大韓不妊學會誌 : 第30卷 第4號 2003
Kor. J. Fertil. Steril., Vol. 30, No. 4, 2003, 12

생쥐 난포 발달 중 Dazla 유전자 발현의 조절에 관한 연구

서울대학교 의과대학 산부인과학교실 서울대학교병원[1],
분당서울대학교병원[2], 의학연구원 인구의학연구소[3]

서창석[2,3]·지병철[2]·구승엽[1,3]·김용범[2]·박교훈[2]
김석현[1,3]·최영민[1,3]·김정구[1]·문신용[1,3]

Hormonal Regulation of Dazla Expression in the Follicle Development of Mouse Ovary

Chang Suk Suh[2,3], Byung Chul Ji[2], Seoung Yup Ku[1,3], Kyo Hoon Park[2], Youn Beom Kim[2], Seok Hyun Kim[1,3], Young Min Choi[2,3], Jung Gu Kim[2,3], Shin Yong Moon[2,3]

Department of Obstetrics and Gynecology, College of Medicine, Seoul National University Hospital[1], Seoul National University Bundang Hospital[2], Institute of Reproductive Medicine and Population[3], Seoul, KOREA.

목 적: 본 연구에서는 생쥐 난소 주기에 따른 Dazla 유전자의 발현 조절 양상을 관찰하고자 하였다.

연구재료 및 방법: 생후 27일된 암컷 미성숙 생쥐 (total 33EA; each 3 EA)로 부터 Pregnant Mare Serum Globulin (PMSG) 투여전, PMSG 투여 3시간, 6시간, 12시간, 24시간, 48시간 후 난소 조직과 hCG 투여 3시간, 6시간, 12시간, 24시간, 48시간 후 난소 조직을 획득하였다. 암컷 성숙 생쥐 난소와 (n=3) 수컷 성숙 생쥐 (n=3)로부터 고환 조직을 획득하여 대조군으로 사용하였다. 각각의 획득된 조직에서 Dazla mRNA 의 발현 여부를 RT-PCR과 in situ hybridization (ISH) 방법으로 확인하였다. 호르몬 투여 시기에 따른 Dazla 유전자의 발현 강도를 ISH 방법으로 비교하여 정상 난소주기에서 PMSG와 PMSG+hCG에 의한 Dazla 유전자의 발현 조절 양상을 관찰하였다.

결 과: 미성숙 생쥐, 성숙 생쥐 난소 내 난자 모두에서 Dazla 유전자의 발현이 관찰되었다. 성숙 생쥐 고환 내 정자에서도 Dazla 유전자의 발현이 관찰되었다. 미성숙 및 성숙 생쥐의 난포에서는 난포의 성장에 따라 Dazla 유전자의 발현 강도가 강하게 관찰되었다. 미성숙 생쥐에 투여된 PMSG와 PMSG+hCG에 의한 유전자의 발현 강도는 난포의 크기가 같을 경우 호르몬 투여전과 비교하여 차이가 없었다.

결 론: Dazla 유전자는 난소내 난자에서 특이하게 발현되며, 난포의 크기에 따라 난자에서의 dazla유전자 발현의 강도도 증가한다. 그러나 PMSG와 PMSG+hCG에 의하여 유전자 발현이 조절되지 않은 것으로 보아 난소의 배란주기에 따른 유전자의 발현 양상에 변화가 없는 것으로 생각된다.

