Caloric Restriction Promotes the Reserve of Follicle Pool in Adult Female Rats by Inhibiting the Activation of Mammalian Target of Rapamycin Signaling

Reproductive Sciences
2015, Vol. 22(1) 60-67
© The Author(s) 2014
Reprints and permission:
sagepub.com/journalsPermissions.nav
DOI: 10.1177/1933719114542016
rs.sagepub.com

Li Li, MD¹, Yu-cai Fu, PhD², Jin-jie Xu, MD², Xuan-hao Lin, MD³,
Xiao-chun Chen, MD¹, Xing-mei Zhang, MD⁴, and Li-li Luo, MD¹

Abstract

Caloric restriction (CR) is known to increase the number of primordial follicles and prolong the reproductive life span. However, how CR modulates follicular development is not well understood. In the present study, we examined the effects of CR on follicular development in rats and investigated the underlying mechanism. After 10 weeks of CR or high-fat diet, ovarian follicles at different developmental stages were examined by histological analysis. Plasma levels of luteinizing hormone (LH), follicle-stimulating hormone (FSH), and estrogen (ESG) were measured, and the levels of mammalian target of rapamycin (mTOR), p70S6 kinase (p70S6K), and phosphorylated p70S6K in the ovary were detected by Western blot. The results showed that the reserve of follicle pool in CR rats was increased, accompanied by decreased level of phosphorylated p70S6K in the ovary, and decreased serum LH, FSH, and ESG levels. Taken together, these results suggest that CR may suppress ovarian follicular development and enhance the follicle pool reserve by inhibiting mTOR signaling.

Keywords

caloric restriction, mTOR, primordial follicle, luteinizing hormone, estrogen

Introduction

Restricting caloric intake has been practiced as a method for increasing both the length and quality of life for more than 500 years.¹ It is now established that caloric restriction (CR) prolongs life span and delays the aging process. In addition, CR could increase the number of primordial follicles and the duration of fertility in mice.² Furthermore, CR could increase the number of primordial follicles and reduce the number of atretic follicles, suggesting that CR inhibits the transition of primordial follicles to primary follicles, resulting in delayed follicular depletion.^{3,4}

Ovarian follicles are important components of a mammalian ovary. Exhaustion of the follicle pool results in menopause and the end of female reproductive life. Therefore, to maintain the length of female reproductive life, the majority of primordial follicles must be preserved in a quiescent state, with a limited number of primordial follicles being recruited from the resting follicle reservoir into the growing follicle pool.⁵ Consequently, the reproductive life span of a female depends on both the size of the resting pool of ovarian primordial follicles and the rate of their activation and depletion.⁶ The activation of primordial follicles is a highly regulated process, and the underlying mechanisms are not fully understood.⁷⁻⁹

Recent studies suggest that the mammalian target of rapamycin (mTOR) signaling pathway is implicated in the activation of primordial follicles.^{10,11} Upon the disruption of either tuberous sclerosis complex 2 or tuberous sclerosis complex 1 gene in mice, the primordial follicle pool was activated prematurely due to an elevated mTOR complex (mTORC1) and phosphorylated p70S6 kinase (p70S6K) activity in the oocytes, resulting in the depletion of follicles in early adulthood and premature ovarian failure.¹⁰

Mammalian target of rapamycin is a ubiquitous serine/threonine kinase regulated by nutrients, different stressors, growth factors, and hormones, and it participates in the control

¹ Department of Gynaecology and Obstetrics of the First Affiliated Hospital, Shantou University Medical College, Shantou, China

² Laboratory of Cell Senescence, Shantou University Medical College, Shantou, China

³ Department of Pathology of Shantou Central Hospital, Shantou, China

⁴ Department of Gynaecology and Obstetrics of HuiZhou Municipal Central Hospital, Huizhou, China

Corresponding Author:

