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FERTILITY PRESERVATION

Altered expression of activator proteins that control follicle reserve after ovarian tissue cryopreservation/transplantation and primordial follicle loss prevention by rapamycin

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Abstract

Purpose We investigated whether expression of activator proteins that control follicle reserve and growth change after ovarian tissue vitrification and re-transplantation. Moreover, we assessed whether inhibition of mTOR signaling pathway by rapamycin would protect primordial follicle reserve after ovarian tissue freezing/thawing and re-transplantation.

Methods Fresh control, frozen/thawed, fresh-transplanted, frozen/thawed and transplanted, rapamycin/control, rapamycin fresh-transplanted, and rapamycin frozen-thawed and transplanted groups were established in rats. After freezing and thawing process, two ovaries were transplanted into the back muscle of the same rat. After 2 weeks, grafts were harvested, fixed, and embedded into paraffin block. Normal and atretic primordial/growing follicle count was performed in all groups. Ovarian tissues were evaluated for the dynamic expressions of Gdf-9, Bmp-15, KitL, Lif, Fgf-2, and p-s6K using immunohistochemistry, and H-score analyses were done.

Results Primordial follicle reserve reduced almost 50% after ovarian tissue re-transplantation. Expression of Gdf-9 and Lif increased significantly in primordial and growing follicles in frozen-thawed, fresh-transplanted, and frozen/thawed and transplanted groups, whereas expression of Bmp-15, KitL, and Fgf-2 decreased in primordial follicles. Freezing and thawing of ovarian tissue solely significantly increased p-s6K expression in primordial follicles, and on the other hand, suppression of mTORC1 pathway using rapamycin preserved the primordial follicle pool.

Conclusion Altered expressions of activator proteins that regulate primordial follicle reserve and growth may lead to primordial follicle loss and rapamycin treatment can protect ovarian reserve after ovarian tissue cryopreservation/transplantation.

Keywords Ovarian tissue cryopreservation · Transplantation · mTOR · Rapamycin · Primordial follicle reserve · Fertility preservation

Introduction

Destructive cancer treatments negatively affect ovarian physiology, resulting in decreased ovarian reserve, early menopause, and infertility in women. For prepubertal girls, especially those scheduled for bone marrow transplantation, ovarian tissue cryopreservation (OTC) is the gold standard since it is not possible to freeze embryos at this age [1–3]. For adults, embryo and oocyte cryopreservation are options that are more valid, but OTC can be an exceptional fertility preservation method and specifically is indicated for women in whom cancer treatment cannot be postponed [4]. Thanks to more than 130 live births and up to 80% hormonal recovery reported to date [5, 6], ovarian tissue cryopreservation and autotransplantation are no longer labeled as experimental procedures after a recently published committee opinion [7].

The mechanism behind early follicular loss after the transplantation period has been explained poorly. A major problem in OTC is follicular loss immediately after grafting, possibly due to slow neovascularization that leads to ischemia [8, 9]. The neovascularization and angiogenesis may take 3 to 7 days

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to occur post-transplantation, when transplanted tissues face ischemic stress and damage [10–12]. Related to ischemia or not, the balance between activator and suppressor molecules in the ovary that normally keeps the dormant PFs from depletion is suggested to change instantly and a rapid burn-out leads to a significant follicle loss after OTC causing premature ovarian failure (POF) [13]. The authors recently showed that expression of inhibitor proteins, which control primordial follicle reserve, decreases in cryopreserved ovaries after autotransplantation [14]. Primordial follicles are aroused from a quiescence state by the interaction of various molecules to activate the PF growth, such as growth differentiation factor-9 (GDF-9), bone morphogenic protein-4/7/15 (BMP-4/7/15), Kit-ligand (KITL), leukemia inhibitory factor (LIF), and fibroblast growth factor (FGF-2), which are expressed in oocytes, granulosa cells, and stroma cells [13].

mTOR (mammalian target of rapamycin), as an evolutionarily conserved serine/threonine-specific protein kinase, is found in almost every cell and has important roles in cell growth and development [15]. An mTOR activator, MHY1485, has been reported to accelerate follicle development in ovarian grafts and to support preantral follicle development in infertile mice to achieve live birth [16]. Activation or dormancy of PFs is regulated by mTORC1, and mTORC1 activates s6K, which is responsible for the protein translation that finally regulates growth and proliferation of granulosa cells and their metabolic activities [17].

Rapamycin and other mTOR inhibitors are increasingly used in various indications, according to their potent immunosuppressive and anticancer properties [18], as well as their intriguing effects on ovarian follicle growth and ovarian physiology [19]. mTOR signaling plays a vital role in regulating female reproduction, which has been demonstrated based on data from genetic, pharmacological, and clinical studies [20]. mTOR signaling participates in various processes that occur in the ovary, including ovarian reserve, follicle development, oocyte meiotic maturation, ovarian aging, and proliferation and steroidogenesis of ovarian somatic cells [20]. In recent studies on mice, it has been shown that rapamycin inhibits PF growth [21] and reverses the symptoms of ovarian hyperstimulation syndrome (OHSS) [22]. Based on the authors' recent findings about disrupted expression of inhibitor proteins after OTC and transplantation [14], the aim of this study was to investigate the effect of OTC on the expression of activator proteins that control follicle reserve and the protective potential of rapamycin treatment on PFs after vitrification/warming and re-transplantation in rats. As recent studies have demonstrated the importance of mTOR signaling pathway on PF survival and recruitment after various conditions [23], it was investigated whether PF burn-out after post-thaw and post-transplantation could be prevented by inhibiting the mTOR signaling pathway.

Materials and methods

Experimental animals and study design

Six- and eight-week-old Wistar female rats were obtained from the Akdeniz University Animal Research Unit, Antalya, Turkey. The experimental protocol was approved by the Animal Care and Use Committee at Akdeniz University, Faculty of Medicine (Ethical Approval Protocol: 2017.07.005), and was in accordance with the Declaration of Helsinki and the International Association for the Study of Pain guidelines. Rats were housed in a controlled environment with a cycle of 12-h light/12-h darkness with ad libitum access to food.

Six- and eight-week-old Wistar female rats ($n = 72$ for all experiments; $n = 9$ rats for activator protein expression analysis experiments (4 groups in total) and $n = 6$ rats for rapamycin treatment analysis experiments including vehicle treatments (6 groups in total)) were used and seven groups were established as depicted in Fig. 1. Experiments were initiated with younger rats in groups including autotransplantation, in order to compensate the age effect for the time that is spent after transplantation. By doing so, all the ovaries evaluated in each group were at the same physiological age.

For evaluation of activator protein expression studies:

- Fresh control (FC) group ($n = 9$): two ovaries from each 8-week-old rat were immediately fixed in Bouin's solution (75 mL of picric acid, 25 mL of 40% formaldehyde, 5 mL of glacial acetic acid) and embedded in paraffin for tissue sectioning.
- Frozen/thawed (FT) group ($n = 9$): two ovaries from each 8-week-old rat were vitrified and warmed after 2 weeks, and then fixed in Bouin's solution for paraffin block sectioning.
- Fresh-transplanted (T) group ($n = 9$): two ovaries from each 6-week-old rat were directly transplanted into the back muscle (m. trapezius) of the same rat (autotransplanted heterotopic) [24]. Two weeks after autotransplantation, the ovarian grafts were removed, fixed in Bouin's solution, and embedded in paraffin for tissue sectioning.
- Frozen/thawed and transplanted (FTT) group ($n = 9$): two ovaries from each 6-week-old rat were vitrified immediately, warmed after 2 weeks, and transplanted into the back muscle of the same rat. Two weeks after autotransplantation, the ovarian grafts were removed, fixed in Bouin's solution, and embedded in paraffin for tissue sectioning.

For the rapamycin treatment studies, rapamycin was dissolved in ethanol with 5.2% Tween-80 and 10% polyethylene glycol [25], and a vehicle (ethanol with 5.2% Tween-80 and

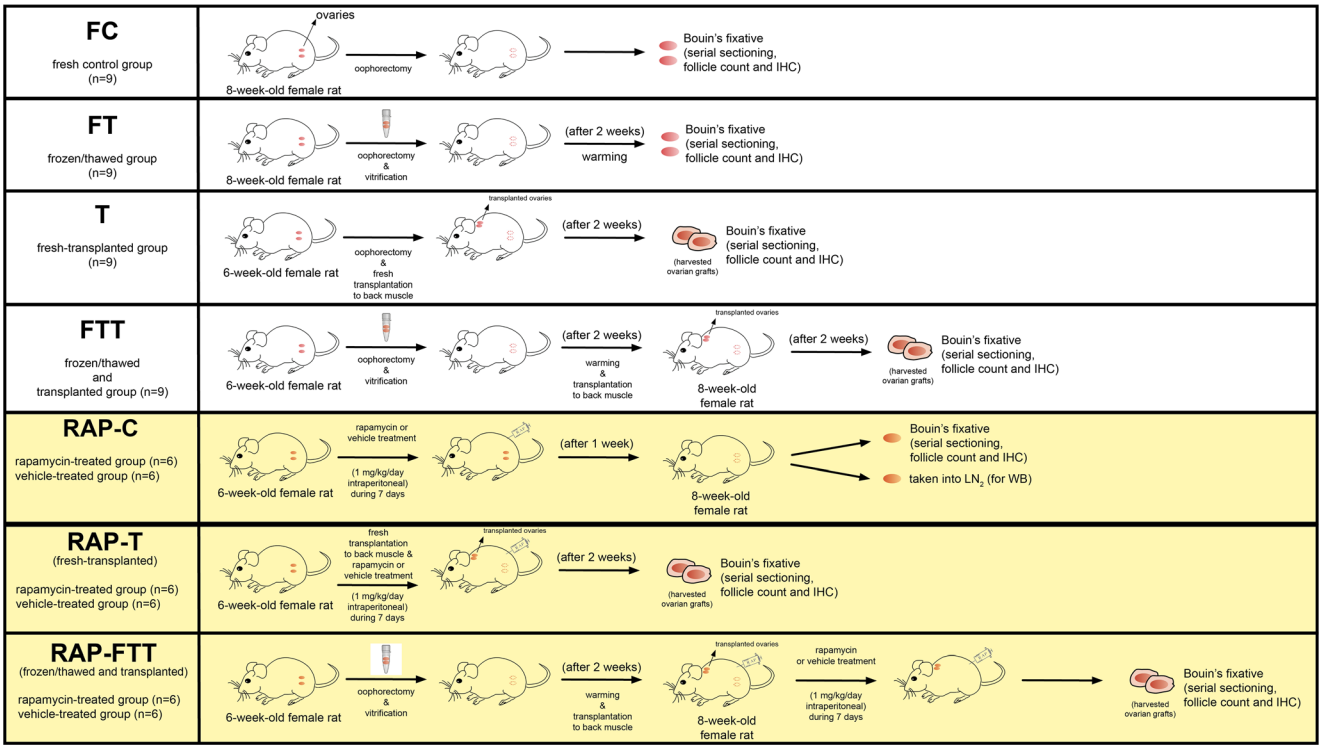


Fig. 1 Experimental study design. IHC, immunohistochemistry; WB, western blot; RAPA, rapamycin; LN_2, liquid nitrogen

10% polyethylene glycol) injection group was designed as control. The reason we performed a control group is to evaluate whether the components of the vehicle group to dissolve rapamycin have alone any adverse effects on the protocol tested.

