Original Article

Differential Expression and Localization of Nanog, Oct 3/4 and C-Kıt in Mouse Ovarian Tissue According to Age

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ABSTRACT

Objective: To investigate the expression of embryonic/pluripotent stem cell markers including Nanog, Octamer – Binding Protein ¾ (Oct 3/4) and c-Kit from the newborn to aging period in the ovary tissues of mouse.
Design: Experimental study using mouse ovary tissues. The expression and localization of Nanog, Oct 3/4 and c-Kit expression were studied in newborn, pubertal, adult and aging ovary.
Setting: Department of Histology and Embryology, Pamukkale University, School of Medicine, Turkey
Subjects: Newborn (n = 6), pubertal (38-day-old) (n = 6), adult (12-week-old) (n = 6) and aged (24-week-old) female Balb/c mice were used in this study.
Intervention : No intervention
Main Outcome Measure: The expression of Nanog, Oct 3/4 and c-kit was evaluated by immunohistochemistry and reverse transcriptase chain reaction (PCR).
Results: Nanog, Oct 3/4 and c-Kit expression were positive in oocytes of newborn, pubertal and adult ovary. But they were negative in granulosa cells in newborn groups. The expression of these markers in adult period was increased. In addition, positive reaction for Nanog, Oct 3/4 and c-Kit was observed in granulosa cells in secondary and tertiary follicles in pubertal and adult ovary. Ovarian surface epithelium were positive for all stem cells markers in adult and aged. In addition to that, only c-kit positive expression was found in theca cells.
Conclusion: According to our findings, each of the three stem cell markers may play an important role in folliculogenesis and ovarian pathology. However, c-kit may be more effective than others because stromal cells were positive in adult and pubertal ovaries as well as in aged ovary.

KEY WORDS: immunohistochemistry, ovary, stem cells,

INTRODUCTION

The most fundamental dogma of reproductive medicine in the ovary is a certain number of oocytes and their possibility to increase. The basis of this doctrine was, first time taken in the 1870s[1].This doctrine was further strengthened in the 1950s[2]. But in the recent years in a serial study the accuracy of this dogma began to be questioned. The first concept of postnatal oogenesis in mice was emerged in 1923[3] and then in humans in 1932[4]. Tilly and his team reviewed the idea of postnatal oogenesis showing oocytes regeneration using peripheral blood and bone marrow[5,6]. They counted healthy and atretic follicles in mouse ovary sterilized with chemotherapeutic agents and they showed a rapid increase in the number of follicles. By the end of two months, they showed that no difference in follicle number was found between control and administered chemotherapeutic agents ovary. Moreover, they observed that the construction of oocytes re-formed in ablated ovary was not found after bone marrow transplant. In later years, oocytes were produced by using the stem cells derived from skin epithelium and amniotic epithelial cells[7,8]. Until now, oogonial stem cells (OSCs) have been isolated from the ovaries of adult mice[9,10] and rats[11] bovine[12] and humans[10]. These results showed that in vitro culture conditions these stem cells found in mammals can produce oocytes which have the ability to fertilize and result in a live birth.

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Based on previous experience it seems that ovarian surface epithelium stem cells retain the characteristic of embryonic stem cells[13-16]. The ovarian surface epithelium (OSE) obtained from adult and even menopaused mouse and human ovaries showed a difference in oocyte-like cells under defined in vitro culture conditions by several groups[13,15,17].

Biological importance of ovarian stem cells and their relation with ovarian function are not clear. However, ovarian stem cells can be considered as a remedy for prevention of reproductive aging, preovarian failure, polycystic ovary syndrome (PCOS) and ovary cancer[16,18,19].

In this study we have studied expressions, locations and distrubition of Nanog, Oct 3/4 and c-Kit markers of pluripotent /embryonic stem cells in newborn, pubertal, adult and aged ovarian tissues using the immunohistochemical technique and reverse transcriptase polymerase chain reaction analysis. So, it has been identified that the embryonic/ pluripotent stem cell markers expression is consistent during ovary development throughout the life. This determination is important to find causes of diseases of the reproductive system, especially in the ovary the pathophysiology and treatment of ovarian cancer are quite significant and greatly contribute in a better understanding of the ovarian functions, fertility and causes of diseases such as PCOS, preovarian failure and ovary cancer, and of course in developing new treatment approaches.

SUBJECTS AND METHODS
Animals

Newborn (n = 6), pubertal (38-day-old) (n = 6), adult (12-week-old) (n = 6) and aged (24-week-old) female Balb/c mouse were used in this study. They were kept at a constant temperature (21 ± 1 °C) and controlled light conditions (light 07.00 – 19.00). Food (standard pellet diet) and tap water were supplied ad libitum. All the animals in whole groups were anaesthetized via intramuscular injection of xylazine (5 mg/kg) and ketamine (90 mg/kg) and were killed by cervical dislocation for collection of ovary. Right ovaries were prepared for immunohistochemistry, left ovaries were prepared for RT-PCR. All studies with animals used in this study were reviewed and approved by the University of Pamukkale Animal Ethics committee.