Li-li Luo, Department of Gynaecology and Obstetrics of the First Affiliated Hospital, Shantou University Medical College, Shantou, 515041, China.
Email: luolili6606@163.com

of key cellular functions such as cell proliferation, growth, and metabolism. Mammalian target of rapamycin exists in 1 of 2 complexes, mTORC1 and mTORC2.¹²⁻¹⁶ Mammalian target of rapamycin complex 1 is responsible for the integration of nutritional and metabolic cues, as well as different stressors, mostly by targeting p70S6K and 4E-binding protein 1. Mammalian target of rapamycin complex 1 directly phosphorylates p70S6K, which is strictly rapamycin sensitive.^{17,18}

Based on these studies, we speculate that mTOR signaling pathway may play a crucial role in the activation of primordial follicles and regulation of follicular development under CR conditions. In the present study, we subjected adult female rats to CR to investigate the effects of CR on the ovarian follicle development and follicle pool reserve. Furthermore, we explored the association of CR-mediated effects on ovarian follicle development with mTOR signaling.

Materials and Methods

Animals and Groups

Thirty-seven 8-week-old Sprague-Dawley female rats (185-220 g) were obtained from the Animal Center of Shantou University Medical College. All of the rats were housed individually in steel cages with free access to tap water. Rats were maintained under a 12/12-hour dark–light cycle at 22°C to 24°C. After a 2-week adaptation period, rats were randomly divided into the following 3 groups: (1) rats in the control group were fed a standard rodent chow (purchased from Shanghai Laboratory Animal Research Center, Shanghai, China) with 24% protein, 4% fat, 4.5% crude fiber, and gross energy (GE) content of 17.3 MJ/kg; (2) rats in the CR group were fed 55% amount of the standard chow consumed by the control group; and (3) rats in the high-fat diet (HF) group were fed high-fat food prepared by our laboratory by adding 10% lard (wt/wt), 3% yolk (wt/wt), 1% cholesterol (wt/wt), and 0.5% sodium cholate (wt/wt) to the standard rodent chow, with a GE content of 19.3 MJ/kg (the overall fat content of this chow was 13.42%). The food intake of the control rats was recorded daily, and the food supply of rats in the CR group was adjusted accordingly.

All rats were handled daily, weighed weekly, and sampled after 10 weeks of dietary treatment. No tumors were observed after dissecting the abdominal cavities. The experimental protocols were approved by the Animal Care and Use Committee of Shantou University Medical College.

Assessment of the Estrous Cycle

The estrous cycles were determined daily, between 8:00 AM and 9:00 AM, by inserting a stick 0.5 cm into the rat vagina, rotating 2 to 3 revolutions against the vaginal wall, and taking out and rolling on a clean glass slide to form 2 parallel tracks of smears. Unstained materials were observed under a light microscope to analyze the proportion of 3 major cell types (epithelial cells, cornified cells, and leukocytes) as described previously.¹⁹ A

4- to 5-day estrous cycle was determined to be a regular cycle, and a cycle duration of >5 days or <4 days was considered to be an irregular cycle.²⁰ Rats displaying at least 4 consecutive regular cycles a month were defined as regular cycling rats, while those displaying constant estrus, intermittent regular, or intermittent irregular estrous cycles were defined as irregular cycling rats. Rats with few or no cells in vaginal secretion for at least 20 days were defined as having ceased their estrous cycle.

Preparation of Ovary Sections

Rats were anesthetized by intraperitoneal injection of pentobarbital at 0.3 mg/100 g body weight. Both ovaries from each rat were isolated and weighed. One ovary was stored at -80°C for further analysis, and one was fixed in 4% paraformaldehyde at 4°C overnight and subsequently dehydrated using a series of ethanol washes, cleared in xylene, and embedded in paraffin. Ovarian tissue sections of 6-μm thickness were prepared, deparaffinized in xylene, hydrated through an ethanol series of 100%, 90%, 80%, 70%, and 50%, and stained with hematoxylin and eosin.

