group compared to the other group ($P < 0.05$), while the expression levels of *P53* and *CASP3* were significantly lower ($P < 0.05$). Also, the concentrations of estradiol, progesterone, testosterone, and androstenedione were significantly higher in the co-culture group compared to the other group ($P < 0.05$).

Conclusion The present study results provided novel evidence on the direct role of hTPCs in the growth and development of human primordial follicles. However, there is a need for future studies to illustrate the underlying mechanisms.

Keywords Human primordial follicles · Co-culture system · Human theca progenitor cells · Human ovarian cryopreservation · Ovarian cortical fragments

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Introduction

The use of in vitro culture for developing human primordial follicles has several potential applications, such as fertility preservation [1]. Therefore, many researchers have tried to optimize the current methods used in in vitro culture systems. For example, Liu et al. treated the strips of human ovarian cortical tissue with PTEN inhibitor for 24 h, leading to primordial follicle activation and mature oocyte formation following xenotransplantation to immunodeficient mice [2]. However, another study showed that PTEN inhibitors might compromise the development of growing follicles despite their effects on the activation of the primordial follicles [3]. Therefore, an alternative approach using mTOR-PI3K stimulators was suggested by Sun et al., which increased the number of growing human follicles in the ovarian cortex [4]. Similarly, another study reported that treating the ovarian fragments of POI patients with an Akt stimulator promoted follicular growth, leading to live births following IVF-ET [5]. Also, Xu et al. reported the most promising results in this area on human pre-antral follicles, obtaining mature oocytes with normal morphology from in vitro culture of primordial follicles [6].

According to evidence, the use of feeder layers secreting different growth factors can stimulate the growth of follicles, particularly during the early phases of follicle growth [7]. A study by Piton et al. reported several critical factors for early follicle development, including extracellular matrix, Leukemia inhibitory factor (LIF), basic fibroblast growth factor (bFGF), kit ligand (KL) or stem cell factor, insulin-like growth factor (IGF), bone morphogenetic proteins (BMPs), and activin A [8]. Moreover, Wu et al. showed that the extracellular matrix and cytokines secreted by fibroblasts in a 3D alginate matrix may create a desirable microenvironment for the growth of primordial/primary follicles. Also, it seems that the unidirectional paracrine signaling from human dermal fibroblasts (HDFs) to follicles is the underlying mechanism of the stimulatory effect of HDFs on follicular growth [9]. A study reported the improving effect of co-culture with hUC-MSCs on the activation of primordial follicles in mouse ovaries [10]. Similarly, Mi et al. reported that the secretome of hUC-MSCs could activate the primordial follicles under both in vitro and in vivo conditions, with the hepatocyte growth factor (HGF) secreted from hUC-MSCs playing a potential role in this process [11]. It has been shown that the HGF produced by theca cells in the ovary can trigger the proliferation and growth of granulosa cells and steroidogenesis [12]. Moreover, it promotes the activation of primordial follicles via kit ligand [11]. Also, Tingen et al. created a co-culture system for follicles that simulated the in vivo environment of the ovary by providing a feeder layer of theca cells and macrophages [7], showing that the co-culture of small follicles with stroma could improve their growth and survival [7].

As the activated follicles are thought to recruit the precursor theca cells from the stromal layer that surrounds the granulosa cells and oocyte, it has been hypothesized that the theca progenitor cells in the stroma are involved in early follicle development. Therefore, the present study aimed to investigate the effect of a feeder layer of human theca progenitor cells (hTPCs), PTEN inhibitors, and Akt stimulators on the development of primordial follicles in human ovarian tissue.

Materials and methods

Study design

The present study was approved by the Ethics Committee of Royan Institute (ethics number: EC/93/1059). Moreover, all the study participants gave informed consent for participation.

The frozen ovarian fragments obtained from 6 patients were thawed, cut into $1 \times 1 \times 0.5$ mm pieces, and divided into 3 groups: the non-culture, co-culture, and mono-culture groups. Each group included 72 ovarian tissue fragments from 6 patients (12 fragments from each patient).

The samples of the co-culture and mono-culture groups underwent incubation for 24 h in 15 mM of bpV (HOpic; CAYMAN Chemical Company, Ann Arbor, USA) and 100 µg/ml of KL at a temperature of 37° C in humidified air with 5% CO_2. Afterward, the media were removed, and the samples of the co-culture group were cultured for 6 days with hTPCs based on the instructions of the previous studies [13]. Moreover, the samples of the mono-culture group were cultured for the same duration without the hTPCs. Afterward, the tissue samples of both groups were processed for histological and gene expression analyses. Also, the levels of the related hormones were assessed in the culture media (Fig. 1).

On the other hand, the samples of the non-culture group were not cultured and were destined for histological and gene expression assays immediately after thawing.

hTPC preparation

Small antral follicles, sized 3–5 mm, were collected from 2 patients. The first patient was a 19-year-old girl referred for ovarian cryopreservation due to malignancy of the right ovary. The follicles were obtained from the left ovary, which was healthy without any sign of malignancy. Moreover, the