- Rapamycin control group (RAP-C) ($n = 6$): 6-week-old rats were injected with 1 mg/kg rapamycin [26] or vehicle daily for 7 days, intraperitoneally. Two weeks after the first rapamycin or vehicle ($n = 6$) injection, ovaries were removed, fixed in Bouin's solution, and embedded in a paraffin block for sectioning.
- Rapamycin fresh transplanted (RAP-T) ($n = 6$): 6-week-old rat ovaries were directly transplanted into the back muscle of the same rat. After transplantation, 1 mg/kg rapamycin or vehicle ($n = 6$) were injected daily for 7 days, intraperitoneally. Two weeks after autotransplantation, ovarian grafts were removed, fixed in Bouin's solution, and embedded in a paraffin block for sectioning.
- Rapamycin frozen/thawed and transplanted groups ($n = 6$) (RAP-FTT): two ovaries from each 6-week-old rats were vitrified immediately, warmed after 2 weeks, and transplanted into the back muscle of the same rat. After transplantation, 1 mg/kg rapamycin or vehicle ($n = 6$) was injected daily for 7 days, intraperitoneally. Two weeks after autotransplantation, ovarian grafts were removed, fixed in Bouin's solution, and embedded in a paraffin block for sectioning.

Ovarian tissue vitrification and warming

Ovaries were cryopreserved by the vitrification method, which is a challenging, cheaper, and faster method than slow freezing, described by Choi et al. [27]. Briefly, ovaries were immersed in an equilibration solution composed of 7.5% ethylene glycol (EG, Sigma, MO, USA) and 7.5% dimethyl sulfoxide (DMSO, Merck, Darmstadt, Germany) in M-199 basal medium (Biowest, Nuaillé, France) with 20% fetal bovine serum (FBS) for 25 min at room temperature. After incubation in the final solution composed of 20% EG, 20% DMSO, and 1.0 M sucrose (Merck, Darmstadt, Germany) in M-199 basal medium with 20% FBS for 5 min, the ovaries were loaded onto the electron microscopic (EM) grids (IGC 400; Pelco International, CA, USA) and excess cryoprotectant solution was removed by touching with blotting paper [28]. EM grids carrying the ovaries were directly plunged in liquid nitrogen (LN_2) for 30 s, gently placed in cryovials under LN_2, and the vial caps were sealed. After 2 weeks, the EM grids were gently removed from the cryovials under LN_2 and directly placed in the thawing solution, composed of 1.0 M sucrose in M-199 basal medium with 20% FBS for 3 min at 37 °C. Subsequently, the ovaries were transferred into a diluting solution composed of 0.5 M sucrose in M-199 basal medium with 20% FBS for 5 min at room temperature. Finally, the ovaries were incubated in M-199 basal medium with 20% FBS for 10 min before transplantation at room temperature.

Histological analysis and follicle count

Ovaries were embedded in paraffin, 5-μm-thick sections were obtained, and morphological grading and follicle counts were done on hematoxylin and eosin-stained slides. Every fifth section obtained from each ovarian paraffin block was used for evaluation to avoid a repeated assessment of the same follicle. Follicle assessment and follicle counting were performed according to Gandolfi et al. [29]. Primordial follicles were defined as having an oocyte surrounded by a single layer of squamous granulosa cells. All other follicles at more advanced stages of maturation were grouped and defined as growing follicles. All primordial, primary, secondary, and tertiary follicles were classified as normal or atretic according to the authors' previous publication [14].

Immunohistochemistry

Paraffin sections, selected for immunohistochemical analysis, were initially deparaffinized in xylene and then rehydrated using decreasing ethanol concentrations. Antigen retrieval was performed with citrated buffer (pH = 6) for 5 min and sections were treated with 3% hydrogen peroxide prepared in methanol for 5 min to block endogenous peroxidase activity. Then, the sections were incubated in blocking buffer (Thermo Scientific TA-125-UB) for 7 min to block nonspecific antigen binding. After blocking, the sections were incubated overnight at 4 °C with the following primary antibodies: rabbit Gdf9 (1:250 dilution, polyclonal, product no. ab93892, Abcam, Inc.), rabbit Bmp15 (1:250 dilution, polyclonal, product no. 247897, Biorbyt, Inc.), mouse KitL (1:250 dilution, monoclonal, product no. 13126, Santa Cruz, Inc.), rabbit Lif (1:100 dilution, polyclonal, product no. 29846, St Jones, Inc.), mouse Fgf2 (1:50 dilution, monoclonal, product no. 75512, Santa Cruz, Inc.), and rabbit p-s6K (p70-s6 Kinase-1) (1:250 dilution, polyclonal, product no. PA5-38307, Invitrogen, Inc.). The following morning, the slides were rinsed with phosphate-buffered saline. After washing out the primary antibodies, slides were incubated for 60 min at room temperature with anti-rabbit (1:1000 dilution, product no. ba1000, Vector Inc.) and anti-mouse (1:1000 dilution, product no. ba2000, Vector Inc.) secondary antibody followed by incubation with streptavidin complex (Thermo Fisher) for 30 min. Appropriate rabbit IgG control or mouse isotype control, in the same amount of protein concentrations for each primary antibody, was used to validate nonspecific background staining. All incubation steps for primary antibodies and negative controls were performed in a humidified chamber to avoid dehydration of the slides. Immunohistochemical reactions were visualized using diaminobenzidine chromogen (product no. D4168, Sigma-Aldrich, Inc., MO, USA), which was oxidized by hydrogen peroxide to give a dark-brown color. For nuclear counterstaining, Mayer's hematoxylin (Merck Inc., Darmstadt, Germany) was applied for 40 s. Gdf-9, Bmp-15, Fgf-2, Lif, KitL, and p-S6K expressions were photographed under a light microscope (CX43, Olympus Inc., Japan) and were evaluated by H-score analysis [14].

H-score analysis

All healthy follicles were evaluated with respect to the intensity of their cytoplasmic and/or nuclear expressions (0 (no staining), + 1 (weak staining), + 2 (moderate staining), and + 3 (intensive staining)) in oocytes and granulosa cells. H-score formula [$\sum Pi\ (i+1)$: "I" score of staining density, "*Pi*" rate of stained cells] was used to calculate the expression levels of the aforementioned proteins [30]. In brief, H-score; $Pi\ (i+1)$, where i = intensity of staining with a value of (±), (+), (++), or (+++) (null/minimal, mild, moderate, or strong, respectively), and Pi is the percentage of oocyte and granulosa cells stained with each intensity, varying between 0 and 100%. A final score, ranging from 0 to 300, was obtained for each follicle type in each group from $n = 9$ rats per group and in each group more than 115 primordial, 90 primary, 70 secondary, and 55 tertiary follicles for each protein in average. Finally, for each group, a mean score for every follicle type was acquired.

Western blot

After homogenization and sonication, the OT samples were centrifuged at 10,000*g* for 30 min. Supernatants were collected and protein concentrations were determined using a standard bicinchoninic acid assay [31]. Prior to electrophoresis, samples were heated for 5 min at 95 °C and 20-μg protein was applied per lane. Samples, subjected to SDS polyacrylamide gel electrophoresis under standard conditions, were transferred onto a polyvinylidene difluoride membrane (Biorad Inc.) in a buffer containing 0.2 mol/L glycine, 25 mM Tris, and 20% methanol and incubated overnight at 4 °C. After blocking with 5% nonfat dry milk, membranes were treated with p-s6K primary antibody (1:500 dilution, product no. PA-38307, Thermo Fisher, Inc.) and GAPDH (1:1000 dilution, product no. 2118S, Cell Signaling, Inc.) overnight at 4 °C. After washing, the membranes were incubated for 1 h at room temperature with horseradish peroxidase–conjugated secondary anti-rabbit IgG (1:1000 dilution, product no. PI-1000, Vector Inc.). The reaction was visualized using a chemiluminescence-based Super Signal CL HRP substrate system (product no. 34080, Thermo Scientific Inc.), and the membranes were exposed to Hyperfilm. As an internal standard to confirm the equal loading of the proteins, GAPDH was used as an internal control. ImageJ (NIH, Bethesda, MD, USA) was used to quantify the density of the western blot bands. Because of the loading order in the same gel, vehicle-treated and rapamycin-treated p-s6K and corresponding

GAPDH bands were put side by side by cutting and pasting them next to each other to show the expression level of p-s6K in the two groups for three different samples. In total, $n = 7$ ovaries from rapamycin-treated and $n = 7$ ovaries from vehicle-treated rats were evaluated for western blot analysis.

Statistical analysis

Experiments were performed at least three times for each group. Data was reported as mean ± SEM. Group comparisons were done with one-way ANOVA followed by Tukey's post hoc test for follicle count and H-score results to describe any differences between the groups for parametric data. For the western blot band density results, the *t* test was used to describe the differences among the groups for parametric data. All statistical analysis was performed utilizing Sigma Stat 3.5 software (Jandel Scientific Corp.) and $p < 0.05$ was considered as statistically significant.

Results

Primordial follicle numbers decrease and number of atretic follicles increases significantly after ovarian re-transplantation

Normal and atretic primordial, primary, secondary, and tertiary follicles were evaluated and counted in all groups. The mean number of PFs was significantly lower in the T and FTT groups when compared with the FC and FT groups ($p < 0.05$) (Fig. 2I). When the number of growing follicles were evaluated, the mean number of primary follicles in the FT, T, and FTT groups was significantly lower than that in the FC group ($p < 0.05$) (Fig. 2II). There was no significant difference in the mean number of secondary follicles between all groups (Fig. 2II). The mean number of tertiary follicles in the T and FTT groups was significantly lower than that in the FC and FT groups ($p < 0.05$) (Fig. 2II).

The mean number of atretic PFs was significantly higher in the FT and FTT groups when compared with the FC and T groups ($p < 0.05$) (Fig. 2III). The mean number of atretic primary follicles in the FT and FTT groups was significantly higher than that in the FC group ($p < 0.05$) (Fig. 2IV). There was no significant difference in the mean number of secondary follicles between all groups (Fig. 2IV). The mean number of atretic tertiary follicles in the T group was significantly lower than that in the FT group ($p < 0.05$) (Fig. 2IV). These results showed that the transplantation process reduced the number of PFs independent of the vitrification and warming effect while the vitrification and warming procedure has a detrimental effect on PFs and increases atretic primordial and primary follicle numbers.

Expression of Gdf-9 increased significantly in primordial and growing follicles after OTC and re-transplantation

The expression of Gdf-9 protein in primordial and growing (primary, secondary, and tertiary) follicles was evaluated in the FC, FT, T, and FTT groups and its expression was assessed with an H-score (Fig. 3). In the FC group, Gdf-9 expression was present in the cytoplasm of both oocytes and granulosa cells in the PFs while its expression slightly increased in the cytoplasm of the granulosa cells in the growing follicles (Fig. 3I). Gdf-9 expression significantly increased in oocyte and granulosa cells of PFs in the FT, T, and FTT groups when compared with the FC group ($p < 0.05$) (Fig. 3I, II). In growing follicles, Gdf-9 expression significantly increased in the FT, T, and FTT groups when compared with the FC group ($p < 0.05$) (Fig. 3I, II). Figure 3 III shows the specific localization of Gdf-9 protein expression in ovarian follicles.