Follicular Classification

Seven representative sections, each separated by 100 μm (to avoid counting the same follicles twice), were selected from each ovary. A primordial follicle was defined as an oocyte surrounded by a layer of squamous (flattened) granulosa cells. A follicle was determined to be a primary follicle if it contained an oocyte that was surrounded, in part or entirely, by a single layer of cuboidal granulosa cells. A follicle was determined to be a secondary follicle if it was surrounded by more than one layer of cuboidal granulosa cells, with no visible antrum. A follicle was determined to be an antral follicle if it possessed a clearly defined antral space and a cumulus granulosa cell layer. Atretic follicles had abnormal structures, such as inspissated follicular fluid, degenerated oocytes, or thickened granulosa layers, or were filled with organized fibrous material in the antrum (Figure 1).²¹ The number of follicles at each stage and the percentage of the total number of follicles were examined and calculated. All counts were repeated 3 times.

Immunohistochemical Analysis

Deparaffinized ovarian sections were blocked in 2% bovine serum albumin in phosphate-buffered saline (PBS) and then incubated with primary antibody against mTOR (1:50, Santa Cruz, California), p70-S6K (1:50, Santa Cruz, California), or phosphorylated p70S6K (1:1000, Santa Cruz, California) overnight at 4°C. Sections were subsequently incubated with biotinylated antirabbit immunoglobulin G (1:200 or 1:2000, Jackson Immuno Research, West Grove) for 1 hour at room temperature, avidin–biotin complex reagent (Vector Laboratories, California) for 30 minutes at room temperature, and

Figure 1. Representative hematoxylin and eosin-stained histological images of follicles at different developmental stages. A, Primordial follicle, with 1 oocyte and surrounded by 1 layer of flattened somatic cells; (B) primary follicle, with 1 layer of cuboidal granulosa cells; (C) secondary follicle, with 2 to 4 layers of granulosa cells, without antral space; (D) antral follicle, with 3 or more layers of granulosa cells with 1 or more antral space; (E) corpus luteum; and (F) atretic follicle, with apoptotic bodies of granulosa cells and fragmentation of the oocyte nucleus.

developed in PBS containing 0.5 mg/mL 3,3-diaminobenzidine tetrahydrochloride. For the negative controls, PBS was used instead of primary antibody. Sections were then counterstained with hematoxylin and analyzed under light microscopy.

Enzyme-linked Immunosorbent Assay

To avoid the effects of cyclical variations in the estrous cycle, blood samples were collected at diestrus, using a tail bleed from living rats or directly from the hearts of killed animals. Blood samples were centrifuged at 4000 rpm for 15 minutes, and serum was stored at -80°C . The serum concentrations of luteinizing hormone (LH), follicle-stimulating hormone (FSH), and estrogen (ESG) were measured using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Shanghai, China) according to the manufacturer's instructions.

Western Blot Analysis

Ovaries were homogenized in radio-immunoprecipitation assay lysis buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1% sodium deoxycholate, 1%NP-40, 1 mmol/L phenylmethylsulfonyl fluoride and 1 mmol/L EDTA) with a Teflon-glass homogenizer on ice, and centrifuged at 12 000 rpm for 20 minutes at 4°C . The supernatants were collected and protein concentrations were determined by Bradford assay. The protein samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. Membranes were incubated with 10% nonfat milk in tris-

buffered saline containing 0.005% Tween 20 at room temperature for 30 minutes. Next, the membranes were incubated with a primary antibody against mTOR (1:500, Santa Cruz), p70-S6K (1:500, Santa Cruz), or phosphorylated p70S6K (1:1000, Santa Cruz, California) overnight at 4°C and then incubated with peroxidase-conjugated secondary antibody at room temperature for 1 hour. The bands were visualized with a chemiluminescence reagent (Thermo Fisher Scientific, Waltham, Massachusetts). Band intensities were analyzed using Quantity One software (Bio-Rad Laboratories, Berkeley, California).

Statistical Analysis

Data were presented as mean $\pm$ standard error of the mean (SEM) and analyzed by SPSS 13.0 software. A one-way analysis of variance was used to compare the data among groups. The chi-square test was used to compare the percentage of follicles at each stage. $P < .05$ was considered statistically significant.

Results

Effects of Different Dietary Regimens on Rat Body Mass and Ovarian Weight

No differences in body mass and ovarian weight were observed among the 3 groups before the initiation of dietary regimens. The mean body mass increased by 27% after 10 weeks of treatment (199.7 ± 5.0 g at the beginning vs 252.7 ± 10.3 g at the

Table 1. Characteristics of Vaginal Smears at Different Stages of a Normal 4-Day Estrous Cycle.

