Immunohistochemical determination of mTOR pathway molecules in ovaries and uterus in rat estrous cycle stages

Authors: Gulcin Ekizceli, Sevinc Inan, Gulperi Oktem, Ece Onur and Kemal Ozbilgin

DOI: 10.14670/HH-18-258
Article type: ORIGINAL ARTICLE
Accepted: 2020-09-17
Epub ahead of print: 2020-09-17

This article has been peer reviewed and published immediately upon acceptance. Articles in “Histology and Histopathology” are listed in Pubmed. Pre-print author’s version
Title: Immunohistochemical Determination of mTOR Pathway Molecules in Ovaries and Uterus in Rat Estrous Cycle Stages

Gulcin Ekizceli 1, Sevinc Inan 2, Gulperi Oktem 3, Ece Onur 4, Kemal Ozbilgin 5

Affiliations:

1 PhD. Gulcin Ekizceli; Department of Histology and Embryology, Faculty of Medicine, Istanbul Health and Technology University, Istanbul, Turkey.

2 Prof. Dr. Sevinc Inan; Department of Histology and Embryology, Izmir Economy University, School of Medicine, Izmir, Turkey.

3 Prof. Dr. Gulperi Oktem; Department of Histology and Embryology, Ege University School of Medicine, Izmir, Turkey.

4 Prof. Dr. Ece Onur; Department of Medical Biochemistry, Manisa Celal Bayar University, School of Medicine, Manisa, Turkey.

5 Prof. Dr. Kemal Ozbilgin; Department of Histology and Embryology, Manisa Celal Bayar University, School of Medicine, Manisa, Turkey.

* Corresponding author Gulcin Ekizceli at: Department of Histology and Embryology, Faculty of Medicine, Istanbul Health and Technology University, Istanbul, Turkey.

E-mail: ekizceli.g@gmail.com

Running Title: The importance of mTOR pathway in estrous cycle

* This research was presented as a poster presentation at the 38th FEBS Congress, held on July 6th - 11th, 2013 in St. Petersburg, Russia.
** This research was granted by Celal Bayar University, Scientific Research Project Committee (#2011/038).

Abstract

mTOR is a member of the PI3K/Akt/mTOR signaling pathway that participates in cell growth, proliferation, protein synthesis, transcription, angiogenesis, apoptosis and autophagy. mTOR and MAPK pathways are two important key signal pathways which are related to each other. We investigated the role of mTOR and other signaling molecules in rat ovaries and uteruses in stages of the estrous cycle. Young adult female rats were divided into four groups as proestrous, estrous, metestrous and diestrous according to vaginal smears. Immunohistochemical staining of mTORC1, IGF1, PI3K, pAKT1/2/3, ERK1 and pERK1/2 was performed and pAKT1/2/3 and ERK1 were also analyzed using western blotting on ovarian and uterine tissue samples. According to our results, PI3K/Akt/mTOR and ERK/pERK showed an increase in the rat ovulation period. When all the groups were evaluated the immunoreactivities for all of the antibodies were detected in the oocytes, granulosa and theca cells, corpus luteum and stroma of ovary and lamina propria, surface and glandular epithelium of uterus with the strongest observed with anti-ERK1 antibody and then with a decreasing trend with anti-mTORC1, anti-pAkt1/2/3, anti-IGF1, anti-PI3K and anti-pERK1/2 antibodies in the proestrus and estrus stages. Differently from other parts of the ovary, highest antibody expression in the corpus luteum was observed in the metestrous stage. Moreover, the existence of pAKT1/2/3 and ERK1 proteins was confirmed with the Western blotting technique. We suggest that mTOR and mTOR-related ERK signaling molecules may participate in the rat ovulation process.

Key words: ERK, estrous cycle, IGF1, mTORC1, ovulation, pAKT.
Introduction

Infertility is a global health problem affecting millions of estimated worldwide couples. Female factors are fully responsible for one third of the cases (Jumayev et al., 2012; Mascarenhas et al., 2012). Abnormalities in ovarian function, including defective oogenesis and folliculogenesis, represent a significant failure of female reproduction (Hillier et al., 2010). The two main functions of the ovary are the production of reproductive hormones that synchronize the reproductive cycle, and the feeding and release of oocytes that are capable of fertilization and development (Robker et al., 2009). Fertile adult women who plan to become pregnant may have ovulation and conception on any day of the menstrual cycle, although maximum likelihood is reached in the middle of the menstrual cycle (Wilcox et al., 2001). Although mechanisms that support and regulate the follicular system have been extensively studied, they are largely unclear, especially in humans (Skinner, 2005; Maheshwari and Fowler, 2008). The follicular microenvironment appears to be stimulated by close interaction of many growth factors (Maheshwari and Fowler, 2008; Dissen et al., 2009). Ovarian function in mammals is primarily regulated by endocrine factors, mainly gonadotropins (FSH and LH), their receptors (FSHR and LHR), and ovarian steroids (Ferreira et al., 2011). In addition to gonadotropins, several growth factors, including insulin-like growth factor (IGF), and intraovarian regulator play a critical role in the development and function of follicles (Mani et al., 2010; Piotrowska et al., 2013). It is known that IGF stimulates proliferation of granulosa and theca cells and increases the ability of gonadotropins to induce steroidogenesis in these cell populations. Furthermore, IGF has a direct antiapoptotic effect and is selectively expressed in healthy follicles as compared to atretic follicles. The Akt and ERK pathways are key signaling means that mediate the effects of IGF (Ryan et al., 2008).
The PI3K/mTOR/Akt pathway is the main regulator of many normal cellular processes, including many downstream targets, including cell proliferation, survival, growth, mobility, rearrangement of the cell skeleton, and metabolism (Thomas et al., 2006). At the membrane, PI3K catalyzes the phosphorylation of phosphatidylinositol-4,5-bisphosphate (PIP2) and converts it to phosphatidylinositol-3,4,5-trisphosphate (PIP3) which recruits downstream molecules with pleckstrin homology domains, particularly the serine-threonine kinases Akt (protein kinase B, PKB) and phosphatidylinositide-dependent kinase 1 (PDK1) to the membrane (Brown et al., 2010; McLaughlin et al., 2014).