Bmp-15 and KitL expression decreased significantly in primordial and growing follicles after OTC and re-transplantation

The expression of Bmp-15 protein in primordial and growing follicles was evaluated in the FC, FT, T, and FTT groups and its expression was assessed with an H-score (Fig. 4). In the FC group, Bmp-15 expression was present in the cytoplasm of both oocytes and granulosa cells in PFs and growing follicles (Fig. 4I). Bmp-15 expression decreased significantly in oocytes and granulosa cells of PFs and growing follicles in the FT, T, and FTT groups when compared with the FC group ($p < 0.05$) (Fig. 4I, II). Figure 4 III shows the specific localization of Bmp-15 protein expression in ovarian follicles.

Expression of KitL protein in primordial and growing follicles was evaluated in the FC, FT, T, and FTT groups and its expression was assessed with an H-score (Fig. 5). In the FC group, KitL expression was present in the cytoplasm of both oocytes and granulosa cells in PFs while its expression was present in the cytoplasm of granulosa cells of the growing follicles (Fig. 5I). KitL expression decreased significantly in oocytes and granulosa cells of PFs and growing follicles in the FT, T, and FTT groups when compared with the FC group ($p < 0.05$) (Fig. 5I, II). Figure 5 III shows the specific localization of KitL protein expression in ovarian follicles.

Lif expression increased significantly in primordial and tertiary follicles after OTC and re-transplantation

Expression of Lif protein in primordial and growing follicles was evaluated in the FC, FT, T, and FTT groups and its expression was assessed with an H-score (Fig. 6). In the FC group, Lif expression was weak in the cytoplasm of both

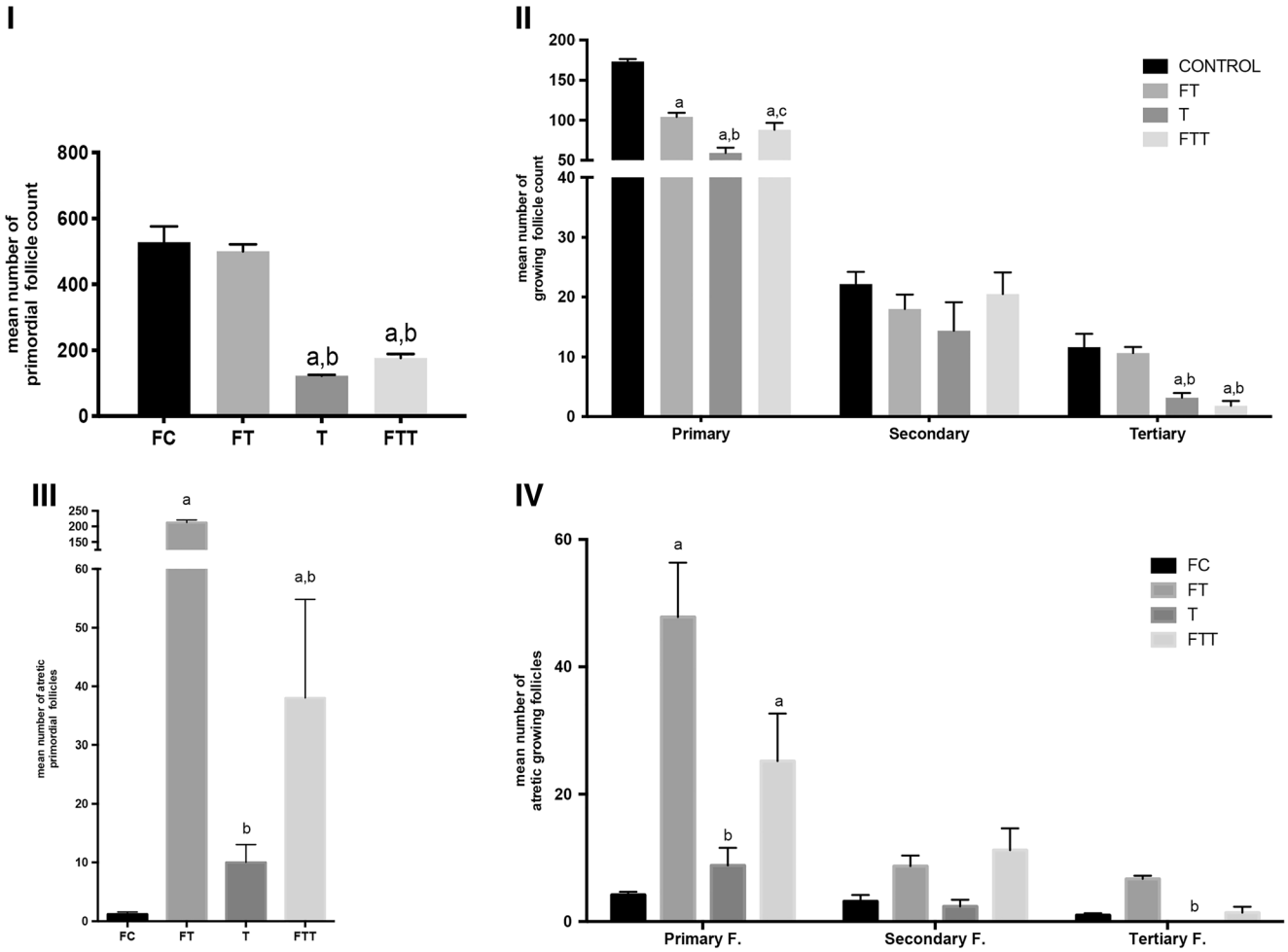


Fig. 2 Mean number of total primordial follicles (I), mean number of total growing follicles (II), mean number of atretic primordial follicles (III), and mean number of atretic growing follicles (IV) in the fresh control (FC) group, frozen and thawed group (FT), fresh-transplanted group (T), and frozen/thawed and transplanted group (FTT). (a) Statistically significant from the FC group ($p < 0.05$). (b) Statistically significant from the FT group ($p < 0.05$). (c) Statistically significant from the T group ($p < 0.05$)

oocytes and granulosa cells in PFs and its expression was present in the cytoplasm of granulosa cells of growing follicles (Fig. 6I). Lif expression increased significantly in oocytes and granulosa cells of PFs in the FT, T, and FTT groups when compared with the FC group ($p < 0.05$) (Fig. 6I, II). In tertiary follicles, Lif expression increased significantly in the FT, T, and FTT groups when compared with the FC group ($p < 0.05$) (Fig. 6I, II). Figure 6 III shows the specific localization of Lif protein expression in ovarian follicles.

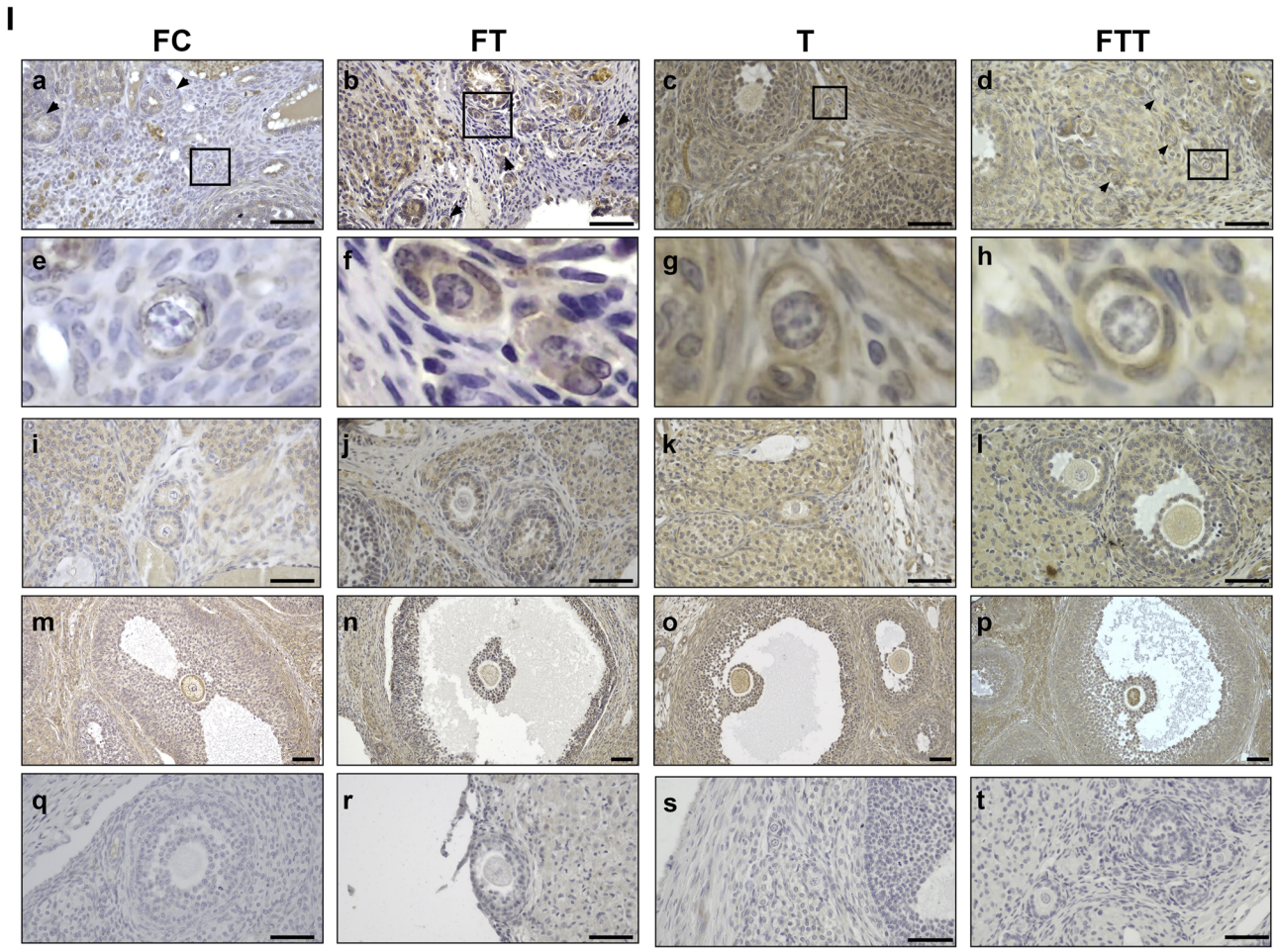
Fgf-2 expression decreased significantly in primordial follicles after OTC and re-transplantation

Expression of Fgf-2 protein in primordial and follicles was evaluated in the FC, FT, T, and FTT groups and its expression was assessed with an H-score (Fig. 7). In the FC group, Fgf-2 expression was present in the cytoplasm of both oocytes and granulosa cells in PFs while its expression was weak in the cytoplasm of granulosa cells of the tertiary follicles (Fig. 7I). Fgf-2 expression decreased significantly in oocyte and granulosa cells of PFs in the FT, T, and FTT groups when compared with the FC group ($p < 0.05$) (Fig. 7I, II). In tertiary follicles, Fgf-2 expression increased significantly in the FT, T, and FTT groups when compared with the FC group ($p < 0.05$) (Fig. 7I, II). Figure 7 III shows the specific localization of Fgf-2 protein expression in ovarian follicles.