Estrous Stage	Duration (day)	Appearance
Diestrus	1.5	Primarily consists of a predominance of leukocytes
Proestrus	1.0	Consists of a predominance of nucleated epithelial cells
Estrus	1.0	Primarily consists of anucleated cornified cells
Metestrus	0.5	Consists of the same proportion of leukocytes, cornified cells, and nucleated epithelial cells

Table 2. The Percentages of Rats at Different Estrous Cycle Stages Among Treatment Groups.

Time and Estrous Cycle Stage	45% CR Group (n = 15)	Control Group (n = 12)	High-Fat Group (n = 10)
1 week			
Regular cycling	15	12	10
Irregular cycling	0	0	0
4 weeks			
Regular cycling	13 (86.67%)	12	10
Irregular cycling	2 (13.33%)	0	0
6 weeks			
Regular cycling	12 (80%)	12	10
Irregular cycling	3 (20%)	0	0
8 weeks			
Regular cycling	10 (66.67%)	12	8 (80%)
Irregular cycling	5 (33.33%)	0	2 (20%)
10 weeks			
Regular cycling	6 (40%)	12	7 (70%)
Irregular cycling	9 (60%)	0	3 (30%)

Abbreviation: CR, caloric restriction.

end) in the control group, increased by 38% in the HF group (from 198.5 ± 2.8 g to 273.8 ± 6.2 g), but decreased by 10% in the CR group (from 198.3 ± 4.04 g to 178.1 ± 2.40 g). The gross ovarian weight of control rats was twice that of the CR group (0.063 ± 0.005 g vs 0.032 ± 0.002 g, $P < .01$) but similar to that of the HF group (0.068 ± 0.004 g). There were no significant differences in body mass and ovarian weight between the control group and the HF group ($P > .05$).

Assessment of Estrous Cycles in Different Groups of Rats

The estrous cycle is a convenient parameter to indicate ovarian activity, which is regulated through the hypothalamus–pituitary–gonadotrophic axis.²² Table 1 shows the criteria for the determination of the phase of a normal 4-day estrous cycle. Table 2 shows the estrous cycle status of rats in different groups. During the study, some rats gradually lost their regular estrous cycles.

After 4 weeks of dietary treatment, 13.3% (2 of 15) of the CR rats exhibited irregular estrous cycles. After 8 weeks of dietary treatment, 33.3% (5 of 15) of the CR rats and 20.0% (2 of 10) of the HF rats exhibited irregular estrous cycles. At the end of the experiment, 60.0% (9 of 15) of CR rats and 30.0% (3 of 10) of the HF rats exhibited irregular estrous cycles. Over half of the vaginal smears from irregular estrous cycles of rats were leukocytic (ie, diestrus). The others showed a constant estrus, among which 1 rat maintained a 22-day estrus state. In contrast, control rats had regular estrous cycles during this study. These data indicate that dietary treatment affects ovary activity.

Effects of Different Dietary Regimens on Rat Ovarian Follicular Reserve

To examine the effects of different dietary regimens on ovarian follicle differentiation and development, we examined the distribution of various follicular stages in adult rats for 10 weeks. Representative images of hematoxylin and eosin staining of follicles at different developmental stages were shown in Figure 1. We characterized the number of ovarian follicles in the primordial, primary, secondary, antral, and atretic developmental stages. The number of primordial follicles was higher in the CR group than in the control and HF groups (129.4 ± 7.8 vs 39.3 ± 3.3 , $P < .01$; 129.4 ± 7.8 vs 29.9 ± 2.0 , $P < .01$; respectively; Figure 2). The percentage of primordial follicles was greater in the CR group ($37.5\% \pm 1.3\%$) than in the control ($14.7\% \pm 1.4\%$; $P < .01$) and HF groups ($11.0\% \pm 0.8\%$; $P < .01$). Although the number and percentage of primary follicles were lower in the CR group, the number of secondary follicles was not significantly different among the 3 groups. However, the percentage of secondary follicles was lower in the CR group. These data suggest that CR may suppress the follicular transformation from primordial follicles to primary and secondary follicles.