색인단어: Dazla, 난소, PMSG, hCG

The ovarian life cycle begins with the formation of a cell, the primordial germ cell that becomes an egg, is fertilized, and then becomes a new female organism in which the events of the ovary life

주관책임자: 서창석, 우) 463-707 경기도 성남시 분당구 구미동 300, 분당서울대학교병원 산부인과
Tel: (031) 787-7251, e-mail: suhcs@snu.ac.kr
본 연구는 서울대학교 신임교수 연구정착금으로 지원되는 연구비에 의하여 수행되었음
This study was supported by the Seoul National University Research Fund.

cycle become progressively expressed in the next generation. The life cycle of the ovary has four major developmental phases[1]: 1. the phase of embryogenesis whereby populations of primordial germ cells and somatic cells become an integrated ovary mass containing oocytes and granulosa cells located within primordial follicles. 2. the phase of folliculogenesis in which oogenesis, granulosogenesis, and thecogenesis occur as a recruited primordial follicle grows and develops to the preovulatory stage or it dies by atresia. 3. the phase of ovulation whereby the oocyte transforms into a mature egg which is secreted into the oviduct to await fertilization. 4. the phase of luteogenesis whereby the follicle, now lacking an oocyte, luteinizes into an endocrine structure, the corpus luteum, which if implantation does not occur, dies by a process termed luteolysis. Many researches in the past century have provided a wealth of important information about the morphological development of the ovary and the regulatory roles of the endocrine and autocrine/ paracrine systems in the mechanism that control its life cycle.

Among this, compelling evidence that oocyte derived growth factors[2], namely growth differentiation factor-9 (GDF-9), and bone morphogenic protein-15 (BMP-15) play a key role in folliculogenesis[3] and female fertility has come from studies in a variety of laboratory animals and human. In situ hybridization studies in a variety of mammals have shown that the GDF-9 and BMP-15 genes are selectively expressed in the developing oocytes during folliculogenesis[4,5]. In GDF-9 deficient female mice, a wide range of reproductive abnormalities appear, including arrested follicle at the primary stage, reduced granulosa proliferation, inappropriate theca development, cyst formation, and infertility[6,7].

These kinds of knock-out mice study demonstrated new genes related with infertility. Dazla is one of the new genes suggested to be active involvement in female folliculogenesis[8,9]. Recently, we demonstrated that dazla mRNA is also exclusively expressed in the oocytes suggesting strong involvement in the ovary folliculogenesis[10]. Here, we provide the regulatory role of gonadotropin hormones on the dazla mRNA expression in the follicle development of mouse ovary.

MATERIALS AND METHODS

1. Source of ovary tissues, oocytes, and embryos

Ovaries were collected from immature mice (n=33) after injecting PMSG (10 IU), which is followed by hCG injection (10 IU). The time of ovary retrieved after PMSG and hCG injection was 3, 6, 9, 12, 24, 48 hours, respectively. The tissues were immediately fixed under 10% neutral formalin. Ovaries from adult female mice (n=3) and testis from adult mice (n=3) were collected and used as control tissues. Animal experiment was performed under observance of AFSG (animal facility service guidelines).

2. Semiquantitative RT-PCR analysis

Primer design was based on the most homologous mRNA sequences among human, mouse, and rat within exonal region. The forward primer was 5'-TCA CTG ATC GAA CTG GTG TG-3', and reverse primer was 5'-TCA GCT CCT GGA TCA ACT TC-3'. RT-PCR was performed as previously described[11]. In brief, 500 ng of extracted RNA was subjected to the RT reaction using first strand cDNA synthesis system (Life Technologies Inc.) with random hexamer (2ng/μl), reverse transcriptase (200 U), and deoxynucleotide triphosphate (dNTP, 0.5 mM) at 42°C for 50 min, 70°C for 10 min. Serial diluation of cDNA (1/2, 1/4, 1/8, 1/16) corresponding to

the different experimental time points (20, 25, 30, 35 cycles of amplification) was made to determine the log phase of amplification reaction. After resuspending first strand cDNA in 50 µl of water, PCR was performed under following conditions: 30 cycles of denaturation at 94°C for 30 sec, annealing temperature at 55°C for 30 sec and extension at 72°C for 30 seconds.