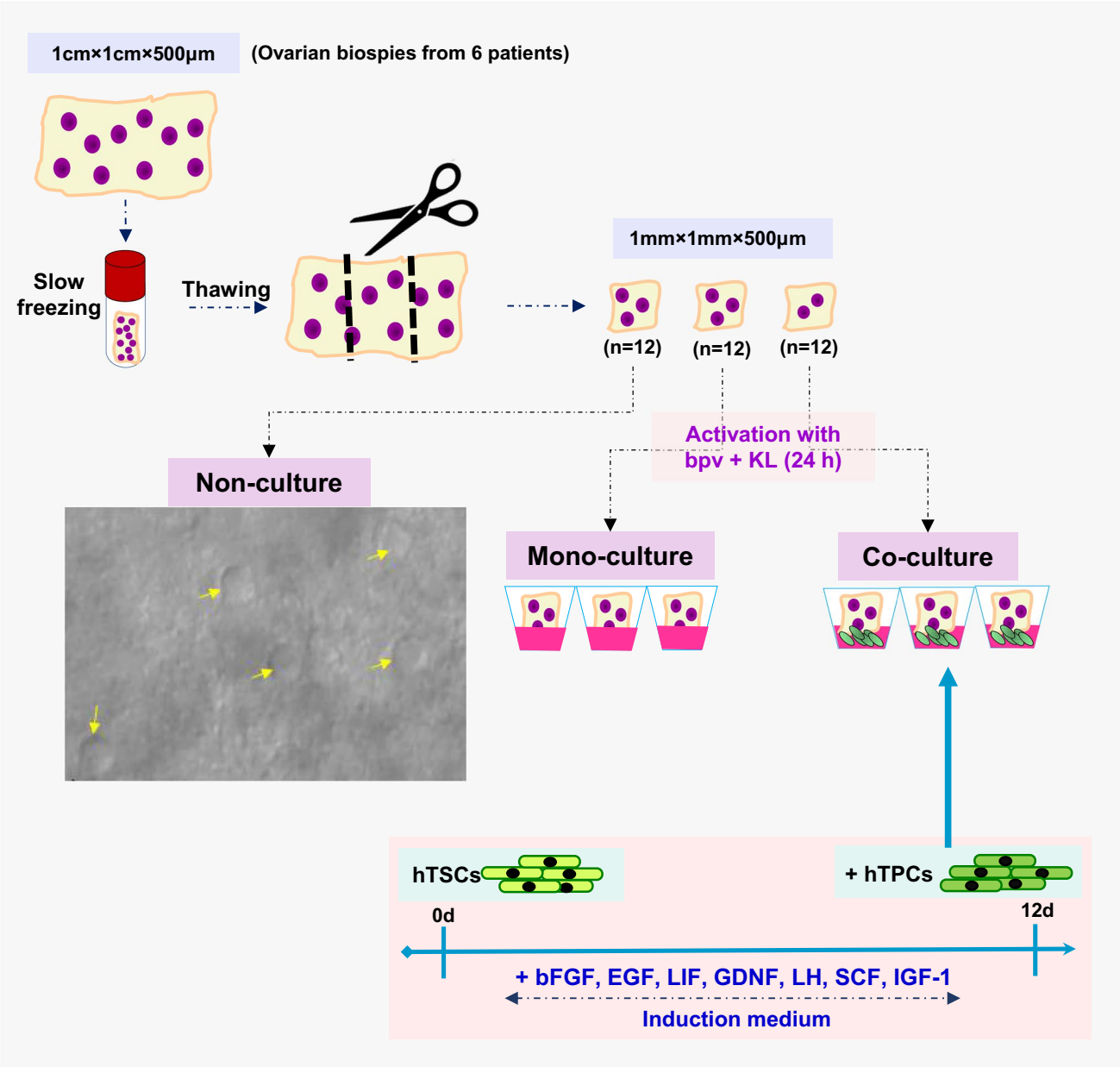

Fig. 1 Study design. Activation and development of primordial follicles in the ovarian tissue fragments of 6 patients in the mono-culture, co-culture, and non-culture groups. Ovarian critical tissue was cut into sections with a thickness of 500 μm using a tissue slicer. Yellow arrows show the primordial follicles in a sample from the non-culture group

second patient was a 36-year-old woman who underwent a laparoscopic procedure for a non-malignant gynecological condition not related to ovaries.

The hTPCs were prepared based on the method suggested by the previous studies [14, 15]. A total of 4 follicles were placed in Dulbecco's modified eagle's medium F12 (DMEM/F12; Gibco, Paisley, UK). Then, they were cut in half under a stereomicroscope, and granulosa cells and oocytes were removed by scraping. The remaining tissue containing cells was dissected into small pieces and incubated with 0.5% collagenase type I (Sigma Chemical Co., St. Louis, MO, USA) for 45 min in a water bath at 37 °C. Afterward, the collagenase activity was neutralized using a medium containing 10% fetal bovine albumin (FBS; Gibco). The cell suspension was passed through 100- and 40-μm filters (BD, Falcon, Mexico). Afterward, the cells were cultured in DMEM/F12 supplemented with 10% FBS, 2 mM glutamine (Gibco), 100 U/mL penicillin G (Sigma Chemical Co.), 100 μg/mL streptomycin (Sigma Chemical Co.), 20 ng/ml epidermal growth factor (EGF; Royan

Biotech, Tehran, Iran), 10 ng/ml bFGF (Royan Biotech), 20 ng/ml LIF (Royan Biotech), and 10 ng/ml glial-derived neurotrophic factor (GDNF; Royan Biotech) at 37 °C in a 5% CO_2 incubator. Moreover, the human Theca Stem Cells (hTSCs) were induced to differentiate into hTPCs by incubation in the DMEM/F12 medium supplemented with 20 ng/ml EGF, 10 ng/ml bFGF, 20 ng/ml LIF, 10 ng/ml GDNF, 110 mIU human chorionic gonadotrophin (hCG; Choriomon, IBSA, Luzern, Switzerland), 100 ng IGF-1 (Royan Biotech), and 100 ng/ml KL (Sigma Chemical Co.) for 12 days.