In addition, we found that there was a greater number of atretic follicles in the HF group compared to the control group (79.4 ± 3.2 vs 61.6 ± 1.5 , $P < .01$). The percentage of atretic follicles was greater in the HF group than in the control group ($29.1\% \pm 1.0\%$ vs $23.0\% \pm 0.7\%$, $P < .05$) but was lower in the CR group than in the control group ($16.7\% \pm 0.6\%$ vs $23.0\% \pm 0.7\%$, $P < .05$). The total number of surviving follicles in the CR group (231.5 ± 12.0) was more than that of the control (154.8 ± 8.1 ; $P < .05$) or HF group (141.7 ± 5.2 ; $P < .05$). These results indicate that an HF may facilitate follicular atresia.

Effects of CR on Serum Levels of Gonadal Hormones

To examine the effects of CR on serum levels of gonadal hormones, we measured serum levels of LH, FSH, and ESG by ELISA. The results showed that ESG and LH levels decreased in the CR group and increased in the HF group compared to the control group (Figure 3A and B). The FSH level decreased in the CR group but not changed in the HF group (Figure 3C). Taken together, these data suggest that CR may inhibit the

Figure 2. Analysis of the number of follicles at each stage and the percentage of different follicles in 3 groups. A, The number of follicles at each stage. Differences among groups were analyzed by one-way analysis of variance (ANOVA). B, The percentage of different follicles. Differences among groups were analyzed by chi-square test. Data were presented as means $\pm$ standard error of the mean (SEM). * $P < .05$ versus the control group.

release of gonadal hormones, while HF may promote the release of LH and ESG.

Status of mTOR Signaling During Ovarian Follicle Development

To determine whether mTOR signaling is involved in ovarian follicle development, we performed immunohistochemical

Figure 3. Changes in serum levels of LH, FSH, and ESG in 3 groups. Blood samples were collected from rats in the control, CR, and HF groups via the tail veins every 3 weeks and serum levels of ESG (A), LH (B), and FSH (C) were measured by ELISA. The values were represented as means $\pm$ standard error of the mean (SEM). LH indicates luteinizing hormone, FSH, follicle-stimulating hormone; ESG, estrogen; CR, caloric restriction; HF, high-fat diet; ELISA, enzyme-linked immunosorbent assay.

analysis of mTOR, p70-S6K, and phosphorylated p70S6K in the ovaries. Mammalian target of rapamycin was extensively expressed in surviving follicles in every stage but not in atretic follicles. Mammalian target of rapamycin was strongly stained in both the nucleus and the cytoplasm of the oocyte and weakly stained in the granulosa cells of the surviving follicles. With the development of a follicle, mTOR staining gradually weakened (Figure 4). The staining of p70-S6K and phosphorylated p70S6K showed similar patterns as that of mTOR (data not shown). These data suggest that mTOR signaling may regulate follicular development.

Figure 4. Staining of mTOR during follicle development. mTOR was present in both the nucleus and the cytoplasm of the oocytes. A, Primordial follicle (indicated by arrows); (B) secondary follicle; (C) preantral follicle; (D) antral follicle (indicated by arrows); (E) atretic follicle; and (F) negative control. Scale bar: 10 μ m. mTOR indicates mammalian target of rapamycin.

Caloric restriction Inhibits the Activation of mTOR Signaling in Rat Ovary

To determine whether mTOR signaling is involved in the effects of CR on ovarian follicle development, we performed Western blot analysis of mTOR, p70-S6K, and phosphorylated p70S6K proteins in the ovaries. The results showed that the levels of mTOR and p70S6K did not differ significantly between the control group and 2 experimental groups ($P > .05$). However, phosphorylated p70S6K level was decreased in the CR group compared to the control group ($P < .01$; Figure 5). These data suggest that CR may inhibit the activation of mTOR signaling in the ovary.