Fixation and Tissue Preparation

Right ovaries were removed and were put in 10% neutral buffered formalin for 72 hours and then embedded in paraffin. Paraffin sections (5 mm) were deparaffinized in xylene and rehydrated through a graded series of ethanol solutions. Three sections from each animal were processed for Nanog, Oct 3/4 and c-Kit immunocytochemistry. Negative controls were performed by omitting the primary antibody.

Antibodies and Staining Procedure

Endogenous peroxidase activity was blocked in 3% hydrogen peroxidase for 10 minutes and sections were incubated with sponin to help easy binding of primer antibody to antigenic areas. Epitopes were stabilized by application of serum blocking solution (Goat serum, Lot# 20570999, Zymed Laboratories INC) for 20 minutes. Sections were incubated with primer antibody Oct 3/4 (C0411, Lot # C01411, Santa Cruz), Nanog (Lot # 823892, Abcam) and c-Kit (C-19, Lot # B2410 Santa Cruz), (diluted 1:100 in PBS) at +4 ^{0}C overnight. After applying with anti rabbit Ig, avidin-biotin complex peroxidase (ABC, Lot# 20570999, Zymed Laboratories INC) was applied to slides. Diaminobenzidine (DAB, Lot# 10163354, Zymed Laboratories INC) was used as chromogen. Afterwards, slides were counterstained with hematoxyline for 1 minute, dehydrated in graded ethanol and mounted in conventional medium. Slides were examined by three experienced histologists.

The intensity of immunoperoxidase reaction was classified as follows: negative (-) when the cells were devoid of any detectable Oct 3/4, Nanog and c-Kit expressions, slightly positive (+), moderately positive (++), and strongly positive (+++). Estimation of intensity of immunoperoxidase was blind with respect to the status of the animal (ovariectomized, or not, or phase of cycle). Negative controls were performed by omitting the primary antibodies resulting in no staining.

RNA Isolation and Semiquantitative RT–PCR Analysis

Nanog, Oct 3/4 and c-Kit mRNA expression profiles in the mouse ovary tissue were examined by RT-PCR. In semiquantitative RT-PCR reaction, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is used as a housekeeping gene. Isolation of RNA from fresh frozen tissue using Trizol (Sigma), was processed according to the manufacturer's protocol. All the animals in whole groups were anaesthetized via intramuscular injection of xylazine (5 mg/kg) and ketamine (90 mg/kg). Ovarian tissues were taken and they were put on an ice-cold glass stage. Total RNA was extracted from the tissues using an RNA isolation reagent, Tri-Reagent (Sigma, St. Louis, MO, USA). The single-tube one-step RT-PCR was standardized using the one-step RT-PCR kit (Qiagen, USA). Briefly, one-step RT-PCR was carried out in a 50 μL reaction mixture containing 1 μg total RNA, 10 pmol each primer, 10 μL 5X buffer (12.5 mM MgCl$_2$), 2 μL dNTPs mix (containing 10 mM of each dNTP), and 2 μL of a mixture

Table 1: Expression and distribution of Nanog in developing mouse ovary

Nanog expression	Newborn groups	Pubertal groups	Adult groups	Aged groups
Surface Epithelium	+++	+++	+++	+++
Oocytes of primordial follicles	-	+	+	
Granulosa cells of primordial follicles	-	-	-	
Oocytes of primary follicles	+++	+	++	-
Garanulosa cells of primary follicles	-	+	-	
Oocytes of secondary follicles	Not avaliable	++	+++	-
Granulosa cells of secondary follicles	Not avaliable	++	++	Not avaliable
Oocytes of tertiary follicles	Not avaliable	++	+++	Not avaliable
Granulosa cells of tertiary follicles	Not avaliable	++	+++	Not avaliable
Corpus Luteum	Not avaliable		+++	-
Theca cells	Not avaliable	-	-	
Blood vessels	++++	++	+++	+++
Ovarian stroma	-	++	+++	+++
OSE	+++	+++	+++	+++