3. In situ hybridization

Paraffin-embedded mouse ovarian tissue sections (10 µm) were used for these experiments. The sections were taken randomly from the ovary pieces, and no effort was made to standardize where the sections were taken (i.e. maximal cross-sections or whole-thickness slices). The relevant in situ hybridization procedures have been described previously[11]. In brief, after dewaxing, sections were treated with proteinase K, acetylated, washed, dehydrated, and hybridized with radioactive probe (10^6cpm/µl). Dazla probes were designed to contain homologous region for mouse, rat, and human. Plasmids constructed for the probe preparation were as follows: Dazla-457 for Dazla, a DNA segment (457 bp) of mouse Dazla exonal region was amplified by RT-PCR from ovarian total RNA and cloned into a pCR II vector (Invitrogen, Carlsbad, CA). Antisense and sense RNA probes were synthesized by transcription using 35^S- UTP and T3 or SP6 polymerase after digestion with BamHI and XhoI restriction enzymes. After hybridization, sections were treated with ribonuclease A, washed in decreasing concentrations of saline sodium citrate (SSC), and finally in 0.1X SSC at 65°C for 40 min. After dehydration in graded ethanol solutions, slides were exposed X-ray film for 3 days. After obtaining adequate X-ray film images, the sections were treated with xylene, rinsed in 100% ethanol, air-dried, and then coated with Kodak NTB-2 liquid autoradiographic emulsion. Slides were exposed for 2 weeks at 4°C in a desiccated dark box. After exposure, the slides were developed (Kodak D19, 3.5 min at 14°C), rinsed briefly in distilled water, and fixed. After washing, slides were lightly stained with hematoxylin and eosin. In each experiment, duplicate slides from all ovaries studied were processed in the same in situ hybridization run using the same probe, conditions of hybridization, and length of autoradiographic exposure. The sections from all ovaries in all experiments were scored and the data were repeated at least three times with similar results.

4. Morphometry

Follicles in the ovary were classified into 5 groups as previously described[11]: primordial (I: the oocyte was surrounded by a single layer of squamous granulosa cells), primary (II: the oocyte surrounded by a single layer of cuboidal cells), early preantral (III: the oocyte was surrounded by one to two layers), preantral (IV: the oocyte was surrounded by more than two to eight layers), small to large Graafian (V-VI: the follicle with fluid filled antrum). The entire section of each ovary was scanned under dark/bright field and only those follicles sectioned through the oocyte nucleolus (the largest follicle cross section) were analyzed. The intensity of the in situ hybridization signals was evaluated on a scale of 1 to 4 as previously described. The following rate was used: +; silver grains were sparse but clearly above background, ++; silver grains were numerous but did not cover the whole area, +++; silver grains were very numerous and began to merge in some places, ++++; silver grains were very dense and formed a near uniform mass. The intensity was recalculated into HSCORE as previously described[12]. In brief, HSCORE is calculated from the following equation: HSCORE = Σ Pi(i=1),

where i=intensity of percentage of stained cells for each intensity, varying from 0% to 100%. The results of the HSCORE will be expressed as mean±SE of at least three separate experiments, with triplicate determinations for each treatment.

5. Statistical analysis

Data were analyzed for statistical significance between ovaries, and signal intensities by Mann Whitney U test, ANOVA, chi-square tests. P-values less than 0.05 were accepted as statistically significant. Analysis was performed using SPSS 8.0 (SPSS Inc., Chicago, IL).

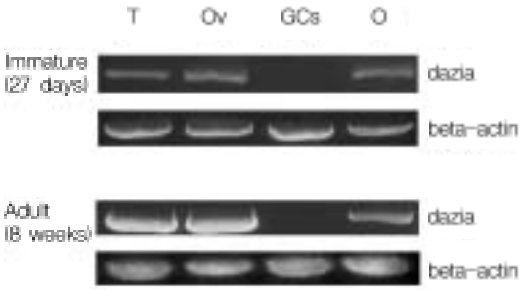

Figure 1. RT-PCR of Dazla in the ovary and testis obtained from immauture and adult testis (T), ovary (Ov), granulosa cells (GCs), oocytes (O). GCs and O were prepared from follicular puncture which was followed by mechanical dissection under dissecting microscope.