mTOR is a serine threonine protein kinase that interacts with several proteins to form two different complexes, namely mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). Especially, mTORC1 integrates inputs from growth factors, stress, energy, oxygen and amino acids into control processes such as cell growth, proliferation and metabolism, including protein and lipid synthesis and autophagy (Hay, 2004; Wullschleger et al., 2006a). MTOR provides increased cellular survival by inhibiting autophagosom formation after a certain point (Munson et al., 2015). mTOR is known to play a role in primordial follicle activation through Akt in mice (Tong et al., 2013). The mTOR molecule is thought to be effective in folliculogenesis, especially at the stage of primordial follicle (Guo et al., 2019).

In this study, we wanted to search the possible role of mTOR pathway molecules in the rat reproductive process, we have analyzed the expression pattern and localization of mTOR pathway molecules such as mTORC1, IGF1, PI3K, pAKT1/2/3, ERK1 and pERK1/2 in the ovary and uterus during the rat estrous cycle using immunohistochemistry, and confirmed key molecules such as pAKT1/2/3 and ERK1/2 with Western blotting method.

The mechanisms that control and regulate the ovulation process and signaling pathways are essential for successful pregnancy, but also for the potential development of embryos to occur. In this study, the expression of key molecules of mTOR and ERK signaling pathways...
in ovarian and uterine tissues, which are important molecules in successful ovulation mechanisms and in obtaining healthy pregnancy, were investigated. In our study, rat ovarian and uterine specimens were used because of their similarity to human ovulation and short estrous cycle. These processes were clearly determined. To ensure successful ovulation, hypothalamic-pituitary-gonadal hormonal control is required, including growth factors (EGF, IGF), adhesion molecules (integrins, selectins, I-CAM, V-CAM) and various signaling pathways (mTOR, PI3K, AKT, ERK, RHO/ROCK, PTK2) in the regulation and integration of these processes.

The aim of this study was to research the expression and protein localization of these mTOR pathway molecules, such as PI3K, IGF1, pAKT1/2/3, MTORC1, and MAPK signalling pathway molecules, such as ERK1/2 and pERK1/2 in normal rat ovaries and uterus according to stages in the rat estrous cycle, such as proestrous, estrous, metestrous and diestrous, to understand possible roles of these molecules in follicular development.

**Material And Methods**

**Animals**

Our study was approved by Ege University Ethical Committee (29/07/2011; 2011-118). We obtained 12-week-old 200-225g Wistar albino female rats (n=28) from Ege University Animal Research Laboratories, Izmir. All animals were housed under standard laboratory conditions with a 12 h light:12 h dark cycle at 22±1° C and 50-60% humidity for at least 4 days before starting the experiments. Animals were provided standard chow and water ad libitum. All procedures were performed in accordance with guidelines and the ethical rules of the Ege University Ethical Committee on the “Guide for Care and Use of Laboratory Animals.
This study was approved by Ege University Ethical Committee (29/07/2011; 2011-118). Ovulation cycles were confirmed by vaginal smears as previously described by Marcondes et al. (MARCONDES et al., 2002). Rats were sacrificed after confirmation of estrous cycle at around 10:00 AM. Seven rats were used to study each period of the estrous cycle. We put the rats in the same cage in groups of seven to synchronize their cycles a week before the experiment. Each rat was examined by vaginal smear method one by one to verify their estrous cycle.

**Groups and vaginal smears**

Vaginal smears were obtained using a vaginal smear brush. The smears were fixed with methanol for 5 min, then stained with Giemsa solution for 10 min (Ozbilgin et al., 2012). The slides were washed under running tap water and air dried. The samples were viewed by light microscopy and rats were divided according to their estrous cycle for evaluation of ovulation cycle. Four groups were created based on estrous cycle stages as proestrous (Group 1, n= 7), estrous (Group 2, n= 7), metestrous (Group 3, n= 7) and diestrous (Group 4, n= 7).