Preparation of hTPC feeder layer

A total number of 6×10^5 hTPCs were cultured in 300 µl of α-MEM medium (Gibco) supplemented with 10% FBS in humidified air containing 5% CO_2 at 37 °C until the feeder layers were formed. Afterward, the feeder layers underwent co-culture with ovarian tissue fragments.

Ovarian cortical tissue biopsy and preparation

The ovarian cortical tissues were obtained from 6 patients aged 27–38 years during elective Caesarean sections and were immediately transferred to the laboratory in PBS (Gibco) at a temperature of 4 °C. Afterward, the tissue samples were placed in McCoy medium (Gibco) supplemented with 3 mg/ml human serum albumin (HSA; Biotest Dreieich, Germany), 75 mg/ml penicillin G (Sigma Chemical Co.), and 50 mg/ml streptomycin (Sigma Chemical Co.) to remove the medulla and uppermost cortex. The remaining ovarian cortical tissue was sliced into sections with a thickness of 500 µm (Thomas Stadie Riggs, Thomas Scientific, Swedesboro, New Jersey, USA) to allow examination under the stereomicroscope. Then, the fragments with at least four primordial/transitional follicles observed using a stereomicroscope were selected for the present study (Fig. 1) and were cut into 1 × 1 cm squares.

Afterward, they were frozen and stored in liquid nitrogen for at least 1 month.

Ovarian tissue freezing and thawing

The ovarian cortical fragments were frozen using the method described by Dalman et al. [16]. According to the method, the tissue slices were immersed in a cryoprotective solution containing 2% HSA and 10% dimethyl sulfoxide (Sigma Chemical Co.) in McCoy medium at a temperature of 4 °C. Afterward, the samples were transferred to 2-mL cryovials containing the same solution. The cryovials were cooled down from 0 to − 8 °C at a rate of 2 °C/min. Afterward, manual seeding was performed. Then, the cryovial was cooled down to − 40° C at a rate of 0.3° C/min and − 70° C at a rate of 5 °C/min. They were kept at − 70° C for 6 min. Then, they were transferred to liquid nitrogen (− 196 °C).

For thawing, the cryovials were exposed to room temperature for 2 min. Afterward, they were kept in a water bath at 37° C until the ice was completely melted. Then, the samples were washed thrice (5 min each) in McCoy medium at room temperature to remove the cryoprotectant.

Method of *in vitro* culture of follicles

According to Labrune et al. [17], early follicle culture should be performed using small-sized tissues with the lowest possible thickness. Therefore, frozen-thawed ovarian fragments were cut into 1 × 1 mm squares using a pair of scissors. Moreover, each piece was evaluated using a dissecting microscope for the presence of follicles, and only the fragments containing follicles with 40-µm diameter (primordial/transitory follicles) in the histological analysis were selected for the study. Finally, a total of 12 tissue samples from each patient were selected for each group ($n = 72$ samples for each group), and each sample was placed in a well of 48-well plates (TPP, Trasadingen, Switzerland) and cultured at 37 °C in humidified air with 5% CO_2 (Fig. 1). The culture media included 500 µl of α-MEM medium supplemented with 3 mM glutamine, 0.1% HSA, 0.1 mg/ml penicillin G, 0.1 mg/ml streptomycin, 1% ITS, and 50 mg/ml ascorbic acid (Sigma Chemical Co.). Also, 15 mM of bpV (HOpic) and 100 µg/ml KL were added to the medium in the first 24 h of the culture [18].

Finally, the culture media were removed, and tissue fragments of the co-culture and mono-culture groups were cultured for 6 days with and without the feeder layer of hTPCs, respectively. Moreover, half of the medium was replaced every other day.

Tissue processing for histological investigations

All the ovarian tissue fragments were placed in 2% agarose (Sigma Chemical Co.) and fixed in 10% formalin. Afterward, the fragments were dehydrated in ascending ethanol concentrations (Merck, Darmstadt, Germany). Then, each cortical fragment was embedded in paraffin, sliced into serial sections with 6-µm thickness, mounted on slides, and left to dry overnight. Afterward, the samples underwent staining with hematoxylin (Baharafshan, Tehran, Iran) and eosin (Sigma Chemical Co.). Finally, the follicles were counted and classified in all tissue sections.

Follicle classification

The follicles with normal morphology of oocyte and granulosa cells were identified and classified as follows: (1) primordial and transitional follicles—a complete or incomplete

single layer of flattened granulosa cells surrounding the oocyte; (2) primary follicles—a single layer of cuboidal granulosa cells surrounding the oocyte; (3) secondary follicles—two or more layers of cuboidal granulosa cells surrounding the oocyte; and (4) pre-antral follicles—the follicles without an antrum.

Follicles with irregular oolema and zona pellucida, extended subzonal space, granulosa cells with pyknotic nuclei, and a shrunken oocyte were considered degenerated and were not counted or classified. Moreover, only the follicles with clear oocyte nuclei were counted to avoid repetition in follicle numbers.