RESULTS

1. RT-PCR analysis of dazla mRNA expression

Dazla mRNA was detected in the adult testis, immature ovary, and adult ovary tissues by RT-PCR (Figure 1). Both oocytes from mature and immature mice showed dazla mRNA expression, while granulosa cells did not show any signal of dazla mRNA expression.

2. In situ hybridization of dazla mRNA

In situ hybridization of dazla in the mice showed strong positive signals both in testis and

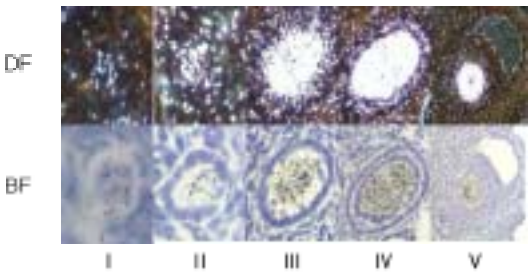

Figure 2. In Situ Hybridization of Dazla in the adult female mice. Each follicles were examined under the dark field (DF) and bright field (BF). Follicles were classified into the Type I (primordial follicle), II (primary follicle), III-IV (secondary follicle), and V (Graafian follicle).The signal intensity was positive exclusively in the cytoplasm of oocyte. And the signal was significantly increased as the size of follicle grows.

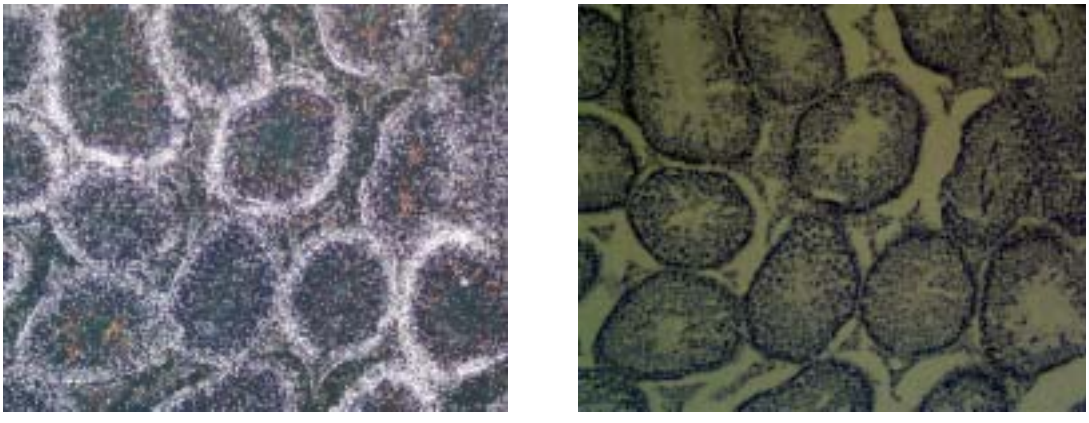

Figure 3. In Situ Hybridization of Dazla in the adult male mice. Examination under the dark filed (DF) and bright field (BF) showed strong signals within the cytoplasm of spermotocytes and spermatozoa.

ovary regardless of their age (Figure 2, Figure 3). The location of expression was cytoplasm of oocyte in the ovaran follicle and spermatocytes in the seminiferous tubule of testis, respectively. The signal intensity was increased by the size of ovary follicle. The average intensity of HSCORE in follicle stage I to V was increased both in the adult and immature mice (Figure 4).

3. Regulation of dazla mRNA by gonadotropin

Dazla mRNA expression showed increased signal in the control group as the size of follicle grows (pre-injection immature mice). However, the intensity signals of dazla mRNA was not changed after the injection of PMSG and PMSG+hCG in the immature mouse compared to immature control if follicle type is the same as the other study group (Table 1).