**Tissue samples, processing and immunohistochemistry**

Female rats were sacrificed under general anesthesia and the right ovary and uterus were removed. After dividing into 1 cm³ pieces, the samples were fixed in 10 % formalin solution, dehydrated through increasing concentrations of ethanol, cleared in xylene and embedded in paraffin. Sections were cut at 5 µm and mounted on poly-L-lysinecoated slides, deparaffinized and rehydrated through descending concentrations of ethanol. Sections were heated in 10 mM citrate buffer, pH 6, containing triton X-100 0.1 % (Sigma-Aldrich, St.
Louis, MO) for antigen retrieval. After three washes with phosphate-buffered saline (PBS), sections were incubated with 0.3 % hydrogen peroxide in methanol for 30 min. to quench endogenous peroxidase activity. After washing with PBS, sections were incubated with a blocking serum (Histostain Plus Bulk Kit; Invitrogen Life Technologies, Camarillo, CA) at room temperature for 1 h. Primary antibodies, anti mTORC1 goat polyclonal antibody (SC-27744; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-IGF1 rabbit polyclonal antibody (SC-9013), anti-PI3K mouse monoclonal antibody (SC-1637), anti-pAKT1/2/3 rabbit polyclonal antibody (SC-35561), anti-ERK1 rabbit polyclonal antibody (SC-94) and anti-pERK1/2 rabbit polyclonal antibody (SC-101761); all antibodies were diluted 1:100 in blocking serum for immunohistochemistry, then added to the slides and incubated at 4° C overnight in a humidified chamber. After washing for 5 min. in PBS, sections were incubated with biotinylated antibody (Histostain Plus Bulk Kit, 85-9043; Invitrogen Life Technologies) for 30 min. Sections then were washed with PBS and incubated with streptavidin-conjugated horseradish peroxidase (Histostain Plus Bulk Kit, 85-9043; Invitrogen Life Technologies) for 30 min. Sections were washed with PBS for 5 min and DAB (DAB-Plus Substrate Kit; Invitrogen Life Technologies) was applied for color development to visualize antigen-antibody complexes. Sections were counterstained with Mayer’s hematoxylin. Control samples were processed in an identical manner, but without the primary antibody. Sections were dehydrated through a graded ethanol series, cleared in xylene, mounted in Entellan and analyzed using a BX40 light microscope (Olympus Corp., Tokyo, Japan). Two observers (G.E, S.İ) who were blinded to the treatment of each group, determined the immunohistochemical scores. The histochemical labeling procedure was repeated three times. Immunoreactivity scores were evaluated semiquantitatively as mild (1), moderate (2), strong (3) or very strong (4).
Protein extraction and Western blotting

The left ovaries and uterus were homogenized at 4° C in homogenization buffer (RIPA buffer; Santa Cruz Biotechnology Inc.). The homogenates then were centrifuged at 11269 × g at 4° C for 20 min. The supernatant was collected and protein concentration was determined using a Qubit fluorometer, which measures the unit amount of protein in a sample (Invitrogen Life Technologies). The proteins in the samples were separated according to their molecular weight using denaturing 4-12% tris acetate polyacrylamide mini gel electrophoresis (Invitrogen Life Technologies), then transferred onto PVDF membranes. Blotted membranes were blocked with 5% skim milk, then incubated with primary antibodies, anti-pAKT1/2/3 diluted 1:500 in blocking solution (SC-135561; Santa Cruz Biotechnology Inc.) and anti-ERK1 diluted 1:500 in blocking solution (SC-94). The membranes then were incubated with horseradish peroxidase (HRP) conjugated secondary antibodies (Invitrogen Life Technologies). Western blot bands were visualized using a chemiluminescent substrate Chromogenic Western Blot Immunodetection Kit (Invitrogen Life Technologies). The pAKT1/2/3 and ERK1 protein bands were determined. Ten microliters NOVEX SeeBlue ® Plus2 Pre-stained Standard (Invitrogen Life Technologies) was used for visualizing protein molecular weight ranges during electrophoresis and for quickly evaluating western transfer efficiency. pAKT1/2/3 and ERK1 protein bands appeared in PVDF membranes. Each data point was repeated in triplicate.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 software (La Jolla, CA). Data are means ± SEM. Statistical significance was determined by one-way ANOVA, then pairwise
comparisons were performed using the Tukey test. Values for $p \leq 0.05$ were considered significant.

Results

We identified mTORC1, IGF1, PI3K, pAKT1/2/3, ERK1 and pERK1/2 in normal rat ovaries and uterus according to rat estrous cycle as proestrous (group 1), estrous (group 2), metestrous (group 3) and diestrous (group 4). Intensity scores for all groups are summarized in Table 1 for ovaries and Table 2 for uterus. All antigens were stained in oocytes of primary, secondary and Graaf follicles, granulosa and theca cells, corpus luteum and stroma of the ovaries and the lamina propria, uterus surface and glandular epithelium of the uterus.