Gene expression level assessment using qRT-PCR

The RNAs were extracted from the tissue sections using TRIzol (Thermo Fisher Scientific, Carlsbad, USA) and were converted into cDNA using the QuantiTect Whole Transcriptome Kit (QIAGEN, Hilden, Germany). The oligonucleotide primers used are listed in Table 1. Moreover, expression levels of folliculogenesis marker genes (*ZP1*, *ZP2*, *ZP3*, *GDF9*, *LIF*, *AMH*, *BMP4*, and *BMP7*) and apoptotic genes (*BAX*, *CASP3*, *BCL2*, *P53*) were assessed using the qRT-PCR, while the expression level of *GAPDH* was used as an internal control. The data included at least six independent replicates, and the $2^{-\Delta\Delta Ct}$ method was used to quantify the relative mRNA levels using the following formulas:

$$\Delta CT = Ct_{target\ gene} - Ct_{GAPDH}$$

$$\Delta\Delta Ct = \Delta Ct_{sample} - \Delta Ct_{control}$$

Hormone level assessment using ELISA

The researchers cultured the hTPCs alone without the ovarian fragments in order to determine the difference in the hormone levels between the co-culture media and the media from the only-hTPC culture. The culture media of the co-culture, mono-culture, and only-hTPC groups were collected on days 0, 3, and 6 of culture and assessed for the levels of hormones estradiol, dehydroepiandrosterone (DHEA), androstenedione, testosterone, and progesterone using the ELISA kits (Roche, Basel, Switzerland). It should be noted that the culture media for the samples of each patient were evaluated separately in each group.

The samples were placed in the wells of the plates. Then, the standard solution was added. The samples were exposed to the enzyme and then the biotin reagent. Following the incubation duration, the wells were decanted and washed

Table 1 List of markers used in this study, including symbol, gene name, accession number, primer sequences, annealing temperature, and PCR product size

Gene symbol	Sequence 5´……3´	Size (bp)	Annealing temperature (°C)	Accession number
BMP4	F: GGCCAGCATGTCAGGATTAG R: CACATCGCTGAAGTCCACAT	216	60	NM_001347913.1
BMP7	F: ACGCTACCACCATCGAGAG R:AAGAGATCCGATTCCCTGCC	180	60	NM_001719.2
AMH	F: CTACACCTGGAGGAAGTGAC R:GGTCCACCGCTAACACC	208	60	NM_000479.4
ZP1	F: ACACCTTTCCAGTCGCACTA R:GCAGAACAAGTAAACCAGTCCT	102	60	NM_207341.3
ZP2	F: AGCATTGAGGTTGGTGATGG R: ACTGTTACCTTGCACATAGTGA	153	60	NM_001290104.1
ZP3	F:GCCAGATACACTCCAGTTCAC R:AGATGTCAGCCGAGCCTT	185	60	NM_001110354.1
GDF9	F:TAGAAGTCACCTCTACAACACTG R:GGTAGTAATGCGATCCAGGTTA	129	60	NM_000546.5
CASP3	F: CATACCTGTGGCTGTGTATCC R: GAGTCCATTGATTCGCTTCC	246	60	NM_004346.3
TP53 (P53)	F: ATTTGCGTGTGGAGTATTTGG R: AGTGTGATGATGGTGAGGATG	172	60	NM_000546.5
BAX	F: TTGCTTCAGGGTTTCATCCA R: GTTGAAGTTGCCGTCAGAA	243	60	NM_001291428.1
BCL2	F: GCCTTCTTTGAGTTCGGTG R: CCAGGAGAAATCAAACAGAG	198	60	NM_000633.2
GAPDH	F: CTCATTTCCTGGTATGACAAC R: CTTCCTCTTGTGCTCTTGCT	122	60	NM_001256799.2

thrice with a washing solution, and the stop solution was added. After 30 min, the results were read at a wavelength of 450 nm (ELISA reader, Hyperion, Germany).

Statistical analyses

The present study was performed using six independent biological replicates. The data were analyzed using the chi-square test for histological findings and one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for gene expression findings and hormone levels. Moreover, statistical analysis was performed using GraphPad Prism version 8. Also, the significance level was considered at 0.05.

Results

hTPC assessment results

The hTPCs were prepared based on our method used in the previous studies on sheep [15] and humans [14]. Following the culture of hTPCs in a differentiation medium for 12 days, they showed accumulated fine lipid droplets in their cytoplasm. Moreover, the levels of DHEA and estradiol were increased significantly in the culture supernatant after 12 days ($P<0.05$). Also, these cells showed significantly increased expression levels of *LHR* and *GLI2* genes ($P<0.05$).

On the other hand, the expression levels of *CYP17a* were increased in the differentiated cells compared to the hTSCs ($P<0.058$). Moreover, the hTPCs were evaluated for the presence of the 3β-HSD enzyme, which catalyzes the conversion of DHEA to androgens in the steroidogenic cells and its activity was approved by the production of a blue insoluble precipitate of Formazan caused by NADH reduction.

Follicle classification results

Following the activation of ovarian tissue fragments with bpv and KL for 24 h and culture for 6 days, the developmental stage of the follicles was evaluated (Fig. 2a–o). According to our results, a total of 330 follicles were counted in all 3 groups, including 107, 103, and 120 follicles in the mono-culture, co-culture, and non-culture groups, respectively. Moreover, most of the follicles had a normal morphology on day 0 of culture (96.66% ± 2.05%), while the rate of follicles with normal morphology was significantly decreased on day 6 in both the mono-culture (79.42% ± 4.21%) and co-culture (80.59% ± 3.50%) groups compared to the non-culture group ($P<0.05$). However, there was no significant intergroup difference in the rate of follicles with normal morphology between the mono- and co-culture groups on day 6 ($P>0.05$, Fig. 3(a)).

Figure 3(b) illustrates the substantial changes in follicular development in the cultures. According to our findings, the percentage of primordial/transitional follicles decreased significantly in the mono-culture (10.28%) and co-culture (5.82%) groups compared to the non-culture group (92.5%) on day 6 ($P<0.05$), while the percentage of primary follicles was significantly lower in the co-culture group (10.68%) compared to the mono-culture group (29.90%, $P<0.05$). However, the percentages of secondary (mono-culture: 28.03%; co-culture: 40.77%) and pre-antral follicles (mono-culture: 14.1%; co-culture: 28.2%) were significantly higher in the co-culture group compared to the mono-culture group ($P<0.05$).