Vitrification and warming of ovarian tissue solely significantly increased the expression of p-s6K in primordial and growing follicles except in tertiary follicles

Expression of p-s6K protein in primordial and growing follicles was evaluated in the FC, FT, T, and FTT groups and its expression was assessed with an H-score (Fig. 8). In the FC group, p-s6K expression was present in the cytoplasm of both oocytes and granulosa cells of PFs and its expression increased significantly in the cytoplasm of both oocytes and

Gdf-9



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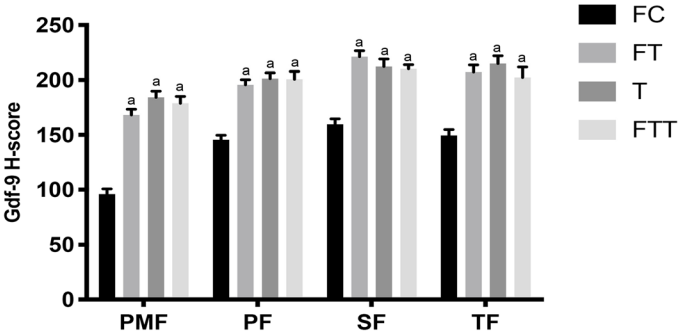


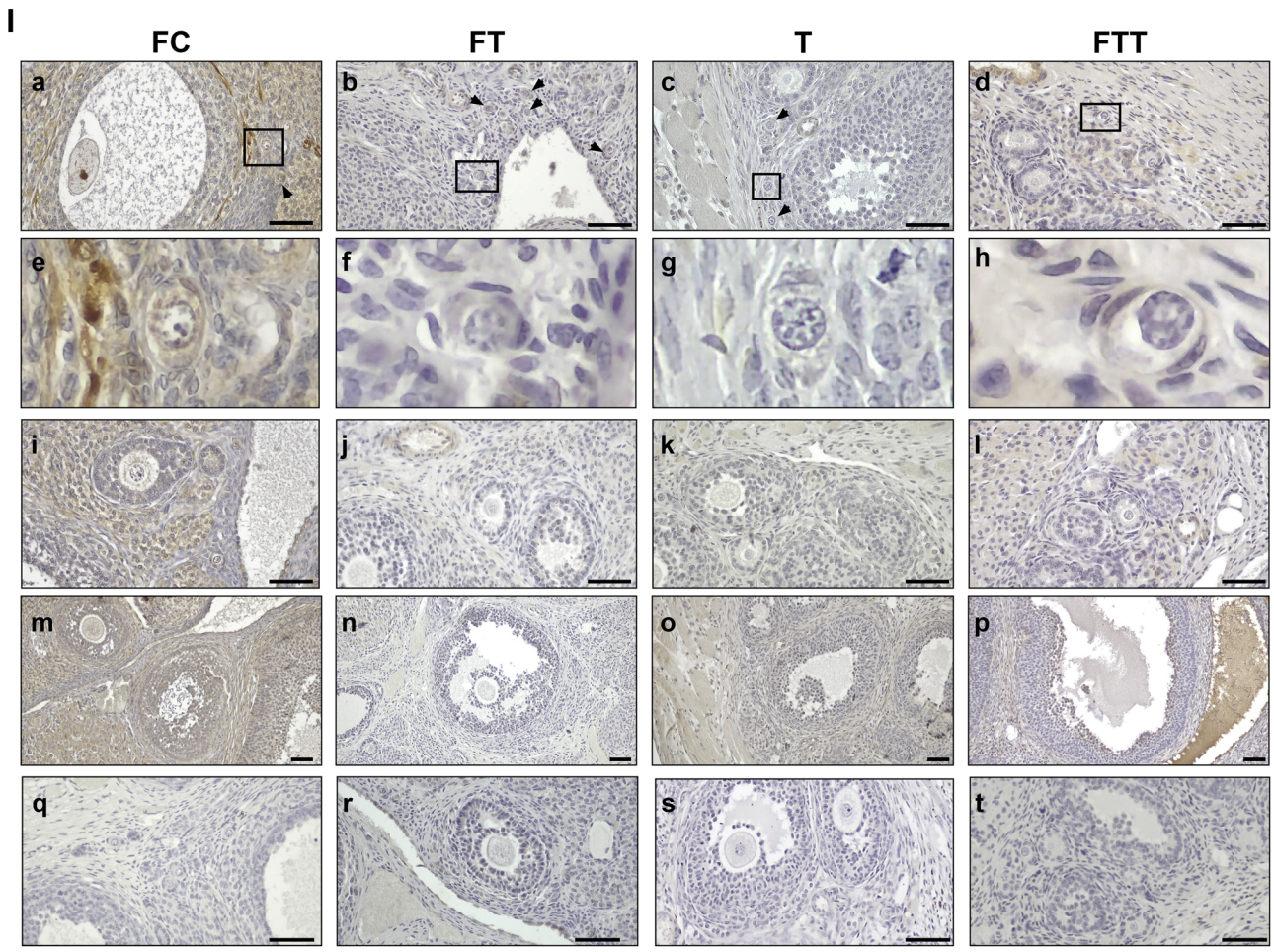
III

Primordial Follicle	Oocyte		Granulosa	
	Nucleus	Cytoplasm	Nucleus	Cytoplasm
FC	-	+	-	+
FT	-	+	-	++
T	-	++	-	+++
FTT	-	++	-	+++

Primary Follicle	Oocyte		Granulosa	
	Nucleus	Cytoplasm	Nucleus	Cytoplasm
FC	-	++	-	++
FT	-	++	-	++
T	-	+++	-	+++
FTT	-	+++	-	+++

Sec. & Ter. Follicle	Oocyte		Granulosa	
	Nucleus	Cytoplasm	Nucleus	Cytoplasm
FC	-	++	-	++
FT	-	++	-	++
T	-	+++		+++
FTT	-	+++	-	+++

Fig. 3 Expression of Gdf-9 in ovarian follicles. (I) Gdf-9 immunohistochemistry results for the fresh control group (FC), frozen/thawed group (FT), fresh-transplanted group (T), and frozen/thawed and transplanted group (FTT). (a–d) Primordial follicles. (e–h) Higher magnification of designated area with the square in a, b, c, and d, respectively. (i–l) Growing follicles. (m–p) Tertiary follicles. (q–t) Negative control images for the FC, FT, T, and FTT groups, respectively. Arrowheads: primordial follicles. (II) H-score results. (a) Statistically significant from the FC group ($p < 0.05$). (b) Statistically significant from the FT group ($p < 0.05$). (III) The distribution of ovarian follicle expression of Gdf-9 (scale bars = 50 μm)



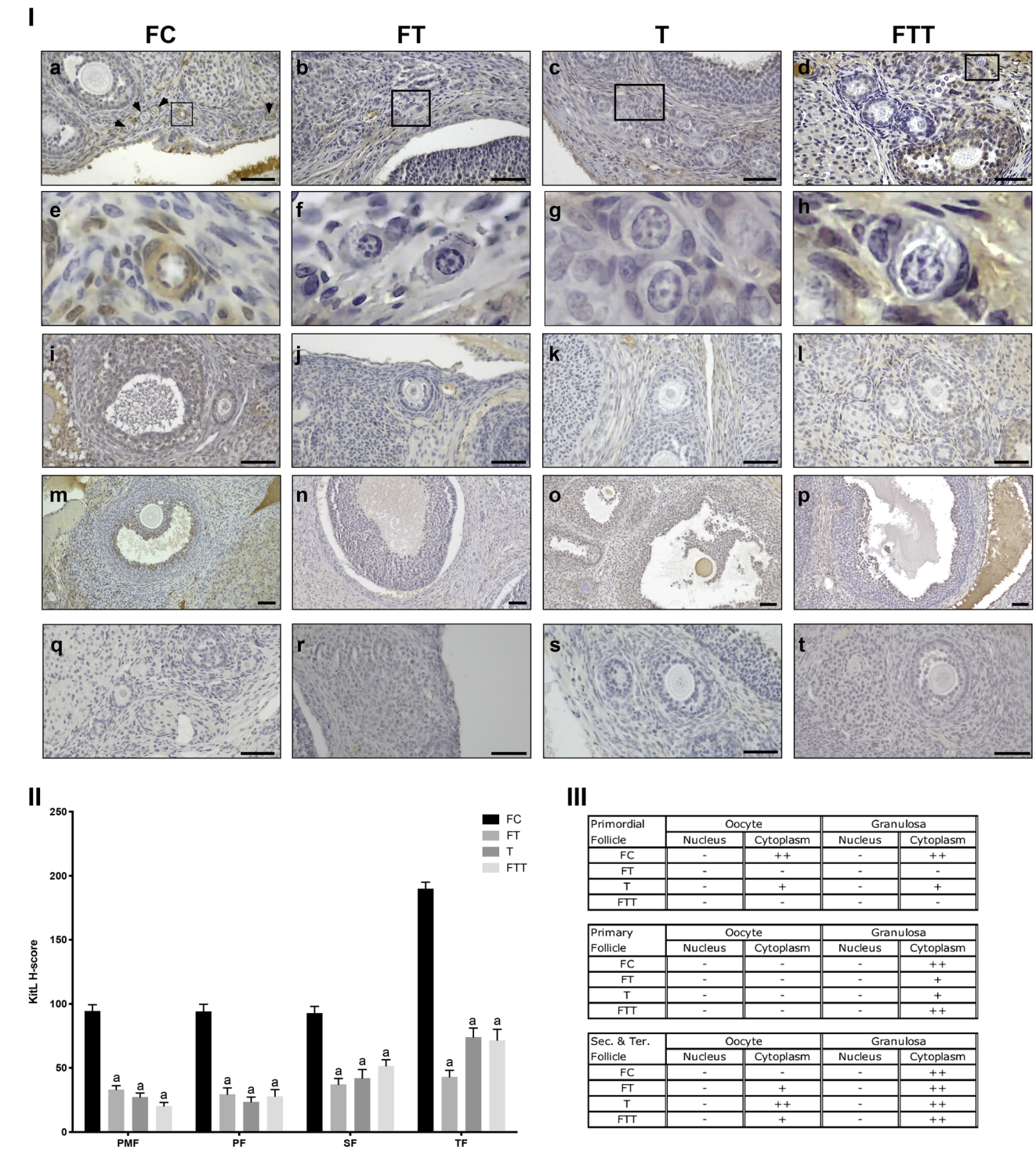
III

Primordial Follicle	Oocyte		Granulosa	
	Nucleus	Cytoplasm	Nucleus	Cytoplasm
FC	-	+	-	+
FT	-	-	-	-
T	-	-	-	-
FTT	-	-	-	-

Primary Follicle	Oocyte		Granulosa	
	Nucleus	Cytoplasm	Nucleus	Cytoplasm
FC	-	+	-	+
FT	-	-	-	+
T	-	-		+
FTT	-	-	-	+