Discussion

Ovarian follicular development is regulated by a variety of endocrine, autocrine, and paracrine factors.⁶ The oocyte reserve is crucial for female reproductive function. Factors that augment primordial follicle reserve and reduce follicle atresia could promote the reproductive potential, prolong the duration of female fertility, and shorten the life proportion after menopause.⁷⁻⁹

As a mediator of dietary effects, mTOR signaling pathway is well positioned to sense cellular nutrient and energy levels to control cellular function.²³ Caloric restriction has been shown to suppress TORC1 expression and the activation of mTOR signaling.^{18,24} Previous findings suggested that suppressed mTOR signaling activity in oocytes could preserve

primordial follicles in a dormant state.¹¹ Consistent with this, we found that rats exposed to CR had suppressed mTOR signaling activity, accompanied by a higher number and percentage of primordial follicles, and a lower percentage of primary follicles, secondary follicles, and atretic follicles. Because of the reduced primordial follicle transformation into primary follicles, the total number of surviving follicles in rats with suppressed mTOR signaling activity was higher, resulting in more oocytes with reproductive potential and prolonged reproductive life span. These data suggest that CR may augment the reserve of oocyte pool in female rats via suppressing the activation of mTOR signaling.

In addition, we found that the number of atretic follicles was higher in the HF group than in the control group, suggesting that an HF can promote oocyte apoptosis. All these data demonstrate that HF and CR have opposite effects on follicular development in female rats. However, we found no difference in the number of primordial follicles between the HF group and the control group. We suspect that this may be due to the exposure time, which was too short to change the oocyte pool.

Mammalian target of rapamycin signaling is known to impact the release of gonadal hormones. Chronic blockade of mTOR by rapamycin has dramatic inhibitory effects on circulating LH and estrogen levels in female rats.²⁵ The gonadotrophic hormones LH and FSH play a primary role in the regulation of peripheral reproductive tissue function and modulate the synthesis and release of estrogens, which are crucially involved in the development and normal physiological function

Figure 5. Western blot analysis of mTOR signaling in the ovaries of 3 groups. A, Representative blots showing the detection of mTOR, p70S6K, and p-p70S6K in the ovary. β -actin was loading control. B, Densitometry analysis of p-p70S6K level in the ovary. Values were expressed as means $\pm$ SEM from 3 independent experiments.

** $P < .01$, CR group versus control group. mTOR indicates mammalian target of rapamycin; p70S6K, p70S6 kinase; p-p70S6K, phosphorylated p70S6K; SEM, standard error of the mean; CR, caloric restriction.

of female reproductive systems.²⁶ Our results showed that CR decreased LH, FSH, and ESG levels. In contrast, LH and ESG levels were increased in the HF group. These data suggest that mTOR signaling may regulate ovarian follicular development by affecting steroidogenesis in rats exposed to CR.

In summary, in this study, we demonstrated that CR can suppress mTOR signaling pathway in the ovary and promote the reserve of follicle pool in female rats. These findings will help develop novel strategies to maintain reproductive health and delay reproductive aging.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by the National Natural Science Foundation of China (No. 81070458, 81270670).