mTORC1 and IGF1 immunoreactivity in the oocytes of primary, secondary and Graff follicles in groups 1 and 2 (Fig. 1-a,c) (Fig. 2-a,c) was significantly greater than for groups 3 and 4 (Fig. 1-e,g) (Fig. 2-e,g) ($p<0.001$). mTORC1 immunoreactivity in the lamina propria, surface and glandular epithelium in groups 1, 2 and 4 was significantly higher than group 3 (Fig. 1-b,d,f,h) ($p<0.001$). IGF1 immunoreactivity in granulosa cells in group 1 was greater than group 3 (Fig. 2-a,e) ($p<0.05$) and theca cells in groups 1 and 2 was higher than group 3 (Fig. 2-a,c,e) ($p<0.01$). The highest IGF1 immunoreactivity in corpus luteum was determined in group 2 and IGF1 immunoreactivity of group 2 was statistically higher than group 4 (Fig. 2-c,g), ($P<0.05$). PI3K immunoreactivity in the oocytes of primary, secondary and Graff follicles in group 1 was significantly greater than for group 3 (Fig. 3-a,e) ($p<0.01$). pAKT immunoreactivity was generally high in all ovaries and uteruses. However, the pAKT immunoreactivity in the theca cells of group 1 was significantly higher than the theca cells of third group (Fig. 4), ($P<0.05$).
The immunoreactivity of ERK1 was determined to be high in the ovaries and uteruses in general, as well as the PAKT immunoreactivity. In oocytes of group 3 ERK1 immunoreactivity was significantly higher than oocytes of groups 1, 2 and 4 (Fig. 5-a,c,e,g) (P<0.001) and granulosa cells of group 1 ERK1 immunoreactivity was significantly higher than of groups 3 and 4 (Fig. 5-a,e,g) (P<0.001). Also in granulosa cells of group 2 ERK1 immunoreactivity was significantly higher than groups 3 (P<0.001) and 4 (P<0.05) (Fig. 5-c,e,g). In theca cells of group 1 ERK1 immunoreactivity was statistically higher than groups 3 and 4 (Fig. 5-a,e,g) (P<0.001). ERK1 immunoreactivity in group 1 was significantly greater than groups 3 and 4 and in group 2 was higher than in group 3 in surface epithelium of uterus (Fig. 5-b,d,f,h), (P<0.001). In glandular epithelium of uterus ERK1 immunoreactivity in group 3 was significantly less than groups 1, 2 (P<0.001) and 4 (P<0.05) also in lamina propria of group 1 ERK 1 immunoreacitivity was determined that significantly higher than groups 3 and 4 (P<0.001) (Fig. 5-b,d,f,h). pERK1/2 immunoreactivity oocytes of group 1 was significantly greater than groups 3 and 4 (Fig. 6-a,e,g) (P<0.001) and in granulosa cells of group1 was higher than group 4 (P<0.05) (Fig. 6-a,g).

We found that PI3K/Akt/mTOR and ERK/pERK immunoreactivities were expressed during the rat estrous cycle. Different amounts of mTORC1, IGF1, PI3K, pAKT1/2/3, ERK1 and pERK1/2 were expressed in oocytes, corpus luteum, stroma, granulosa and theca cells of ovaries and were expressed also lamina propria, surface and glandular epithelium of uteruses. The presence of pAKT1/2/3 and ERK1 proteins was confirmed using western blotting (Fig. 7).

Discussion
mTOR is a member of the PI3K/AKT/mTOR signaling pathway of the serine-threonin protein kinase family, and plays an important role in cell cycle coordination, protein synthesis, transcription and energy metabolism, and is associated with cell proliferation, growth, differentiation, angiogenesis, apoptosis and autophagy (Rohde et al., 2001; Fingar and Blenis, 2004). mTORC1 immunoreactivity in ovarian specimens; oocytes of primary, secondary and Graaf follicles were found to be significantly increased in the proestrous and estrous stages of rat estrous cycle and it was thought that the cause of this increase could be due to ovulation in the rats between the stages of proestrous and estrous. It was proposed that growth factors activate the mTORC1 molecule via the PI3K/AKT pathway (Reuben J. Shaw et al., 2006). Studies show that mTOR mRNA was found in high amounts in normal and pig ovary tissue (Kim et al., 2012), mTOR protein was expressed in mice ovary in different stages of folliculogenesis (Yaba et al., 2012) and it has been shown that mTOR protein acts as a control point that provides mitotic proliferation and survival in ovarian granulosa cells (Yaba et al., 2008, 2012). In contrast, another study showed that mTOR suppression of granulosa cells and ovarian follicles reduced the proliferation of granulosa cells and follicle growth in the ovaries (Yu et al., 2011). It has also been suggested that various signaling pathways that affect not only apoptosis but also autophagy and hormones are effective in the reorganization of luteal cells (Kim et al., 2019). In our study, similar to other studies performed, mTORC1 immunoreactivity was found in oocytes, granulosa and theca cells, corpus luteum and stroma in ovarian tissue it was found that the rat estrous cycle varied in different stages. It was thought that the increase in mTORC1 in proestrous, estrous and diestrous phases could be related with folliculogenesis and the increase in corpus luteum in metestrous stage might be related with luteinization. The presence of the mTORC1 molecule in the uterus is shown in studies (Martin and Sutherland, 2001; H. Gao et al., 2009). mTORC1 mRNA was found in sheep uterine endometrium but did not show cyclic change during estrous cycle (Liu et al.,
In a study that reached similar results to our study, it was reported that Raptor mRNA was found in the luminal and glandular epithelium of uteruses and also its expression was changed during the sheep estrous cycle (H. Gao et al., 2009).