Sec. & Ter. Follicle	Oocyte		Granulosa	
	Nucleus	Cytoplasm	Nucleus	Cytoplasm
FC	+	+	-	+
FT	-	-	-	+
T	-	-	-	+
FTT	-	-	-	+

Fig. 4 Expression of Bmp-15 in ovarian follicles. (I) Bmp-15 immunohistochemistry results for the fresh control group (FC), frozen/thawed group (FT), fresh-transplanted group (T), and frozen/thawed and transplanted group (FTT). (a–d) Primordial follicles. (e–h) Higher magnification of designated area with the square in a, b, c, and d, respectively. (i–l) Growing follicles. (m–p) Tertiary follicles. (q–t) Negative control of the FC, FT, T, and FTT groups respectively. Arrowheads: primordial follicles. (II) H-score results. (a) Statistically significant from the FC group ($p < 0.05$). (b) Statistically significant from the FT group ($p < 0.05$). (III) The distribution of ovarian follicle expression of Bmp-15 (scale bars = 50 μm)



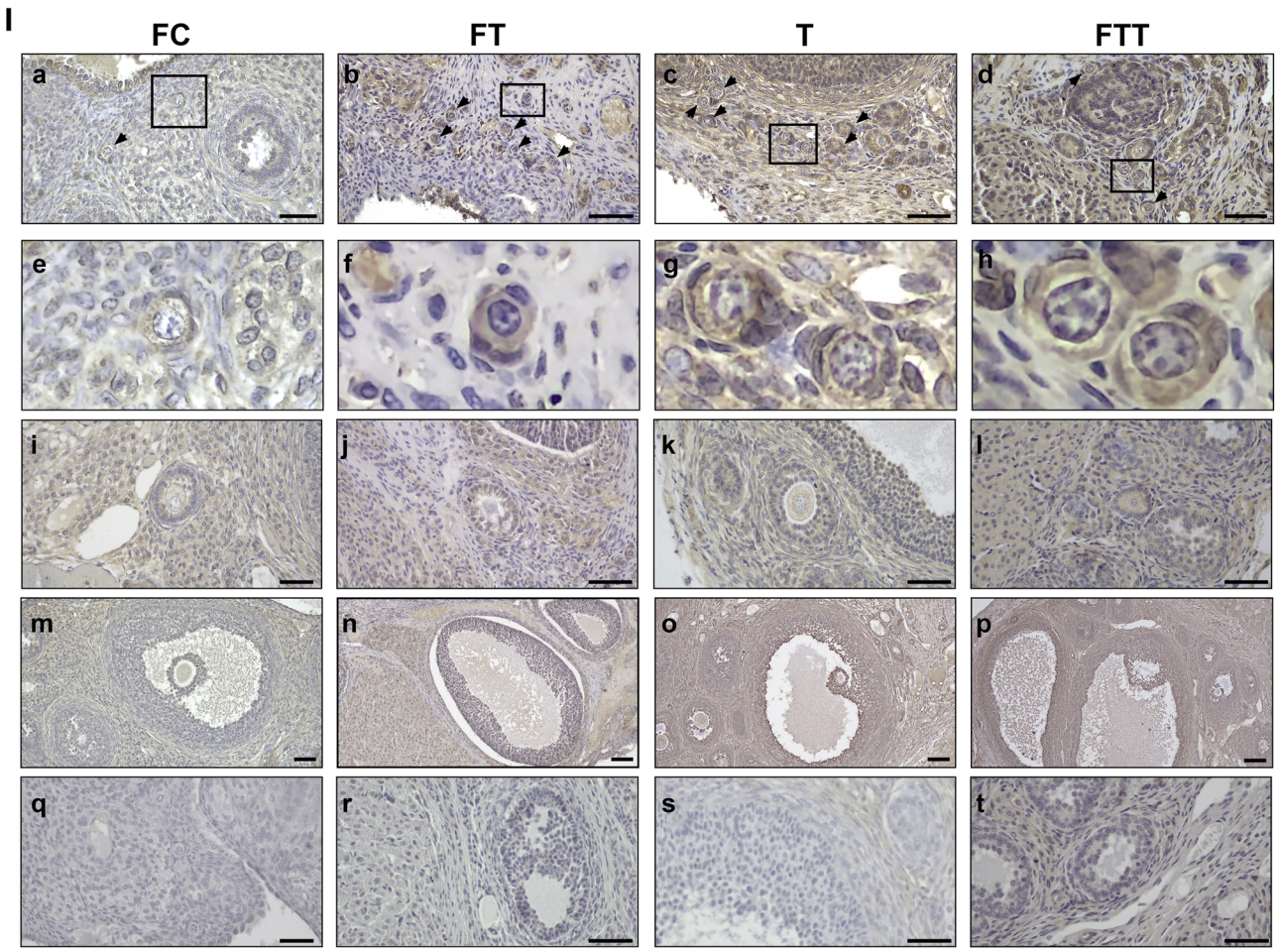
Primordial Follicle	Oocyte		Granulosa	
	Nucleus	Cytoplasm	Nucleus	Cytoplasm
FC	-	++	-	++
FT	-	-	-	-
T	-	+	-	+
FTT	-	-	-	-

Primary Follicle	Oocyte		Granulosa	
	Nucleus	Cytoplasm	Nucleus	Cytoplasm
FC	-	-	-	++
FT	-	-	-	+
T	-	-	-	+
FTT	-	-	-	++

Sec. & Ter. Follicle	Oocyte		Granulosa	
	Nucleus	Cytoplasm	Nucleus	Cytoplasm
FC	-	-	-	++
FT	-	+	-	++
T	-	++	-	++
FTT	-	+	-	++

Fig. 5 Expression of KitL in ovarian follicles. (I) KitL immunohistochemistry results for the fresh control group (FC), frozen/thawed group (FT), fresh-transplanted group (T), and frozen/thawed and transplanted group (FTT). (a–d) Primordial follicles. (e–h) Higher magnification of designated area with the square in a, b, c, and d, respectively. (i–l) Growing follicles. (m–p) Tertiary follicles. (q–t) Negative control images for the FC, FT, T, and FTT groups, respectively. Arrowheads: primordial follicles. (II) H-score results. (a) Statistically significant from the FC group ($p < 0.05$). (b) Statistically significant from the FT group ($p < 0.05$). (III) The distribution of ovarian follicle expression of KitL (scale bars = 50 μm)

Lif



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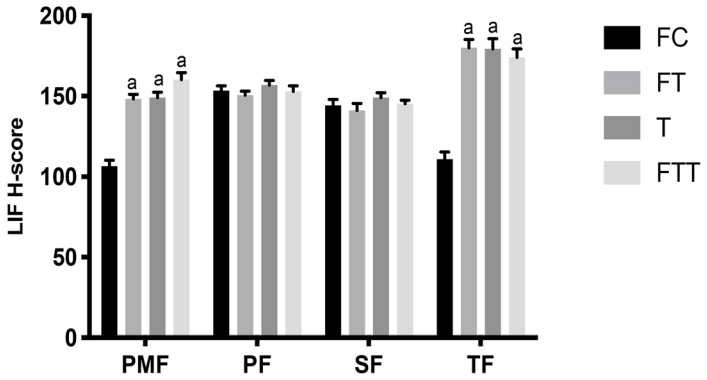


III

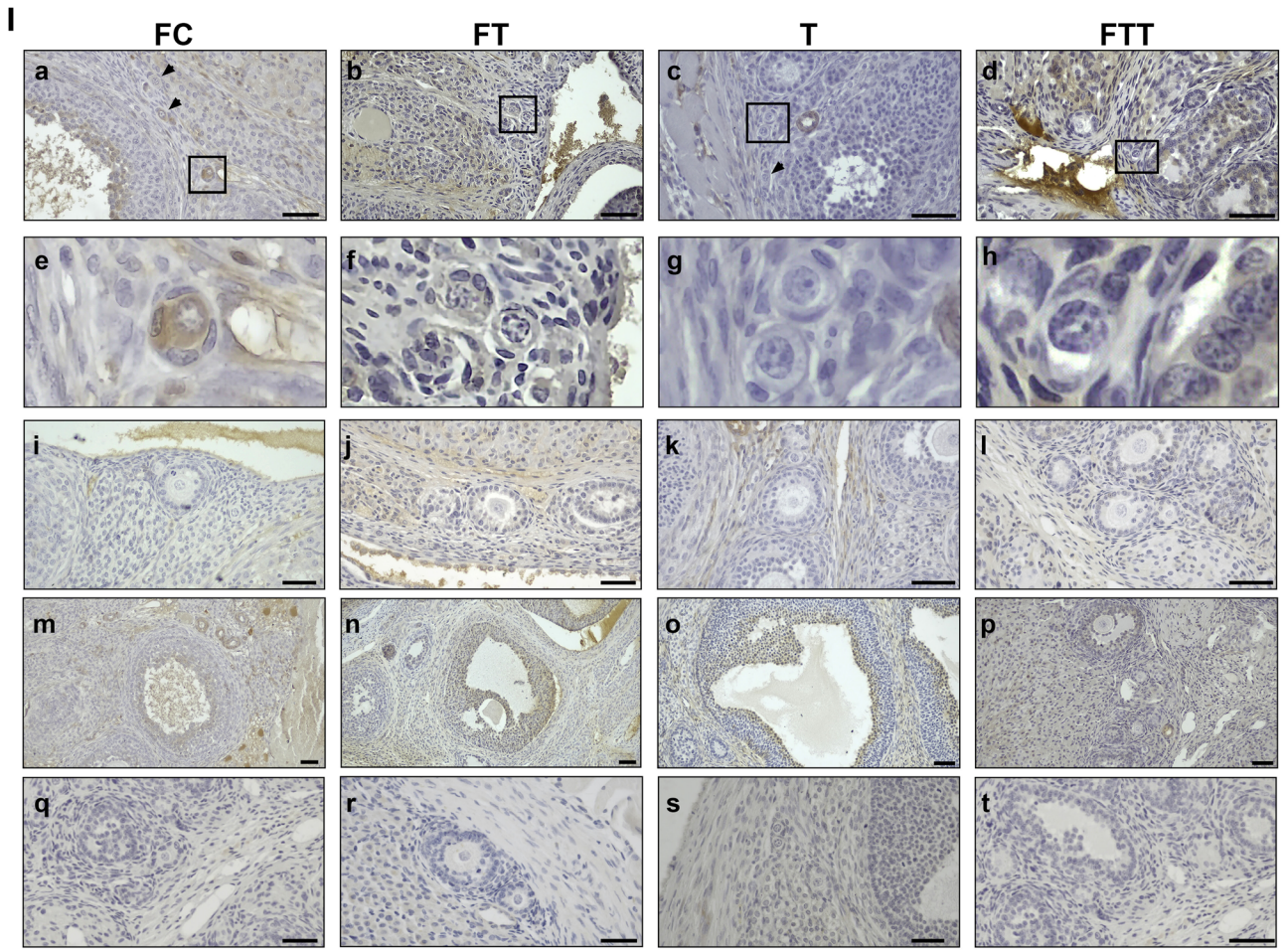
Primordial Follicle	Oocyte		Granulosa	
	Nucleus	Cytoplasm	Nucleus	Cytoplasm
FC	-	+	-	+
FT	-	++	-	++
T	-	++	-	++
FTT	-	++	-	++