DISCUSSION

Primordial follicles represent a pool of non-growing follicles, which is established during oogenesis and used later for folliculogenesis, have two fates: One is recruitment (activation) of follicles to primary and preantral follicles, the other is attrition (apoptosis) that eventually leads to complete depletion of primordial follicle pool resulting in menopause[13]. The follicles on the pathway of apoptosis appear to be always more than the activated follicles. In sheep, 8 primordial follicles disappear everyday from the primordial reserve pool, which was demonstrated by comparing primordial follicle number between 2 and 8-year-old ewes[14]. Everyday 5 to 6 follicles die by apoptosis within the primordial pool, while only 1 or 2 follicles per day show growth initiation. Among growing pool, one follicle can be ovulated, whereas remainder results in apoptosis again. Though we do not precisely understand the mechanism of primordial to primary follicle transition, there have been several reports on the role of autocrine, and paracrine growth factors secreted from ovary itself. IGF system and FSH are not essential to primordial activation, because follicles can develop into preantral follicle without those substances.

Kit ligand (KL) and its receptor kit were

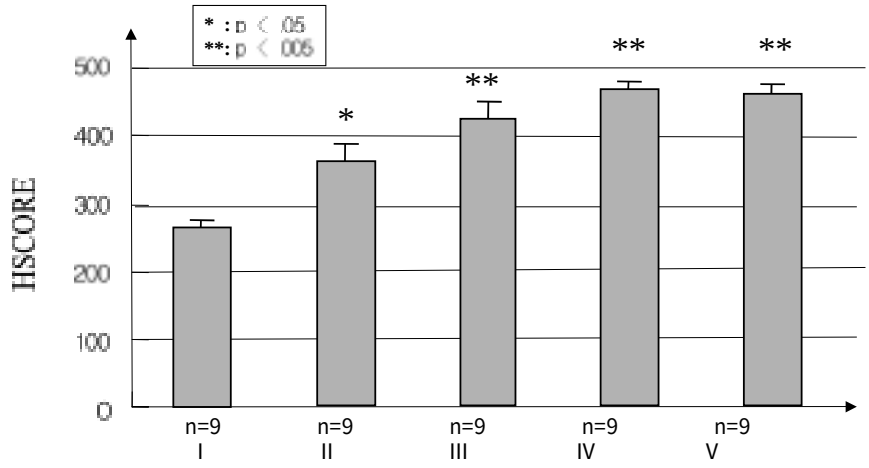

Figure 4. HSCORE of Dazla mRNA according to the follicle stage by In situ hybridization (ISH) in the adult female mice. The HSCORE was increased significantly as the follicle size grows. Follicles were classified into the Type I (primordial follicle), II (primary follicle), III-IV (secondary follicle), and V (Graafian follicle).

Table 1. HSCORE of dazla according to the time sequences of PMSG and hCG

Group \ Follicle	I	II	III	IV	V
Control	150.3±9.7	228.1±20.0*	284.1±21.6**	321.4±30.6**	422.6±15.3**
PMSG					
3hr	156.7±10.7	212.5±14.8*	221.7±19.4**	303.3±34.3**	335.0±10.2**
6hr	132.7±9.1	131.0±18.4	185.3±17.8*	339.1±23.7**	384.3±17.9**
12hr	160.0±5.0	175.0±12.5	214.3±19.9*	325.0±17.3**	378.5±9.6**
24hr	167.4±5.3	165.6±5.8	224.6±17.5*	359.8±11.9**	400.8±9.6**
48hr	167.8±7.0	176.8±11.8	242.3±25.3**	313.8±6.6**	389.2±8.6**
PMSG+hCG					
3hr	161.4±10.4	176.6±8.0	211.4±11.9*	319.0±16.0**	388.8±8.7**
6hr	153.4±9.9	152.5±6.1	172.8±9.0*	211.7±29.1*	214.9±11.2**
12hr	154.7±9.6	173.1±12.2*	182.3±10.7*	285.0±17.9**	259.4±22.5**
24hr	182.2±18.5	206.7±38.2*	264.0±25.2*	346.7±22.0**	442.5±11.4**
48hr	174.5±8.6	191.1±8.9*	288.7±13.3**	386.7±15.9**	433.9±5.7**