References

1. Speakman JR, Mitchell SE. Caloric restriction. *Mol Aspects Med.* 2011;32(3):159-221.
2. Nelson JF, Gosden RG, Felicio LS. Effect of dietary restriction on estrous cyclicity and follicular reserves in aging C57BL/6 J mice. *Biol Reprod.* 1985;32(3):515-522.
3. Selesniemi K, Lee HJ, Tilly JL. Moderate caloric restriction initiated in rodents during adulthood sustains function of the female reproductive axis into advanced chronological age. *Aging Cell.* 2008;7(5):622-629.
4. Li L, Fu YC, Xu JJ, Chen XC, Lin XH, Luo LL. Caloric restriction promotes the reproductive capacity of female rats via modulating the level of insulin-like growth factor-1 (IGF-1). *Gen Comp Endocrinol.* 2011;174(2):232-237.
5. McGee EA, Hsueh AJ. Initial and cyclic recruitment of ovarian follicles. *Endocr Rev.* 2000;21(2):200-214.
6. Erickson GF. Role of growth factors in ovary organogenesis. *J Soc Gynecol Invest.* 2001;8:S13-S16.
7. Liu K, Rajareddy S, Liu L, et al. Control of mammalian oocyte growth and early follicular development by the oocyte PI3 kinase pathway: new roles for an old timer. *Dev Biol.* 2006;299(1):1-11.
8. Adhikari D, Liu K. Molecular mechanisms underlying the activation of mammalian primordial follicles. *Endocr Rev.* 2009;30(5):438-464.
9. Reddy P, Zheng W, Liu K. Mechanisms maintaining the dormancy and survival of mammalian primordial follicles. *Trends Endocrinol Metab.* 2010;21(2):96-103.
10. Adhikari D, Flohr G, Gorre N, et al. Disruption of Tsc2 in oocytes leads to overactivation of the entire pool of primordial follicles. *Mol Hum Reprod.* 2009;15(12):765-770.
11. Adhikari D, Zheng W, Shen Y, et al. Tsc/mTORC1 signaling in oocytes governs the quiescence and activation of primordial follicles. *Hum Mol Genet.* 2010;19(3):397-410.
12. Schmelzle T, Hall MN. TOR, a central controller of cell growth. *Cell.* 2000;103:253-262.
13. Martin DE, Hall MN. The expanding TOR signaling network. *Curr Opin Cell Biol.* 2005;17(2):158-166.
14. Wullschlegel S, Loewith R, Hall MN. TOR signaling in growth and metabolism. *Cell.* 2006;124:471-484.
15. Chiang GG, Abraham RT. Targeting the mTOR signaling network in cancer. *Trends Mol Med.* 2007;13(10):433-442.
16. Tsang CK, Qi H, Liu LF, Zheng XF. Targeting mammalian target of rapamycin (mTOR) for health and diseases. *Drug Discov Today.* 2007;12(3-4):112-124.
17. Thoreen CC, Sabatini DM. Rapamycin inhibits mTORC1, but not completely. *Autophagy.* 2009;5(5):725-726.
18. Blagosklonny MV. Calorie restriction: decelerating mTOR-driven aging from cells to organisms (including humans). *Cell Cycle.* 2010;9(4):683-688.
19. Marcondes FK, Bianchi FJ, Tanno AP. Determination of the estrous cycle phases of rats: some helpful considerations. *Braz J Biol.* 2002;62(4A):609-614.
20. Schedin P, Mitrenga T, Kaeck M. Estrous cycle regulation of mammary epithelial cell proliferation, differentiation, and death in the Sprague-Dawley rat: a model for investigating the role of

- estrous cycling in mammary carcinogenesis. *J Mammary Gland Biol Neoplasia*. 2000;5(2):211-225.
21. Luo LL, Huang J, Fu YC, Xu JJ, Qian YS. Effects of tea polyphenols on ovarian development in rats. *J Endocrinol Invest*. 2008;31(12):1110-1118.
 22. Tsukamura H, Thompson RC, Tsukahara S, et al. Intracerebroventricular administration of melanin-concentrating hormone suppresses pulsatile luteinizing hormone release in the female rat. *J Neuroendocrinol*. 2000;12(6):529-534.
 23. Tokunaga C, Yoshino K, Yonezawa K. mTOR integrates amino acid- and energy-sensing pathways. *Biochem Biophys Res Commun*. 2004;313(2):443-446.
 24. Tzatsos A, Kandror KV. Nutrients suppress phosphatidylinositol 3-kinase/Akt signaling via raptor-dependent mTOR-mediated insulin receptor substrate 1 phosphorylation. *Mol Cell Biol*. 2006;26(1):63-76.
 25. Roa J, Garcia Galiano D, Varela L, et al. The mammalian target of rapamycin as novel central regulator of puberty onset via modulation of hypothalamic Kiss1 system. *Endocrinology*. 2009;150(11):5016-5026.
 26. Gibson DA, Saunders PT. Estrogen dependent signaling in reproductive tissues - a role for estrogen receptors and estrogen related receptors. *Mol Cell Endocrinol*. 2012;348(2):361-372.