Hormone level assessment results

The levels of hormones were assessed in the co-culture, mono-culture, and only-hTPC groups on days 0, 3, and 6 of culture. According to our results (Fig. 4), the levels of estradiol and testosterone were increased in the only-hTPC group on day 6 of the culture compared to day 0 ($P<0.05$), while the mono-culture group showed significantly increased levels of estradiol and progesterone on day 6 ($P<0.05$), as well as significantly increased levels of testosterone on days 3 and 6 ($P<0.05$). Moreover, the levels of estradiol, progesterone, and androstenedione were significantly increased on day 6 in the co-culture group ($P<0.05$).

On the other hand, the levels of estradiol and progesterone were significantly higher in the co-culture group compared to the mono-culture and only-hTPC groups on days 3 and 6 ($P<0.05$), while the level of androstenedione was only significantly higher on day 6 ($P<0.05$).

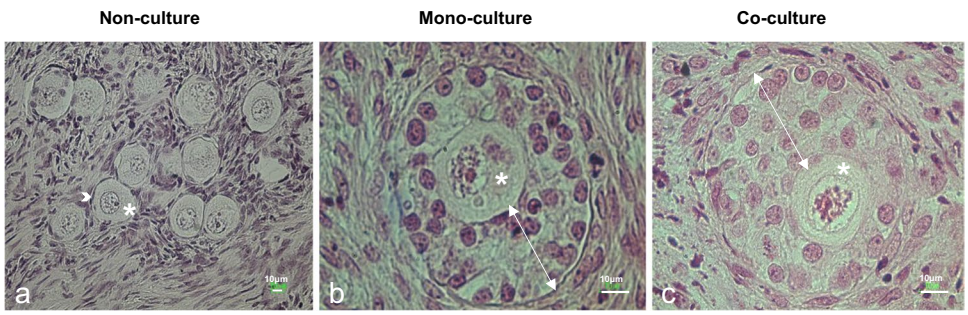

Fig. 2 Histological sections of the human ovarian tissue. **a** Non-culture group, **b** Mono-culture group, **c** Co-culture group. H&E staining. Star: oocyte; Double headed arrow: multilayer of granulosa cells; Arrowhead: pre granulosa cells. Scale bar: 10 µm