Mean±SE, *: p <0.05, **: p <0.005

described as essential for oocyte migration and follicular development in the adult mouse. Kit gene encoding kit is expressed from embryonic day 7.5 in the germ cells of developing gonads, oocyte of every follicle stages and some theca-interstitial cells. Spontaneous development of primordial follicle can be totally inhibited in the mouse ovary by ACK-2, a c-kit antibody that blocks KL actions, while blockade after initiation of c-kit function (5 days after birth), primoridial follicle formation, survival, and further development beyond antral follicles was not impaired. Thus, KL seems to be the first molecule that is related with initiation of primordial follicle development[15]. Recent report showed that the regulation of KL system is closely related to oocyte specific factor GDF-9, which is one of the key molecules regulating ovary folliculogenesis.

As previously described[10], dazla mRNA is also first expressed in the ovary during embryogenesis at between PCD 11.5-12.5, which implies early involvement of ovary folliculogenesis like KL. Dazla is autosomal homologue of DAZ, which is related with male spermatogenesis in the human and primate only. In other species, its autosomal homologue Dazla is regarded as having function of DAZ instead. Dazla is expressed in both male and female gonad with lesser extent of expression in the female. The function of Dazla was identified by mutational study of Dazla gene in the mouse[16]. In homozygous female, killed 6-9wks after birth, had tiny ovaries compared to littermate controls. In homzygous male, size of testis was estimated as 1/3 of the control. Histologic analysis of the ovaries showed that follicles and ova were absent. Testis sections also showed complete

absence of germ cells beyond spermatogonial stage. Thus, it seems clear that Dazla mRNA is related gametogenesis both in male and female.

But, the exact mechanism of regulation on this molecule was not elucidated yet. Before answering this question, we performed several experiments on the life cycle of dazla expression from oocyte, fertilized egg, and gonad from early embryo[10]. And exact location of dazla expression in the ovary was performed. In the initial experiment on the life cycle of dazla showed that it is expressed persistently in the oocyte regardless of their maturity, that is, unstimulated or stimulated oocytes by PMSG and hCG.. However, this oocyte suddenly lost their signal just after fertilization from syngamy and 2-cell embryo stage. That was quite interesting finding because both oocyte and sperm showed strong expression of dazla. Thus, this suggests some genes related to fertilization might negatively control the expression of dazla. But, the meaning and importance of this finding has not been clearly elucidated yet. The turned-off gene keeps going on to the stage of blastocyst, which is followed by the resurgence of that gene expression just after the gonadal formation. The exact location of dazla expression in the ovary was controversial[17]. On the contrary to the previous studies, our study demonstrated clearly the exact location of dazla is oocyte. That experiment was performed several times by procuring oocytes and granulose cell separately under microscope so as not to get false positive signals.

Dazla mRNA expression showed increased signal in the control group as the size of follicle grows (pre-injection immature mice). However, the intensity signals of dazla mRNA was not changed after the injection of PMSG and PMSG+hCG in the immature mouse compared to immature control (Table 1). Because signals observed in the oocytes before and after injection of PMSG and PMSG+hCG was not changed, the role of gonadotropin on the dazla expression in the ovarian follicle was expected that might not have any regulatory role. That is, no difference between mature and immature oocyte of the intensity of dazla in the preliminary study implies that FSH is not a critical factor for the regulation of dazla. Thus, both the ovary from immature and mature mice showed same intensity of signals if the ovary follicle is in the same stage of follicle development.

Taken together, obviously it is expressed in the oocyte cytoplasm of ovarian follicles, however, still the regulatory molecule of dazla gene and exact roles in the oocyte maturation or follicle development should be further investigated.