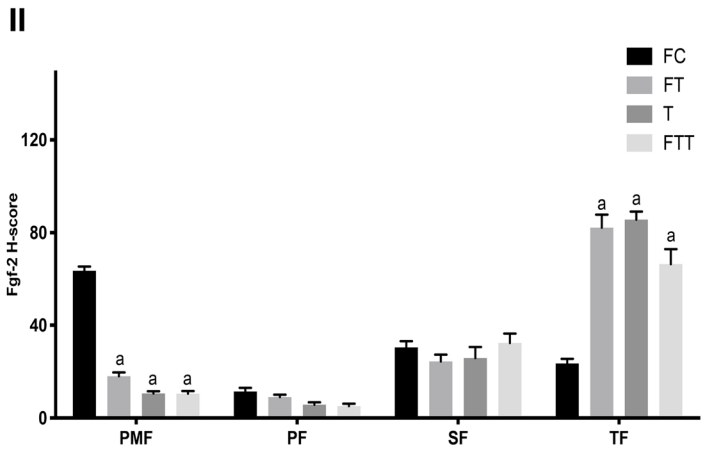
Primary Follicle	Oocyte		Granulosa	
	Nucleus	Cytoplasm	Nucleus	Cytoplasm
FC	-	-	-	++
FT	-	+	-	+
T	-	-		++
FTT	-	+	-	+

Sec. & Ter. Follicle	Oocyte		Granulosa	
	Nucleus	Cytoplasm	Nucleus	Cytoplasm
FC	-	+	-	+
FT	-	+	-	+
T	-	+	-	+
FTT	-	+	-	+

Fig. 6 Expression of Lif in ovarian follicles. (I) Lif immunohistochemistry results for the fresh control group (FC), frozen/thawed group (FT), fresh-transplanted group (T), and frozen/thawed and transplanted group (FTT). (a–d) Primordial follicles. (e–h) Higher magnification of designated area with the square in a, b, c, and d, respectively. (i–l) Growing follicles. (m–p) Tertiary follicles. (q–t) Negative control images for the FC, FT, T, and FTT groups, respectively. Arrowheads: primordial follicles. (II) H-score results. (a) Statistically significant from the FC group ($p < 0.05$). (b) Statistically significant from the FT group ($p < 0.05$). (III) The distribution of ovarian follicle expression of Lif (scale bars = 50 μm)

Fgf-2





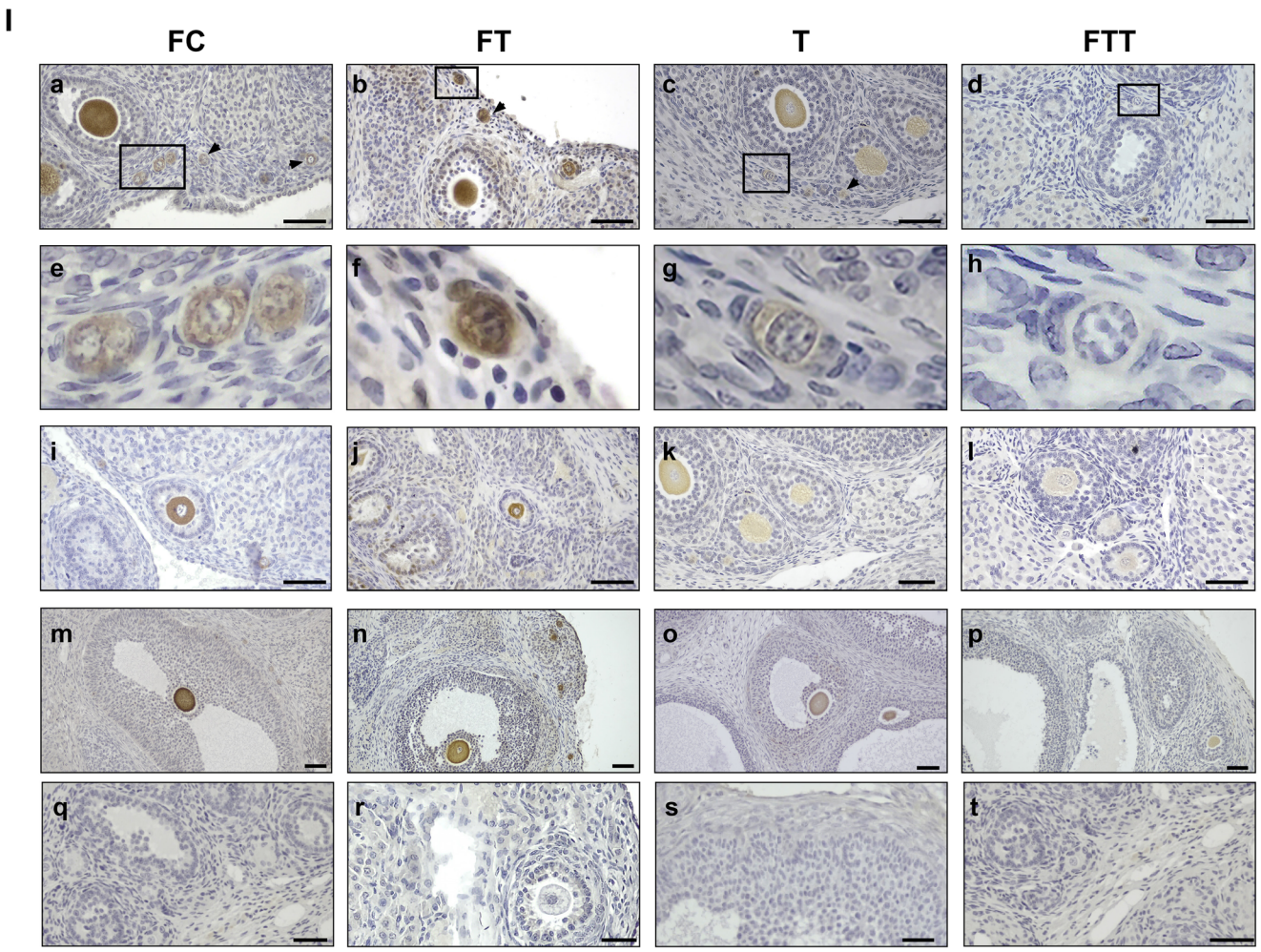
III

Primordial Follicle	Oocyte		Granulosa	
	Nucleus	Cytoplasm	Nucleus	Cytoplasm
FC	-	++	-	+
FT	-	-	-	-
T	-	-	-	-
FTT	-	-	-	-

Primary Follicle	Oocyte		Granulosa	
	Nucleus	Cytoplasm	Nucleus	Cytoplasm
FC	-	-	-	-
FT	-	-	-	-
T	-	-	-	-
FTT	-	-	-	-

Sec. & Ter. Follicle	Oocyte		Granulosa	
	Nucleus	Cytoplasm	Nucleus	Cytoplasm
FC	-	-	-	-
FT	-	-	-	++
T	-	-	+	+
FTT	-	-	+	++

Fig. 7 Expression of Fgf-2 in ovarian follicles. (I) Fgf-2 immunohistochemistry results for the fresh control group (FC), frozen/thawed group (FT), fresh-transplanted group (T), and frozen/thawed and transplanted group (FTT). (a–d) Primordial follicles. (e–h) Higher magnification of designated area with the square in a, b, c, and d, respectively. (i–l) Growing follicles. (m–p) Tertiary follicles. (q–t) Negative control images for the FC, FT, T, and FTT groups, respectively. Arrowheads: primordial follicles. (II) H-score results. (a) Statistically significant from the FC group ($p < 0.05$). (b) Statistically significant from the FT group ($p < 0.05$). (III) The distribution of ovarian follicle expression of Fgf-2 (scale bars = 50 μm)



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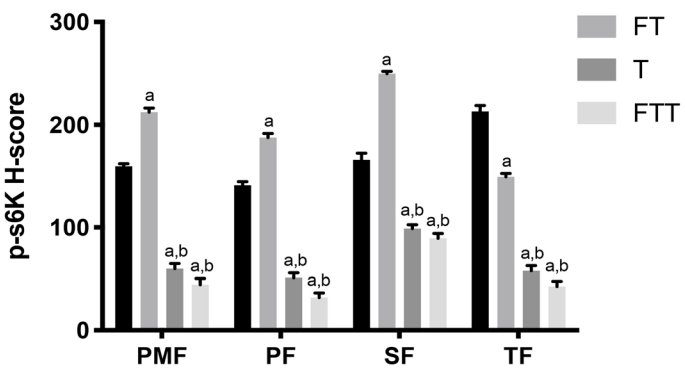


III

Primordial Follicle	Oocyte		Granulosa	
	Nucleus	Cytoplasm	Nucleus	Cytoplasm
FC	-	++	-	-
FT	-	+++	-	++
T	-	+	-	-
FTT	-	-	-	-

Primary Follicle	Oocyte		Granulosa	
	Nucleus	Cytoplasm	Nucleus	Cytoplasm
FC	-	++	-	-
FT	-	+++	-	+
T	-	+	-	-
FTT	-	-	-	-

Sec. & Ter. Follicle	Oocyte		Granulosa	
	Nucleus	Cytoplasm	Nucleus	Cytoplasm
FC	-	+++	-	-
FT	-	+++	-	+
T	-	+	-	-
FTT	-	+	-	-

Fig. 8 Expression of p-s6K in ovarian follicles. (I) p-s6K immunohistochemistry results for the fresh control group (FC), frozen/thawed group (FT), fresh-transplanted group (T), and frozen/thawed and transplanted group (FTT). (a–d) Primordial follicles. (e–h) Higher magnification of designated area with the square in a, b, c, and d, respectively. (i–l) Primary follicles. (m–p) Growing follicles. (q–t) Negative control images for the FC, FT, T, and FTT groups, respectively. Arrowheads: primordial follicles. (II) H-score results. (a) Statistically significant from the FC group ($p < 0.05$). (b) Statistically significant from the FT group ($p < 0.05$). (III) The distribution of ovarian follicle expression of p-s6K (scale bars = 50 μm)

granulosa cells of the FT group ($p < 0.05$) (Fig. 8I, II). However, p-s6K expression decreased significantly in oocytes' cytoplasm of PFs in the T and FTT groups when compared with the FC and FT groups ($p < 0.05$) (Fig. 8I, II). Figure 8 III shows the specific localization of p-s6K protein expression in ovarian follicles.

Suppression of mTOR pathway by rapamycin treatment preserved primordial follicle reserve when compared with vehicle controls

In order to suppress the increased expression of p-s6K due to vitrification and warming, rapamycin treatment was initialized immediately after the autotransplantation of warmed ovaries into the back muscle of the rats. Representative micrographs of ovarian sections for rapamycin-treated and vehicle-treated groups are shown in Fig. 9I. When the number of primordial and growing follicles was counted in the control, fresh-transplanted, and frozen/thawed and transplanted groups, the mean number of PFs in the rapamycin-treated groups was significantly higher when compared with vehicle-treated groups ($p < 0.05$) (Fig. 9II–IV). Moreover, in the control group, the mean number of all growing follicles was significantly less in rapamycin-treated groups when compared with vehicle-treated groups ($p < 0.05$) (Fig. 9II). Both in fresh-transplanted and frozen/thawed and transplanted groups, the mean number of all growing follicles in the rapamycin-treated group was similar to the vehicle-treated group (Fig. 9III, IV).