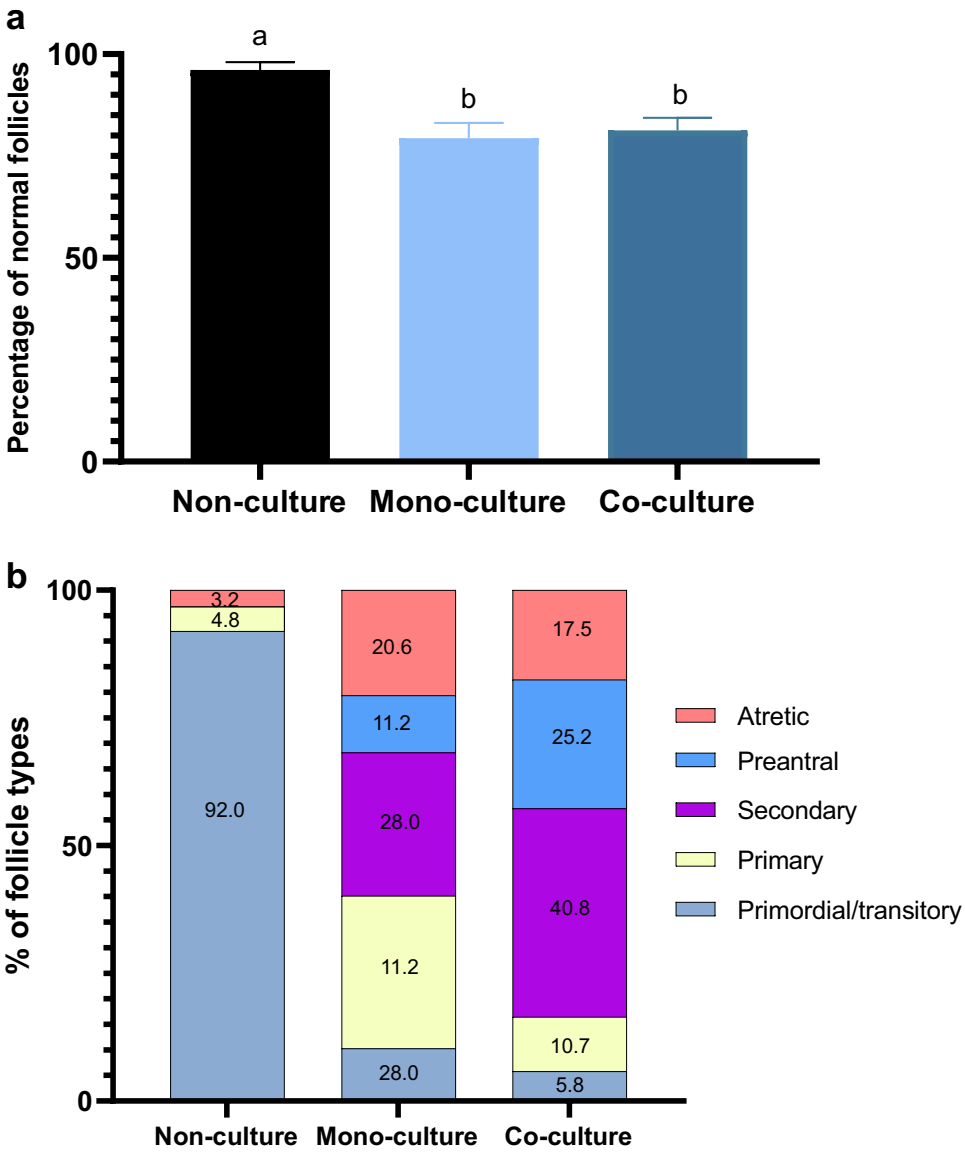

Fig. 3 Follicle classification results. (**a**) Rate of follicles with normal morphology in the non-culture, co-culture, and mono-culture groups after thawing. (**b**) Rate of each class of follicles with normal morphology and their changes in the co-culture and mono-culture groups. Data are presented as means ± SEM and analyzed using the chi-square test. Small letters (**a**, **b**, **c**) represent a significant inter-group difference ($P < 0.05$)

Gene expression assessment results using qRT-PCR

According to our findings, the expression levels of folliculogenesis-related genes of *AMH*, *GDF9*, *ZP1*, *ZP2*, *ZP3*, and *BMP7* were significantly higher in the co-culture group compared to other groups (Fig. 5(a), $P < 0.05$), while the expression levels of *BMP4*, *AMH*, and *ZP1*, *ZP2*, *ZP3* were significantly higher in the mono-culture compared to the non-culture group ($P < 0.05$).

On the other hand, the expression levels of pro-apoptotic genes of *CASP3* and *P53* were significantly lower in the co-culture group compared to the mono-culture group ($P < 0.05$), while the expression level of *BCL2*, an anti-apoptotic gene, was significantly higher (Fig. 5(b); $P < 0.05$). Also, the expression ratio of *BAX* to *BCL2* was significantly higher in the mono-culture group compared to the non-culture group (Fig. 5(c), $P < 0.05$).

Discussion

According to evidence, the oocyte and granulosa cells attract the theca cells, which form an essential structural part of a growing follicle [19]. To the best of our knowledge, the present study was the first to investigate the effect of human ovarian tissue's co-culture with hTPCs on the in vitro development of the primordial follicles, proving the improving effect of hTPCs on the growth of primordial follicles in human ovarian cortical tissue.

Despite the several systems developed for the in vitro culture of follicles, preserving the viability and activity

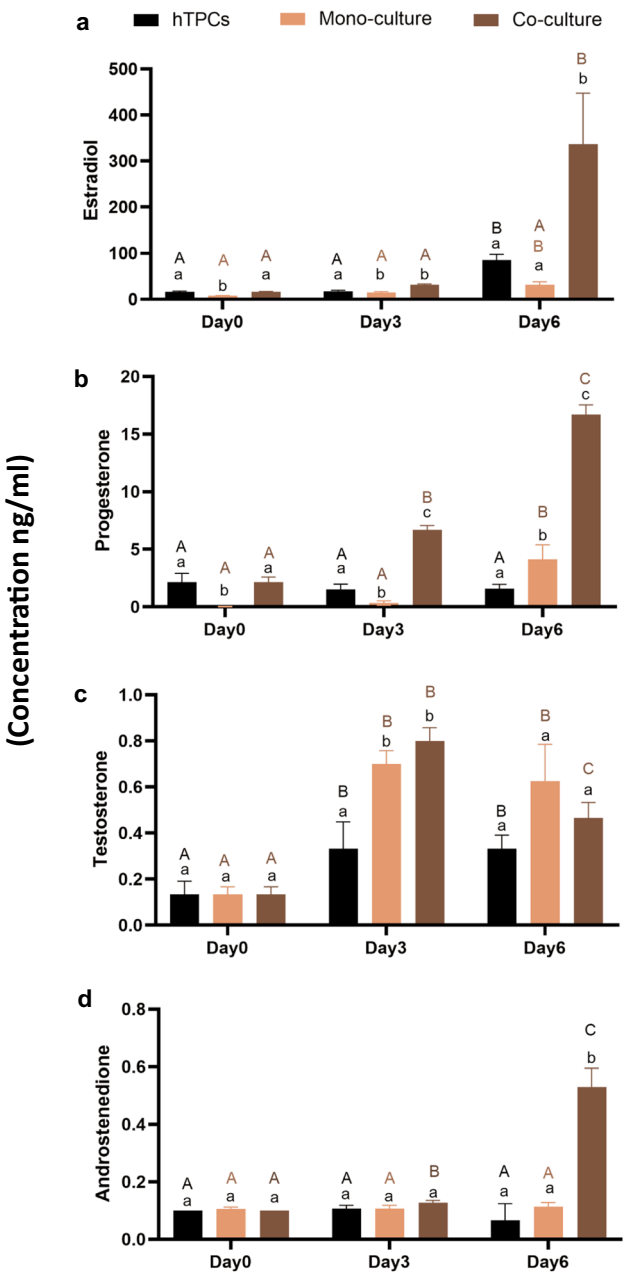

Fig. 4 Hormone assessment results in the co-culture and mono-culture groups. Hormone levels in the culture media on days 0, 3, and 6 of culture. (**a**) Estradiol; (**b**) progesterone; (**c**) testosterone; (**d**) androstenedione. Comparisons were performed using the ANOVA and then Tukey's post hoc test. Small letters (**a**, **b**, **c**) represent a significant intergroup difference ($P < 0.05$), while the capital letters (**A**, **B**, **C**) represent a significant intragroup difference ($P < 0.05$)