IGFs are highly similar to insulin (Annunziata et al., 2011) and IGF2 in humans are primary IGF, while IGF1 plays a major role in ruminants and the reproductive system of rodents (Giudice, 2001). Studies have shown that mTOR-associated IGF1 mRNA is present in large amounts in the ovarian tissue of pigs (Kim et al., 2012). It has also been reported that all components of the signaling systems associated with IGF are produced in a number of tissues including ovarian follicles, corpus luteum and oocyte (Giudice, 2001). It has been reported that IGF1 does not have any effect on the development of primordial follicle, but both IGF1 and IGF2 stimulate the in vivo and in vitro development of secondary follicles, as well as studies on IGFs in Graaf follicles that stimulate proliferation and steroidogenesis of granulosa cells and inhibit apoptosis (Mihm et al., 2008). There are ideas that IGF1 and IGF2 are highly produced in the corpus luteum and that they support luteal development and functions until mid-luteal phase, but there is no clear evidence for this hypothesis (Berisha et al., 2005). In our study, it was speculated that IGF1 increases in proestrous and estrous stages may be related to folliculogenesis and activation of the mTOR pathway. Previous studies have shown that both IGF1 and IGF2 expression play an important role in cyclic growth and differentiation of endometrium of humans and rodents (Gu et al., 1999; McCampbell et al., 2008). Studies of IGF1 and IGF2 have been shown to induce the tyrosine kinase receptor in endometrial stromal and epithelial cells and stimulate the activation of IGF1R signaling via PI3K/AKT pathway (Zhou et al., 1994; Rutanen, 1998). In this sense, it was thought that the determination of the change in different stages of rat estrous cycle of IGF1, already known and effective in ovaries and uterus, would contribute to the literature.
PI3K family are the proteins responsible for the transmission of growth and living signals (Chang et al., 2003). In the mTOR pathway, PI3K is one of the major molecules that induce phosphorylation of the mTOR protein after IGF1. The PI3K pathway continues with activation of AKT. Protein kinase B is a protein encoded by the AKT1 and AKT2 genes. Cytokines and growth factors activate the PI3K and AKT pathway to generate living signals for the cells (Nicholson et al., 2002). AKT stimulation affects the activity of various proteins in the cell. One of them is the mTOR protein (Klos et al., 2006). In proestrous, PI3K immunoreactivity in oocytes was significantly higher than in oocytes in the oestrous phase. It has been interpreted that the reason why IGF1 shows high immunoreactivity in oocytes, especially in the prostrus phase, it may be effective as a molecule that triggers PI3K. Unlike IGF1, PI3K immunoreactivity did not differ significantly in the granulosa cells, corpus luteum and stroma of the ovary. The reason for the high IGF1 immunoreactivity seen in the proostrus stage in oocytes may be due to the activation seen in the proostrus stage in oocytes and other parts of ovary may be affected by different signaling pathways PI3K has been shown to be involved in follicular activation such as mTOR (Reddy et al., 2009). Studies have shown that the functions of the PTEN/PI3K pathway suppress follicular activation in oocytes (Castrillon, 2003), although mTOR mediated events regulate cell growth and proliferation in various cell types, it has also been reported to be closely related to the PI3K pathway (Wullschleger et al., 2006b; Guertin and Sabatini, 2007). In the light of all these data, in our study, PI3K immunoreactivity decreased in the metestrous stage. This may be because the corpus luteum is active during the metestrous stage High pAKT activity in endometrial tissues has been shown to be correlated with PI3K protein (Yin et al., 2012). Human endometrial cells have been shown to exhibit cyclic changes by stimulating the PI3K/AKT signaling pathway (Yoshie et al., 2009). In our study, in addition to the literature, PI3K, one of the molecules of
the mTOR pathway, was found to show cyclic changes in the rat estrous cycle together with IGF1.

It has been shown in studies that cytokines and growth factors activate the PI3K and AKT pathway to generate living signals for cells, while PTEN plays a negative regulatory role by inhibiting the formation of PIP3 on this pathway (Nicholson et al., 2002). AKT stimulation affects the activity of various proteins in the cell. One of them is the mTOR protein (Klos et al., 2006). Phosphorylation of mTOR is very important for cellular survival with cell cycle progression and proliferation (Reuben J Shaw et al., 2006).