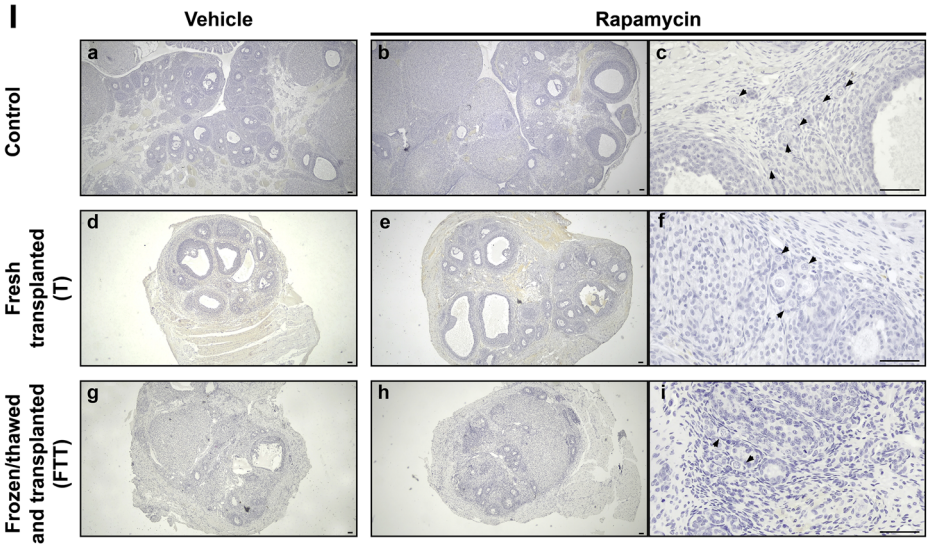
To evaluate mTOR signaling pathway activity, p-s6K protein expression was evaluated in OT both by immunohistochemistry (Fig. 9V, VI) and western blot analysis Fig. 9VII). p-s6K expression decreased significantly in the cytoplasm of both oocytes and granulosa cells in primordial and growing follicles when compared with the vehicle-treated group ($p < 0.05$) (Fig. 9V, VII). Western blot analysis also confirmed reduced expression of p-s6K expression after 1 mg/kg/day rapamycin administration for 7 days (Figs. 7, 8, and 9) which is an indicator for inhibition of mTOR pathway (Fig. 9VII).

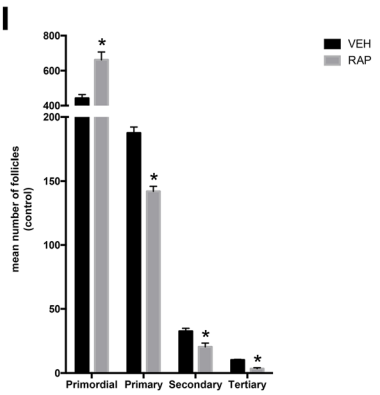
Discussion

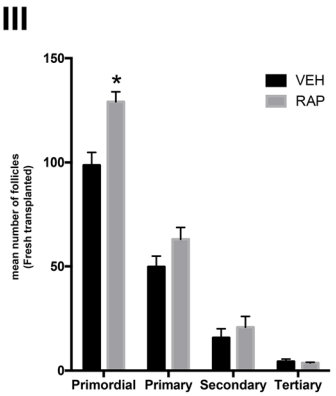
In the present study, the expression of activator proteins, Gdf-9, Bmp-15, KitL, Lif, Fgf-2, and p-s6K, which control follicle reserve and follicle development, were evaluated and it was found that their expression was disturbed after OTC and autotransplantation in rats. Increased expression of p-s6K after vitrification and warming led to the investigation of whether rapamycin treatment would protect PF reserve after OTC and transplantation and it was found that either after fresh or frozen/thawed autotransplantation, the number of PFs decreased more than 50%. These results showed that the transplantation process itself reduced the PF reserve independent of vitrification and warming effect, although vitrification and warming also increased the number of atretic primordial and primary follicles. Moreover, in the rapamycin-treated group, the number of PFs was significantly higher than in in the vehicle-treated groups, suggesting that inhibition of the mTOR pathway protects follicle reserve after OTC and transplantation.

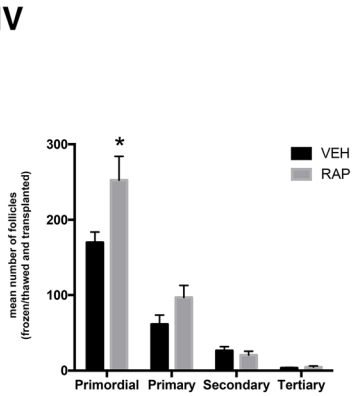
In the present study, we used vitrification method for OTC and Sugishita et al. also showed that histological evaluation of thawed samples from vitrified human ovarian tissues provided similar follicular viability features to slow-frozen ovarian tissues [32]. According to live birth outcomes after OTC and transplantation in humans, slow freezing is still considered as a gold standard method for ovarian tissue cryopreservation. There are contradictory reports on vitrification [6]; however, this is an easy and cheaper protocol to perform in animal research setup as well as in clinic. In a systematic review and meta-analysis [33], it has been concluded that vitrification may well be more effective than slow freezing, but the authors stressed that variations in protocols could negatively impact the dissemination of the technique, and therefore its application in routine clinical practice is not common. In our study, heterotopic OT transplantation to the back muscle of rats was performed. In clinic, the orthotopic transplantation method is more successful than heterotopic transplantation regarding successful live birth rate [34]. In humans, when ovarian grafts are transplanted against a vascular bed on the pelvic sidewall (heterotopic), subcutaneously (heterotopic), or on the remaining menopausal ovary (orthotopic), their survival is acutely dependent on the neovascularization process [35]. Heterotopic autotransplantation of frozen-thawed OT is indicated as an alternative to orthotopic autotransplantation in cases of severe pelvic adhesions or poor pelvic vasculature in humans [36]. In experimental animal models, the orthotopic transplantation method of OT is not feasible to use; thus, heterotopic transplantation methods (e.g., back muscle or kidney capsule) are available options [34]. Although the difference in the local environments in these various sites may impact neovascularization and transplant efficacy, the initial ischemic phase is associated with massive loss of PF reserve and limits the longevity and success of ovarian transplants in both humans and experimental animals. Therefore, there is a great clinical need to develop strategies to enhance neoangiogenesis after ovarian transplantation and to improve the clinical utility of this procedure for understanding the dissimilarities between different transplantation sites [37].

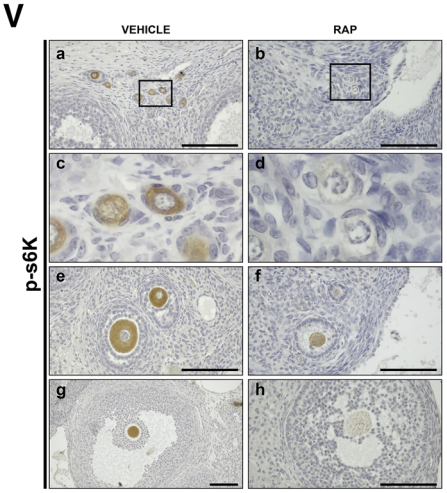
Numerous papers have addressed an over-accelerated and uncontrolled PF recruitment, a burn-out syndrome, which may be due to lack of the protective role of AMH and/or a destructive metabolism caused by ischemia after fertility preservation [38, 39]. The authors have recently reported decreased expression of inhibitor proteins that control primordial follicle reserve after OTC and re-transplantation [14] and











VI

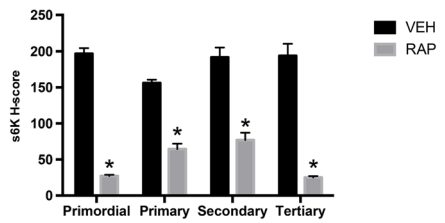
a

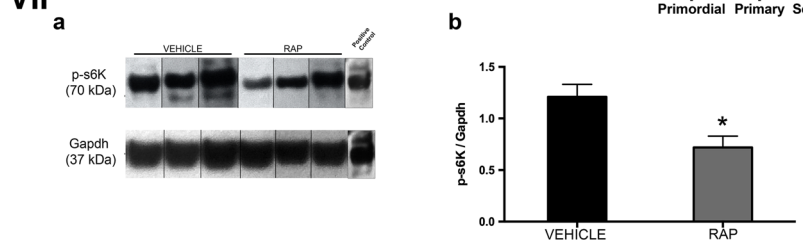
Primordial Follicle	Oocyte		Granulosa	
	Nucleus	Cytoplasm	Nucleus	Cytoplasm
VEH	-	+++	-	++
RAP	-	+	-	-

Primary Follicle	Oocyte		Granulosa	
	Nucleus	Cytoplasm	Nucleus	Cytoplasm
VEH	-	+++	-	+
RAP	-	+	-	-

Secondary & Tertiary Fol.	Oocyte		Granulosa	
	Nucleus	Cytoplasm	Nucleus	Cytoplasm
VEH	-	+++	-	-
RAP	-	+	-	-

b





◀ **Fig. 9** Effect of rapamycin treatment on ovarian follicle number and p-s6K expression. (I) Representative images for vehicle-treated and rapamycin-treated ovary in the control, fresh-transplanted (T), and frozen/thawed and transplanted (FTT) groups. Arrowheads: primordial follicles. (II) Mean number of follicles for the control group in the vehicle-treated and rapamycin-treated groups. (III) Mean number of follicles for the fresh-transplanted group in the vehicle-treated and rapamycin-treated groups. (IV) Mean number of follicles for the frozen/thawed and transplanted group in the vehicle-treated and rapamycin-treated groups. (V) p-s6K protein expression in the vehicle-treated and rapamycin-treated groups. (V-c, V-d) Higher magnification of designated area with the square in (a) and (b) respectively. (VI (a)) The distribution of ovarian follicle expression of p-s6K in the vehicle-treated and rapamycin-treated groups. (VI (b)) H-score results. A single asterisk indicates a statistically significant from the VEH group ($p < 0.05$). (VII (a)) p-s6K protein expression in the vehicle-treated and rapamycin-treated groups and (b) quantification of vehicle-treated and rapamycin-treated groups (in total $n = 7$ ovaries have been evaluated for each group). Figure shows 3 representative bands. Mouse uterus tissue was used as a positive control. *$p < 0.05$. Veh, vehicle; RAP, rapamycin (scale bars = 50 μm)

upon this finding, the expressions of activator proteins, which control primordial follicle reserve and follicle growth in the same model, were evaluated. Expression of Gdf-9 and Lif increased significantly in PFs after OTC and re-transplantation. It has been reported that Gdf-9 protein plays a role as an activator in transition from PF to primary follicle [40]. In this study's model, it was found that its expression increased significantly in both primordial and growing follicles in the FT, T, and FTT groups indicating that increased Gdf-9 expression can be one of the possible mechanisms for primordial follicle activation and eventually follicular burn-out after OTC and transplantation. Similar to GDF-9, LIF plays a paracrine role in primary follicle formation and growth [41] by accelerating oocyte maturation [42]. In this study's model, it was found that Lif expression increased significantly in primordial follicles in the FT, T, and FTT groups indicating that its increase may lead to over-accelerated follicle growth and subsequent PF loss.