REFERENCES

1. Suh, CS, Sonntag B, Erickson GF. The ovarian life cycle: a contemporary view. Rev Endocr Metab Disord 2002; 3(1): 5-12.
2. Erickson, G.F. and S. Shimasaki, The physiology of folliculogenesis: the role of novel growth factors. Fertil Steril 2001; 76(5): 943-9.
3. Otsuka F, Yamamoto S, Erickson GF, Shimasaki S. Bone morphogenetic protein-15 inhibits follicle- stimulating hormone (FSH) action by suppressing FSH receptor expression. J Biol Chem 2001; 276(14): 11387-92.
4. McGrath SA, Esquela AF, Lee SJ. Oocyte-specific expression of growth/differentiation factor-9. Mol

Endocrinol 1995; 9(1): 131-6.
5. Laitinen M, Vuojolainen K, Jaatinen R, Ketola I, Aaltonen J, Lehtonen E et al. A novel growth differentiation factor-9 (GDF-9) related factor is co-expressed with GDF-9 in mouse oocytes during folliculogenesis. Mech Dev 1998; 78(1-2): 135-40.
6. Dong J, Albertini DF, Nishimori K, Kumar TR, Lu N, Matzuk M, Dong J. Growth differentiation factor-9 is required during early ovarian folliculogenesis. Nature, 1996; 383(6600): 531-5.
7. Elvin JA, Yan C, Wang P, Nishimori K, Matzuk M. Molecular characterization of the follicle defects in the growth differentiation factor 9-deficient ovary. Mol Endocrinol 1999; 13(6): 1018-34.
8. McNeilly JR, Saunders PT, Taggart M, Cranfield M, Cooke HJ, McNeilly AS. Loss of oocytes in Dazl knockout mice results in maintained ovarian steroidogenic function but altered gonadotropin secretion in adult animals. Endocrinology 2000; 141(11): 4284-94.
9. Pan HA, Tsai SJ, Chen CW, Lee YC, Lin YM, Kuo P L. Expression of DAZL protein in the human corpus luteum. Mol Hum Reprod 2002; 8(6): 540-5.
10. 서창석, 김용범, 구승엽, 지병철, 최영민, 김정구 등. 생쥐 난소의 발달 중 Dazla 유전자의 발현 양상. 대한불임학회지 2002; 29(3): 159-167
11. Teixeira Filho FL, Baracat EC, Lee TH, Suh CS, Matsui M, Chang RJ et al. Aberrant expression of growth differentiation factor-9in oocytes of women with polycystic ovary syndrome. J Clin Endocrinol Metab 2002; 87(3): 1337-44.
12. Tavaniotou A, Bourgain C, Albano C, Platteau P, Smitz J, Devroey P. Endometrial integrin expression in the early luteal phase in natural and stimulated cycles for in vitro fertilization. Eur J Obstet Gynecol Reprod Biol 2003; 108(1): 67-71.
13. Gougeon A. and D. Busso. Morphologic and functional determinants of primordial and primary follicles in the monkey ovary. Mol Cell Endocrinol 2000; 163(1-2): 33-42.
14. Tisdall DJ, Fidler AE, Smith P, Quirke LD, Stent VC, Heath DA. Stem cell factor and c-kit gene expression and protein localization in the sheep ovary during fetal development. J Reprod Fertil 1999; 116(2): 277-91.
15. Ismail RS, Okawara Y, Fryer JN, Vanderhyden BC. Hormonal regulation of the ligand for c-kit in the rat ovary and its effects on spontaneous

oocyte meiotic maturation. Mol Reprod Dev 1996; 43(4): 458-69.
16. Ruggiu M, Speed R, Taggart M, McKay SJ, Kilanowski F, Saunders P et al. The mouse Dazla gene encodes a cytoplasmic protein essential for gametogenesis. Nature 1997; 389(6646): 73-7.
17. Nishi S, Hoshi N, Kasahara M, Ishibashi T, Fujimoto S. Existence of human DAZLA protein in the cytoplasm of human oocytes. Mol Hum Reprod 1999; 5(6): 495-7.