In our study, as a result of indirect immunohistochemical examination of ovarian samples, pAKT1/2/3 immunoreactivity, oocytes and granulosa cells were found to be high immunoreactivity in the stages of proestrous, estrous and diestrous, and decreased in the metestrous stage compared to the other stages. This was related to the growth and proliferation of oocytes and granulosa cells active in these stages and the correlation with high PI3K expression in these stages was also confirmed. It was thought that the high immunoreactivity seen in the estrous stage may be due to the fact that the LH receptors found in the singleton interna cells were triggered in the direction of androstenedione synthesis due to ovulation between the proestrous and estrous phases. A significant difference was observed between the estrous and metestrous stages in terms of pAKT1/2/3 immunoreactivity in oocytes, granulosa and theca cells. In the corpus luteum and stroma, pAKT1/2/3 immunoreactivity was determined higher in the metestrous stage than in the other estrous cycle stages. Similarly, it was speculated that IGF1 might have triggered the increase of pAKT since IGF1 immunoreactivity increased in the metestrous phase of the corpus luteum and stroma.
Studies have shown that the PI3K/AKT pathway plays a role in the proliferation of granulosa cells during follicular development in the rat ovary (Asselin et al., 2001). The PI3K signaling pathway is a critical regulator for follicle growth, differentiation and survival, and a deletion in the PI3K gene has been reported to cause infertility and POF (Premature Ovarian Failure) (Edson et al., 2009). In a 2007 study, it was reported that AKT1-knockout female mice had decreased fertility, estrous cycles were delayed by up to 5 days, and the age of first fertility increased. AKT3-deficient mice have been reported to have normal fertility but there are no data on the fertility of AKT2-deficient mice. The AKT1 molecule in human ovaries has been reported at every stage of oocytes, granulosa cells and theca cells (Goto et al., 2007). In rodents, AKT1 has been reported in both oocytes and granulosa cells (Reddy et al., 2009). PI3K has been suggested to be the main regulator of AKT activation and myogenesis (Lewis et al., 1998; Matheny et al., 2012). AKT signaling has been reported to affect the release of the mTOR molecule (Rico et al., 2012). AKT activity has been reported to be effective by reducing PI3K (Cecconi et al., 2012). It has been reported that the pAKT molecule increases apoptosis in granulosa cells of Graaf follicles in rat ovaries (Yang et al., 2013). In the light of this information, pAKT1/2/3 activity was thought to play an important role in the proliferation of granulosa cells in ovaries, especially folliculogenesis during ovulation period, which is the determinant of fertility in the female reproductive system. In studies, the presence of AKT protein in the endometrium has been shown (Q. Gao et al., 2009). According to our results, pAKT1/2/3 protein showed an increase in proliferation and growth stages, and showed increases and decreases proportional to PI3K and mTORC1 as a proof of its effect on the mTOR pathway.

MAP kinases control cellular events for many complex short-term changes, such as embryogenesis, cell differentiation, cell proliferation and cell death, which are necessary for homeostasis and acute hormonal responses (Lewis et al., 1998). The MAPK signaling
pathway is composed of RAF, MEK and ERK proteins required for proliferation in normal cells (Pazarbaşi et al., 2011). Recent studies have shown the importance of ERK1/2 (MAPK1/3) signaling in follicular rupture (Fan et al., 2009). Although ERK1/2 has also been shown to play a special role in oocyte maturation, how to transform granulosa cells into luteal cells is not clear. It has been shown that ERK 1 and 2 are expressed in all mammalian cells and are involved in oocyte maturation and cell proliferation and differentiation in cell culture studies (Su, 2002). It was thought that the determination of the distribution of ERK1 and pERK1/2 proteins in the stages of rat estrous cycle would contribute to the literature.

The importance of mTOR signaling in pubertal metabolic regulation and fertility has been the subject of research in recent years. In our study, we think that mTOR and mTOR-related signaling molecules will contribute to the literature in terms of determining the distribution of fertility in ovulation period, which is the main mechanism for female reproductive system. As a continuation of this study, which is the control of mTOR signal pathway molecules related to these mechanisms in the female reproductive system, there is a need for studies in the future by stopping or triggering all these pathways with various signaling molecules or active substances.

**Conclusion**

In this study, mTOR and related molecules were evaluated immunohistochemically in ovarian and uterine samples in the rat estrus cycle in order to investigate the key role of mTOR and MAPK pathways in ovulation, which is one of the key molecular pathways in cell growth, metabolism and proliferation in cells. One of the most important causes of deterioration of infertility is ovulation problems. mTOR and its related molecules, ERK1 and pERK1/2, were found to vary in different stages of the rat estrus cycle. In addition, the key molecules such as...
ERK1 and pAKT in these pathways were confirmed by western blot, a different technique. In experimental animals, the oestrus cycle was examined and it was shown that there were molecules that changed according to the stage active in both ovaries and uterus and showed some cyclic changes. All these findings, if supported by further studies, may contribute to the development of new pharmacological agents to eliminate ovulation problems in women.

Funding
This research was granted by Celal Bayar University, Scientific Research Project Committee. Number 2011/038.

Declaration of interests
The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.
References


Zygote 16, 285-96.


In vitro and in vivo studies in cattle and sheep. J. Ovarian Res. 1, 2.


Yaba A., Bianchi V., Borini A. and Johnson J. (2008). A putative mitotic checkpoint dependent on MTOR function controls cell proliferation and survival in ovarian granulosa


Figure Legends

Figure 1. Immunohistochemical evaluations of anti-mTORC1 antibody in rat ovaries (a,c,e,g) and uterus (b,d,f,h). In the photomicrographs, ovaries are seen partially (400X) in big size micrographs and whole (200X) in small size micrographs. Star shows oocytes, arrow head shows theca cells, GR shows granulosa cells in the ovary and SE shows surface epithelium, LP shows lamina propria, blue arrow shows glandular epithelium in the uterus.
Figure 2. Immunohistochemical evaluations of anti-IGF1 antibody in rat ovaries (a,c,e,g) and uterus (b,d,f,h). In the photomicrographs, ovaries are seen partially (400X) in big size micrographs and whole (200X) in small size micrographs. Star shows oocytes, arrow head shows theca cells, GR shows granulosa cells in the ovary and SE shows surface epithelium, LP shows lamina propria, blue arrow shows glandular epithelium in the uterus.