The authors found that Bmp-15 and KitL expression decreased significantly in primordial and growing follicles after OTC and re-transplantation. Both BMP-15 and KITL are crucial factors that regulate follicle activation and growth [43–45]. It has been suggested that KitL activates the PI3K signaling pathway in oocytes, which results in the activation of GDF-9 and BMP-15 [45]. Moreover, it has been suggested that while GDF-9 inhibits KITL expression, BMP-15 increases KITL expression in granulosa cells [46]. Since GDF-9 and BMP-15 regulate KITL expression in an opposite manner for follicle activation and growth in this study, increased GDF-9 and decreased BMP-15 expression in oocytes and granulosa cells of PFs may be responsible for decreased KITL expression after OTC and transplantation. Altogether, it can be suggested that increased GDF-9 expression and decreased BMP-15 expression may trigger the imbalance of other protein expressions such as KITL, which may lead to PF loss.

In this study, we found that the expression of Fgf-2 decreased significantly in PFs after OTC and re-transplantation in the FT, T, and FTT groups, whereas Fgf-2 expression increased significantly in tertiary follicles in the FT, T, and FTT groups. Fibroblast growth factor-2 acts as a positive regulator in transition from PF to primary follicle [47] and enhances KITL expression in granulosa cells [48]. FGF-2 protein is activated by the PI3K/AKT pathway to protect granulosa cells against FasL-mediated apoptosis and also inhibits p53-induced apoptosis [49]. In a recent study, the effect of FGF-2 supplementation improved revascularization, oocyte quality, and survival of grafted OT after cryopreservation [50]. In the present study, decreased Fgf-2 expression after OTC and re-transplantation processes may be responsible for decreased KitL expression; thus, this could be a mechanism to recompensate PF activation and burn-out. Additionally, decreased Fgf-2 expression in PFs could lead to loss of protection from FasL-mediated and p53-induced apoptosis and thus could lead to subsequent follicle loss. Altogether, this study's results indicate that disturbed expression of Gdf-9, Bmp-15, KitL, Lif, and Fgf-2 proteins after OTC and re-transplantation may contribute to depletion of PF reserve and to follicular growth impairment.

Recently, it has been found that rapamycin maintains PF reserve and prolongs the ovarian lifespan in female rats via modulating mTOR activation. In rats treated with rapamycin, the number of PFs was twice that of the control group, suggesting that rapamycin preserves PF number [51]. Pharmacological inhibition of mTORC1 prevented over-activation of the PF pool in response to elevated PI3K signaling and thus preserved the PF pool and prevented POF [52]. Another recent study showed that short-term (2 weeks) treatment of rapamycin (2 mg/kg) increased ovarian lifespan in young (8 weeks) and middle-aged (8 months) female mice [25]. In 2017, Zhou et al. found that cyclophosphamide-induced over-activation of the PF pool was prevented through the inhibition of the mTOR signaling pathway, suggesting that rapamycin may be an effective protector for ovarian function when used during chemotherapy [53]. In the same year, Goldman et al. found that blocking mTOR with clinically available small-molecule inhibitors (a clinically approved drug everolimus—RAD001—or experimental drug INK128) preserves ovarian reserve and maintains PF counts, serum anti-Mullerian hormone levels, and fertility during cyclophosphamide chemotherapy in mice [54]. In our study, rapamycin was dissolved in ethanol with 5.2% Tween-80 and 10% polyethylene glycol. Although this handling method is used for application of rapamycin to research animals, clinically available small-molecule inhibitors such as RAD001 and INK128 have the potential to be used in humans.

Although recent literature demonstrated the importance of inhibition of the mTOR signaling pathway via rapamycin for PF survival in gonadotoxic treatment models [55], it was not known whether the same approach could preserve ovarian reserve after OTC and re-transplantation. Therefore, this study firstly investigated whether the mTOR signaling pathway was activated after OTC and re-transplantation and found that vitrification and warming of OT alone significantly increased the p-s6K expression in PFs. Secondly, it was aimed to inhibit this pathway by injecting rats with 1 mg/kg rapamycin every day for 7 days, after re-transplantation of OTs to their back muscle immediately. The tissues were harvested 14 days after transplantation, the primordial and growing follicles were counted, and it was found that the PF number was significantly higher in the rapamycin-treated control, fresh-transplanted, and frozen/thawed and transplanted groups, indicating that PF loss could be prevented by rapamycin in an OTC and re-transplantation model. A recent study published in 2020 by a Japanese group indicates that rapamycin treatment maintains developmental potential of oocytes in mice and PF pool in human cortical fragments [56]. They confirmed that rapamycin treatment for 3 weeks can suppress PF development and maintains follicle reserve in mice. They also showed that the developmental potential of oocytes was not affected by rapamycin treatment and the effect of rapamycin to decrease initial follicle recruitment is reversible [56]. Moreover, they used human ovarian cortical fragments and demonstrated that rapamycin treatment can inhibit follicle growth from the primordial stage [56]. Findings of this study well support our results. However, in this study, immature mice were treated with rapamycin (15 mg/kg body weight, i.p.) every other day for 3 weeks [56]. In our study, we showed that even a much lesser dose of rapamycin (1 mg/kg body weight) and less treatment time period (1 week) is effective to decrease p-s6K expression, thus inhibiting mTOR pathway and protecting PF reserve in rats. In a recent study about follicular activation in humans, it has been shown that mTOR levels in primordial follicles did not change after transplantation; however, its expression increased in growing follicles irrespective of grafting time [57]. In a recent study about follicular activation in humans, it has been shown that mTOR levels in primordial follicles did not change after ovarian tissue transplantation; however, its expression increased in growing follicles irrespective of grafting time [57]. Our results are in accordance with this human study since we did not observe an increase in pS6K expression, a well-known final marker of mTOR pathway activation, after fresh or frozen ovarian tissue transplantation. Moreover, a significant mTOR pathway activation was observed, indicated by pS6K expression, in primordial follicles with additional experimental groups in our study where ovarian tissues were solely frozen and thawed without transplantation.

Rats were treated with rapamycin for 7 days since it is known that the hypoxic period ends after 5–7 days after re-transplantation due to neovascularization of the ovarian graft [58–60]. It is known that extensive PF activation and loss is observed as early as 3 days post-transplantation in OT grafts, indicating that follicle loss occurs within a very short time frame after grafting [38]. In this study, although the dose (1 mg/kg/day) and the duration (7 days) of rapamycin treatment were effective to preserve the PF pool, different doses, time points, and/or administration methods of rapamycin can enhance the success of preservation of the PF pool, which needs further investigation. According to the rapamycin treatment experiments, our immunohistochemistry results indicate low expression of p-s6K while there is significant p-s6K expression in western blot analysis. We argue that the two different techniques, immunohistochemistry and western blot, may have different detection levels for the p-s6K antibody which has been used for both methods in our study. Moreover, both techniques noticeably show the difference of p-s6K expression between vehicle-treated and rapamycin-treated groups.

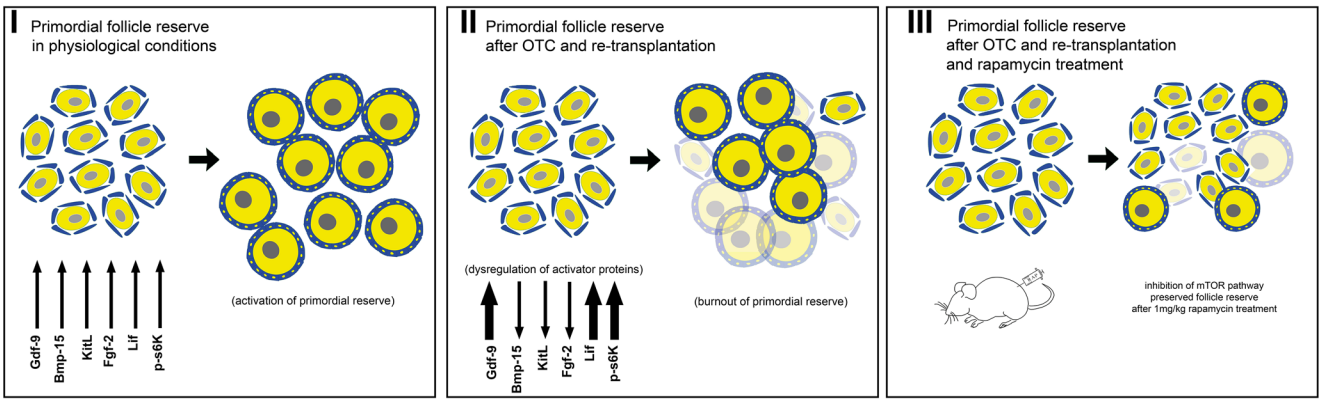


Fig. 10 A hypothetical mechanism regarding the possible roles of the activator proteins that regulate primordial follicle reserve in (I) physiological conditions and after. (II) Ovarian cryopreservation and transplantation. (III) Increased mTOR pathway activation (as determined by up-regulated p-s6K expression in primordial follicles) after OTC can be suppressed by rapamycin treatment in OTC and re-transplantation and rapamycin treated rats. Thin upward arrows: activity of proteins, thick upward arrows: increased expression of proteins, downward arrows: decreased expression of proteins

In conclusion, the results of this study demonstrate that expression of activator proteins that regulate the PF reserve and follicle growth are disturbed following OTC and re-transplantation. Increased expression of Gdf-9 and Lif and decreased expression of Bmp-15, KitL, and Fgf-2 in PF after OTC and transplantation may lead to decreased ovarian reserve and increased atresia of the follicles. Moreover, increased mTOR pathway activity as indicated by increased p-s6K expression after the vitrification and warming process could be one of the key contributors for PF activation and subsequent follicle loss. Rapamycin treatment was effective in the inhibition of the mTOR pathway and accordingly preserved the PF pool after transplantation of OT. Thus, mTOR pathway activation seems to be one of the major causes for PF loss after OTC and transplantation. A hypothetical mechanism related to our findings is illustrated in Fig. 10. This study highlights particular molecular aspects that are related to fertility preservation and follicular loss after OTC. Rapamycin and other mTOR inhibitors are candidate molecules in preserving fertility for patients who undergo OTC and re-transplantation and/or who receive gonadotoxic treatments.

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Compliance with ethical standards

The experimental protocol was approved by the Animal Care and Use Committee at Akdeniz University, Faculty of Medicine (Ethical Approval Protocol: 2017.07.005), and was in accordance with the Declaration of Helsinki and the International Association for the Study of Pain guidelines.

Conflict of interest The authors declare that they have no competing interests.

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Part of this study has been presented as a poster presentation at The International Society for Fertility Preservation (ISFP) Meeting that has been held in NYC, USA, between 14 and 16 November 2019 and Soner Celik received the award for outstanding abstract.