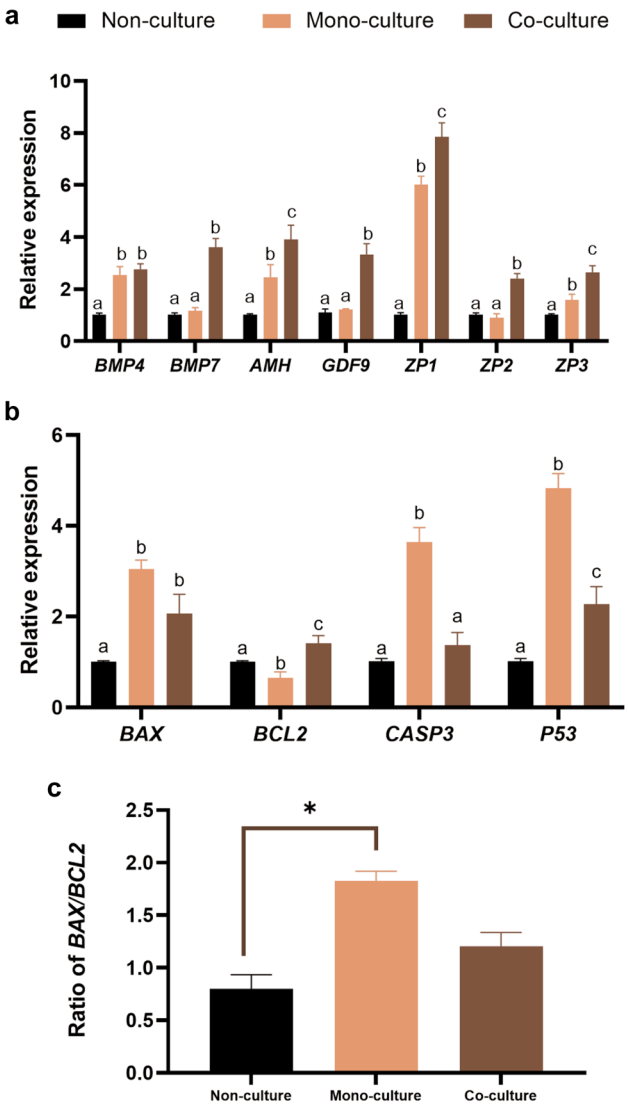

Fig. 5 Gene expression assessment results in the co-culture, mono-culture, and non-culture groups. (**a**) Expression levels of folliculogenesis-related genes, including *BMP-4*, *BMP-7*, *AMH*, *GDF9*, *ZP1*, *ZP2*, and *ZP3*; (**b**) Expression levels of apoptotic genes, including *P53*, *CASP3*, *BAX*, and *BCL2*; (**c**) Expression ratio of *BAX* to *BCL2*. The samples were obtained from 6 patients and included 18 ovarian tissue fragments containing approximately 80 follicles in each group. Comparisons were performed using the ANOVA and then Tukey's post hoc test. Small letters (**a**, **b**, **c**) represent a significant intergroup difference ($P < 0.05$)

of primordial follicles in in vitro cultures is challenging. It has been shown that the first-stage follicles are highly dependent on the paracrine secretion of stromal cells, extrafollicular ovarian tissue, and other neighboring follicles for their development [20]. During every ovulation cycle in an ovary of an adult woman, 15–20 follicles are simultaneously activated. However, only one of the growing follicles eventually undergoes ovulation [21]. Therefore, instead of isolating the single primordial follicles, some researchers have used the small tissue fragments of the ovarian cortex for primordial follicle culture in order to preserve the effect of the ovarian microenvironment [22-24]. However, these tissue fragments lack the theca cells because they usually start to approach the follicles at the late stages of development [19]. In the present study, we also used small pieces of ovarian

cortex with minimum underlying stromal tissue for in vitro culture of primordial follicles.

The role of theca cells has been extensively studied in both the early and late stages of folliculogenesis [25]. These cells are believed to induce the proliferation of granulosa cells and affect the oocyte-granulosa cell crosstalk via the secretion of essential factors, such as bone morphogenetic protein 2 (BMP-2), BMP-7, and transforming growth factor β. Also, they may be indirectly involved in the late stages of follicle development via the induction of connective tissue growth factor secretion by granulosa cells, which increases the matrix remodeling and angiogenesis, essential processes for the formation of the antrum. Furthermore, theca cells produce keratinocyte and hepatocyte growth factors, which make the granulosa cells produce KL [26]. It seems that the secreted KL affects early follicle development by providing a critical stimulus for theca precursor cells to become LH-responsive [27].

In the present study, histological analysis showed remarkable follicular growth and development in tissue fragments co-cultured with hTPCs. According to our findings, the number of secondary and pre-antral follicles was increased in the co-culture group compared to the mono-culture and non-culture groups, while the number of primordial/transitional follicles decreased. Moreover, it was shown that the hTPCs induced the proliferation of granulosa cells. Also, the development of primordial follicles was confirmed by analyzing the expression levels of folliculogenesis-related genes, *BMPs*, *AMH*, *GDF9*, and *ZPs*, in the oocytes, granulosa cells, and theca cells [28].

The *BMPs*, *BMP-4*, and *BMP-7* are expressed in the theca and granulosa cells [29]. Moreover, new biological activities regarding folliculogenesis and ovulation have been suggested for BMP-7 [30]. According to evidence, BMPs improve the stimulatory effects of FSH on steroidogenesis [31]. Furthermore, studies have shown that the number of developing primary follicles and the primordial-to-primary transition is increased in the ovaries exposed to BMP-4 [32], while the BMP-4 protein was detected in the stromal tissue of the ovaries, basement membranes, and the stromal cells (i.e., the precursor of the theca cells) surrounding the follicles [32]. In the present study, the co-culture of hTCPs with ovarian tissue could significantly increase the expression of *BMP-7* compared to culture with hTCPs.