Figure 3. Immunohistochemical evaluations of anti-PI3K antibody in rat ovaries (a,c,e,g) and uterus (b,d,f,h). In the photomicrographs, ovaries are seen partially (400X) in big size micrographs and whole (200X) in small size micrographs. Star shows oocytes, arrow head shows theca cells, GR shows granulosa cells in the ovary and SE shows surface epithelium, LP shows lamina propria, blue arrow shows glandular epithelium in the uterus.

Figure 4. Immunohistochemical evaluations of anti-pAKT1/2/3 antibody in rat ovaries (a,c,e,g) and uterus (b,d,f,h). In the photomicrographs, ovaries are seen partially (400X) in big size micrographs and whole (200X) in small size micrographs. Star shows oocytes, arrow head shows theca cells, GR shows granulosa cells in the ovary and SE shows surface epithelium, LP shows lamina propria, blue arrow shows glandular epithelium in the uterus.

Figure 5. Immunohistochemical evaluations of anti-ERK1 antibody in rat ovaries (a,c,e,g) and uterus (b,d,f,h). In the photomicrographs, ovaries are seen partially (400X) in big size micrographs and whole (200X) in small size micrographs. Star shows oocytes, arrow head shows theca cells, GR shows granulosa cells in the ovary and SE shows surface epithelium, LP shows lamina propria, blue arrow shows glandular epithelium in the uterus.
Figure 6. Immunohistochemical evaluations of anti-pERK1/2 antibody in rat ovaries (a,c,e,g) and uterus (b,d,f,h). In the photomicrographs, ovaries are seen partially (400X) in big size micrographs and whole (200X) in small size micrographs. Star shows oocytes, arrow head shows theca cells, GR shows granulosa cells in the ovary and SE shows surface epithelium, LP shows lamina propria, blue arrow shows glandular epithelium in the uterus.

Figure 7. Western blot analysis of pAKT1/2/3 and ERK1 using ovaries and uterus lysates isolated from female rats. ERK1 (43 kDa) protein is in the first and second panels and pAKT1/2/3 (51 kDa) is in the third and fourth panels. The numbers of the groups are shown at the top of the figure. Also, results of ovaries are seen in the first and third panels. Uterine results are seen in the second and fourth panels. These findings confirm the presence of ERK1 and pAKT1/2/3 in the female rat ovaries and uterus during estrus cycle process.

Table legends

<table>
<thead>
<tr>
<th>OVARY</th>
<th>GROUP 1 PROESTROUS</th>
<th>GROUP 2 ESTROUS</th>
<th>GROUP 3 METESTROUS</th>
<th>GROUP 4 DIESTROUS</th>
</tr>
</thead>
<tbody>
<tr>
<td>mTORC1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GROUP 1</td>
<td>GROUP 2</td>
<td>GROUP 3</td>
<td>GROUP 4</td>
</tr>
<tr>
<td>------------------</td>
<td>---------------</td>
<td>---------------</td>
<td>---------------</td>
<td>---------------</td>
</tr>
<tr>
<td><strong>Oocyte (O)</strong></td>
<td>3,571±0,534</td>
<td>3,285±0,488</td>
<td>1,428±0,378</td>
<td>2,142±0,378</td>
</tr>
<tr>
<td><strong>Granulosa cells (GC)</strong></td>
<td>3,428±0,534</td>
<td>3,142±0,378</td>
<td>1,142±0,378</td>
<td>2,285±0,488</td>
</tr>
<tr>
<td><strong>Theca cells (TC)</strong></td>
<td>3,285±0,488</td>
<td>3,142±0,378</td>
<td>1,285±0,488</td>
<td>1,714±0,488</td>
</tr>
<tr>
<td><strong>Corpus luteum (CL)</strong></td>
<td>2,142±0,378</td>
<td>2,571±0,534</td>
<td>2,571±0,534</td>
<td>2,571±0,534</td>
</tr>
<tr>
<td><strong>Stroma (ST)</strong></td>
<td>2,714±0,488</td>
<td>2,714±0,488</td>
<td>2,285±0,488</td>
<td>2,428±0,534</td>
</tr>
<tr>
<td><strong>IGF1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Oocyte (O)</strong></td>
<td>3,143±0,378</td>
<td>2,429±0,534</td>
<td>1,857±0,378</td>
<td>2,857±0,378</td>
</tr>
<tr>
<td><strong>Granulosa cells (GC)</strong></td>
<td>2,714±0,488</td>
<td>2,571±0,534</td>
<td>1,429±0,534</td>
<td>1,714±0,488</td>
</tr>
<tr>
<td><strong>Theca cells (TC)</strong></td>
<td>2,571±0,534</td>
<td>2,571±0,534</td>
<td>1,571±0,534</td>
<td>1,571±0,534</td>
</tr>
<tr>
<td><strong>Corpus luteum (CL)</strong></td>
<td>2,142±0,378</td>
<td>2,571±0,534</td>
<td>2,143±0,378</td>
<td>2,143±0,378</td>
</tr>
<tr>
<td><strong>Stroma (ST)</strong></td>
<td>2,714±0,488</td>
<td>2,714±0,488</td>
<td>2,285±0,488</td>
<td>2,428±0,534</td>
</tr>
<tr>
<td><strong>PI3K</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Oocyte (O)</strong></td>
<td>2,429±0,534</td>
<td>1,857±0,378</td>
<td>1,286±0,488</td>
<td>1,714±0,488</td>
</tr>
<tr>
<td><strong>Granulosa cells (GC)</strong></td>
<td>1,714±0,488</td>
<td>1,714±0,488</td>
<td>1,429±0,534</td>
<td>1,714±0,488</td>
</tr>
<tr>
<td><strong>Theca cells (TC)</strong></td>
<td>1,429±0,534</td>
<td>1,857±0,378</td>
<td>1,286±0,488</td>
<td>1,714±0,488</td>
</tr>
<tr>
<td><strong>Corpus luteum (CL)</strong></td>
<td>1,429±0,534</td>
<td>1,714±0,488</td>
<td>1,571±0,534</td>
<td>1,571±0,534</td>
</tr>
<tr>
<td><strong>Stroma (ST)</strong></td>
<td>1,571±0,534</td>
<td>1,714±0,488</td>
<td>1,286±0,488</td>
<td>1,714±0,488</td>
</tr>
<tr>
<td><strong>pAKT1/2/3</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Oocyte (O)</strong></td>
<td>3,429±0,534</td>
<td>3,429±0,534</td>
<td>2,714±0,488</td>
<td>3,286±0,488</td>
</tr>
<tr>
<td><strong>Granulosa cells (GC)</strong></td>
<td>3,429±0,534</td>
<td>3,286±0,488</td>
<td>2,571±0,534</td>
<td>3,286±0,488</td>
</tr>
<tr>
<td><strong>Theca cells (TC)</strong></td>
<td>3,143±0,378</td>
<td>3,286±0,488</td>
<td>2,286±0,488</td>
<td>2,429±0,534</td>
</tr>
<tr>
<td><strong>Corpus luteum (CL)</strong></td>
<td>2,714±0,488</td>
<td>2,857±0,378</td>
<td>3,143±0,378</td>
<td>2,714±0,488</td>
</tr>
<tr>
<td><strong>Stroma (ST)</strong></td>
<td>2,857±0,378</td>
<td>2,714±0,488</td>
<td>3,143±0,378</td>
<td>3,143±0,378</td>
</tr>
<tr>
<td><strong>ERK1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Oocyte (O)</strong></td>
<td>3,857±0,378</td>
<td>3,429±0,534</td>
<td>1,857±0,378</td>
<td>3,143±0,378</td>
</tr>
<tr>
<td><strong>Granulosa cells (GC)</strong></td>
<td>3,714±0,488</td>
<td>3,286±0,488</td>
<td>1,429±0,534</td>
<td>3,286±0,488</td>
</tr>
<tr>
<td><strong>Theca cells (TC)</strong></td>
<td>3,143±0,378</td>
<td>3,286±0,488</td>
<td>1,286±0,488</td>
<td>1,857±0,378</td>
</tr>
<tr>
<td><strong>Corpus luteum (CL)</strong></td>
<td>2,286±0,488</td>
<td>2,571±0,534</td>
<td>3,143±0,378</td>
<td>2,429±0,534</td>
</tr>
<tr>
<td><strong>Stroma (ST)</strong></td>
<td>2,286±0,488</td>
<td>2,571±0,534</td>
<td>2,286±0,488</td>
<td>2,429±0,534</td>
</tr>
<tr>
<td><strong>pERK1/2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Oocyte (O)</strong></td>
<td>2,857±0,378</td>
<td>2,143±0,378</td>
<td>1,571±0,534</td>
<td>1,714±0,488</td>
</tr>
<tr>
<td><strong>Granulosa cells (GC)</strong></td>
<td>2,571±0,534</td>
<td>2,143±0,378</td>
<td>1,714±0,488</td>
<td>1,571±0,534</td>
</tr>
<tr>
<td><strong>Theca cells (TC)</strong></td>
<td>2,286±0,488</td>
<td>1,857±0,378</td>
<td>1,429±0,534</td>
<td>1,714±0,488</td>
</tr>
<tr>
<td><strong>Corpus luteum (CL)</strong></td>
<td>2,429±0,534</td>
<td>2,286±0,488</td>
<td>2,429±0,534</td>
<td>2,413±0,378</td>
</tr>
<tr>
<td><strong>Stroma (ST)</strong></td>
<td>2,143±0,378</td>
<td>2,143±0,378</td>
<td>1,857±0,378</td>
<td>1,857±0,378</td>
</tr>
</tbody>
</table>

**Table 1.** Immunostaining intensities of mTORC1, IGF1, PI3K, pAKT1/2/3, ERK1 and pERK1/2 in ovary during rat estrous cycle stages.
### Table 2.

Immunostaining intensities of mTORC1, IGF1, PI3K, pAKT1/2/3, ERK1 and pERK1/2 in uterus during rat estrous cycle stages.