According to our findings, the expression level of *AMH*, as a marker of granulosa cells, was significantly increased in the co-culture and mono-culture groups. However, its expression was significantly higher in the co-culture group compared to the mono-culture group. These findings indicate the increased proliferation and growth of granulosa cells due to co-culture with hTPCs. Moreover, another marker of normal folliculogenesis, GDF9 [33], was significantly increased in the co-culture group, which was compatible with a study by Hreinsson et al. reporting the improving effect of *GDF9* on the growth and development of primordial follicles [34]. Also, the present study showed a significantly higher expression of *ZP1*, *ZP2*, and *ZP3*, which are oocyte-specific genes, in the co-culture group, indicating the development of zona pellucida in the growing follicles [35].

On the other hand, the present study also evaluated the expression levels of pro-apoptotic genes in the ovarian tissues of the non-culture, mono-culture, and co-culture groups. According to our findings, the expression levels of *P53* and *CASP3* were significantly lower in the co-culture group compared to the mono-culture group, while the expression level of *CASP3* was not significantly different between the co-culture and the non-culture groups. Moreover, the *BAX*-to-*BCL2* ratio, which is effective in the ability of a cell to respond to an apoptotic signal [36], was significantly increased in the mono-culture group. Therefore, it can be concluded that co-culture with hTPCs can decrease the apoptotic signals in the culture. This finding is compatible with the study by Tajima et al. [37], showing the decreased incidence of apoptosis in the granulosa cells throughout follicular development in the co-cultured with theca cells [37].

In the present study, the effect of co-culture with hTPCs on the levels of hormones involved in steroidogenesis was investigated. Generally, theca and granulosa cells secrete steroidal and nonsteroidal factors that affect follicular growth and development [38]. These cells secrete androgens like testosterone and androstenedione in response to LH, while these hormones are converted to estrogens by the granulosa cells in response to FSH [38]. Compared to the mono-culture group, co-culture with hTPCs could significantly increase the levels of estradiol, progesterone, and androstenedione on day 6, which highlights a potential relationship between the granulosa and theca cells in the co-culture group. Moreover, the levels of testosterone were increased on day 3, while they decreased on day 6, which shows its aromatization and conversion to estradiol. However, we observed no significant difference in testosterone levels on day 3 between the mono- and co-culture groups. These findings are compatible with a study by Yada et al. [27] that reported no significant difference in hormone production between the co-culture and single-culture groups during the first 48 h of culture. However, hormone production became significantly different during the second 48 h of the culture [27]. According to their findings, co-culture of oocyte-cumulus-granulosa cell complexes with theca cells could promote the in vitro maturation of oocytes and eliminate the need for androgen addition to culture media [27].

To the best of our knowledge, the estradiol production ability of the theca cells is still controversial [39]. Granulosa cells have an active aromatase system that can produce large estrogen quantities (μg per follicle). However, in some species, including humans [40] and pigs [41], theca cells have also been reported to produce limited amounts of estrogen (ng per follicle). Moreover, it was previously reported that hCG significantly increases the

aromatase activity and secretion of estrogen and progesterone in the porcine theca cells [36]. The incidence of such a phenomenon in our study is not impossible as we used hCG in the induction medium during the culture of hTPCs.

Considering the previous studies, we evaluated the progesterone levels as well. We did not expect the high levels of progesterone in both the mono- and co-culture groups on day 6 of culture. However, such findings have been described in the previous studies on the in vitro culture of human ovarian tissue and could be a sign of premature luteinization of the growing follicles [42-44] or the luteinization of the early antral follicles damaged due to the ovarian dissection [44]. Therefore, it should be further investigated in future studies.

Conclusion

According to our findings, the use of a feeder layer of hTPCs could promote the survival and growth of primordial follicles by inducing the proliferation of granulosa cells, formation of the thecal layer, and increasing the levels of androgens and estrogens in the culture medium. These effects can be due to the paracrine secretions of steroids and non-steroidal factors by the hTPCs into the culture medium or the migration of hTPCs to the ovarian cortex tissue because these cells can support the development of primordial follicles. In order to further investigate these possibilities, it is suggested to perform studies using a cell tracer, such as CFDA SE, to mark hTPCs and observe their location in in vitro culture. Moreover, we used bvp for follicle activation in the present study. However, it should be noted that the in vitro recruitment of primordial follicles occurs without the presence of bvp [45, 46], and this PTEN inhibitor has a potentially detrimental effect on follicle growth [47]. Therefore, it is recommended to eliminate this step in future studies. Finally, our results on the supportive role of hTPCs on the growth of follicles at an early stage of development suggest the development of a novel therapeutic strategy for treating ovarian dysfunctions, such as POI, PCOS, or malignancies, using healthy donor TPCs or TSCs, which can differentiate into mature theca cells in the re-aggregated ovary structure.

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Author contribution AD: preparation of samples, experimental design, data collection and analysis, and drafting of the manuscript; SA: data analysis, drafting, and revising the manuscript; CAA: providing critical review; RP: sample preparation; MT: data analysis and drafting the manuscript; MRV: contributed to the conception and design of study and editing and final approval of the manuscript.

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Data Availability However, to achieve the above statements, no datasets were generated or analyzed during the current study and still need more investigations.

Declarations

Conflict of interest The authors declare no competing interests.

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