of Ominiscript and Sensiscripts reverse transcriptases and Hot Star Taq DNA polymerase. Gene expression was presented as the yield of PCR products from target sequences relative to the yield of PCR products from the GAPDH gene. In each instance, the amount of reverse transcription (RT)-PCR product for the gene of interest was normalized to the amount of GAPDH in the same sample. The primer sequences used in this study and cycling conditions are summarized in Table 1. The experiments were repeated two times using duplicates in each group. The RT-PCR products were analyzed by electrophoresis using 2% Molecular Screening Agarose gel (Roche Diagnostics, GmbH, Mannheim, Germany) and visualized by UV light.
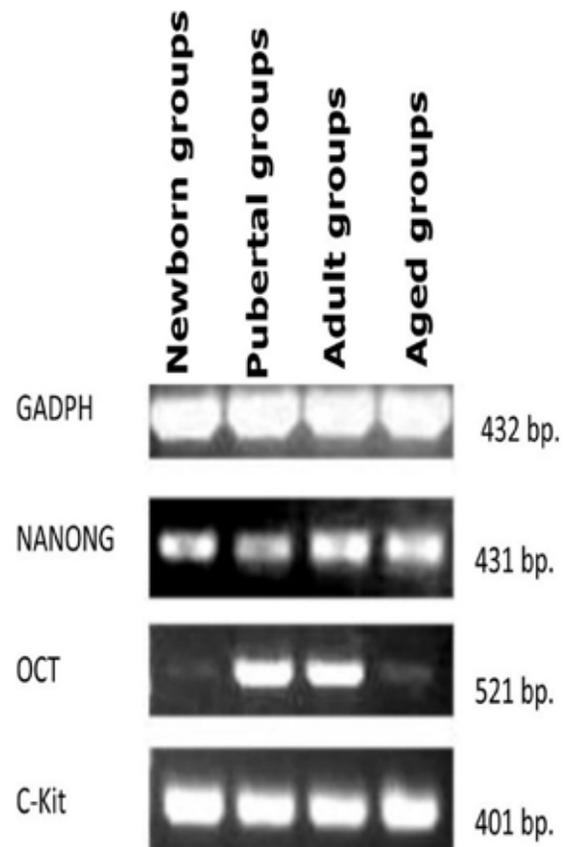
RESULTS
RT-PCR Results
Nanog, Oct 3/4, c-Kit mRNAs expression profiles of mouse ovarian tissue was examined by RT-PCR. Semi quantitative RT-PCR reaction and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression were used as the control of gene expression (housekeeping gene expression).

The results of RNA of Nanog gene expression levels in newborn were similar to pubertal groups. However, Nanog gene expression was increased in adult ovarian tissues when compared with newborn and puberty. Oct 3/4 RNA expression results showed that gene expression in aged and newborn ovarian tissues is much less than adult and pubertal ovarian tissues. RNA expression of c-kit results was found to be similar among the groups. However, it was observed that expression levels of Nanog gene has increased in aged and 2-month-old mouse ovarian tissues as in the tissues of newborn and puberty mice. According to this finding, Nanog expression in mouse ovarian tissue is considered to increase gradually in the developmental period. In newborn and aged groups, Oct 3/4 gene expressions were much less as

compared with adult and pubertal group. The results of the c-Kit mRNA expression were evaluated, and a similar expression has been observed among the groups. According to this finding, the expression of c-Kit gene is considered to be protected from newborn up to the aged group. In this group, the ovarian stroma was found to be strongly positive as compared to the neonatal group (Fig 1).

Fig 1: The RT-PCR gen expression profiles of mouse embryonic stem cells

Immunohistochemical results

Expression of Nanog in Ovarian Tissues: The distribution and localization of nanog expression in newborn, pubertal, adult and aged ovarian tissues are summerized in Table 1.

Newborn groups: In this group, negative reaction for Nanog was observed in follicular epithelial cells. However, oocytes of some primary follicles had positive reactions, and some of them were negative. While blood vessels in ovarian stroma showed a strong positive staining for Nanog, the stromal cells were negative. A strong expression of Nanog was also found in ovarian surface epithelium (Fig 2 A, B).

Pubertal groups: No immunoreactivity was observed in primary and secondary follicles' granulosa cells. Some of the tertiary follicles' granulosa cells had positive reaction, whereas the others had a negative one (Fig 2C). A moderate staining was observed in blood vessels and ovarian stroma. OSE in pubertal group like in the newborn group showed positive staining, however, these cells were negative for Nanog (Fig 2 D).

Adult groups: Primordial follicles cells and oocytes showed negative reactions. Combination of negative and positive reactions was observed in primary follicles cells. Although a weak staining was detected in primary follicles oocytes, an increased expression of Nanog was noted in oocytes of secondary and tertiary follicles. In this group, expression of Nanog was increased in secondary and tertiary follicular cells compared to pubertal groups. Luteal cells cytoplasm showed positive reaction for Nanog but their nucleus had no immunreactions in corpus luteum. In adult groups, OSE, blood vessels and stroma demonstrated strong staining. (Fig 2 E, F)

Aged groups: It was observed that the number of follicles decreased, but the number of corpus luteum increased. It was interesting to observe that stromal cells and atretic follicles had a positive reaction, whereas luteal cells had a negative reaction. Both positive and negative reactions for Nanog were found on ovarian surface epithelial cells in aged mice (Fig 2 G, H).

Expression of Oct 3/4: The distribution and localization of Oct3/4 expression in newborn, pubertal, adult and aged ovarian tissues are summerized in Table 2.
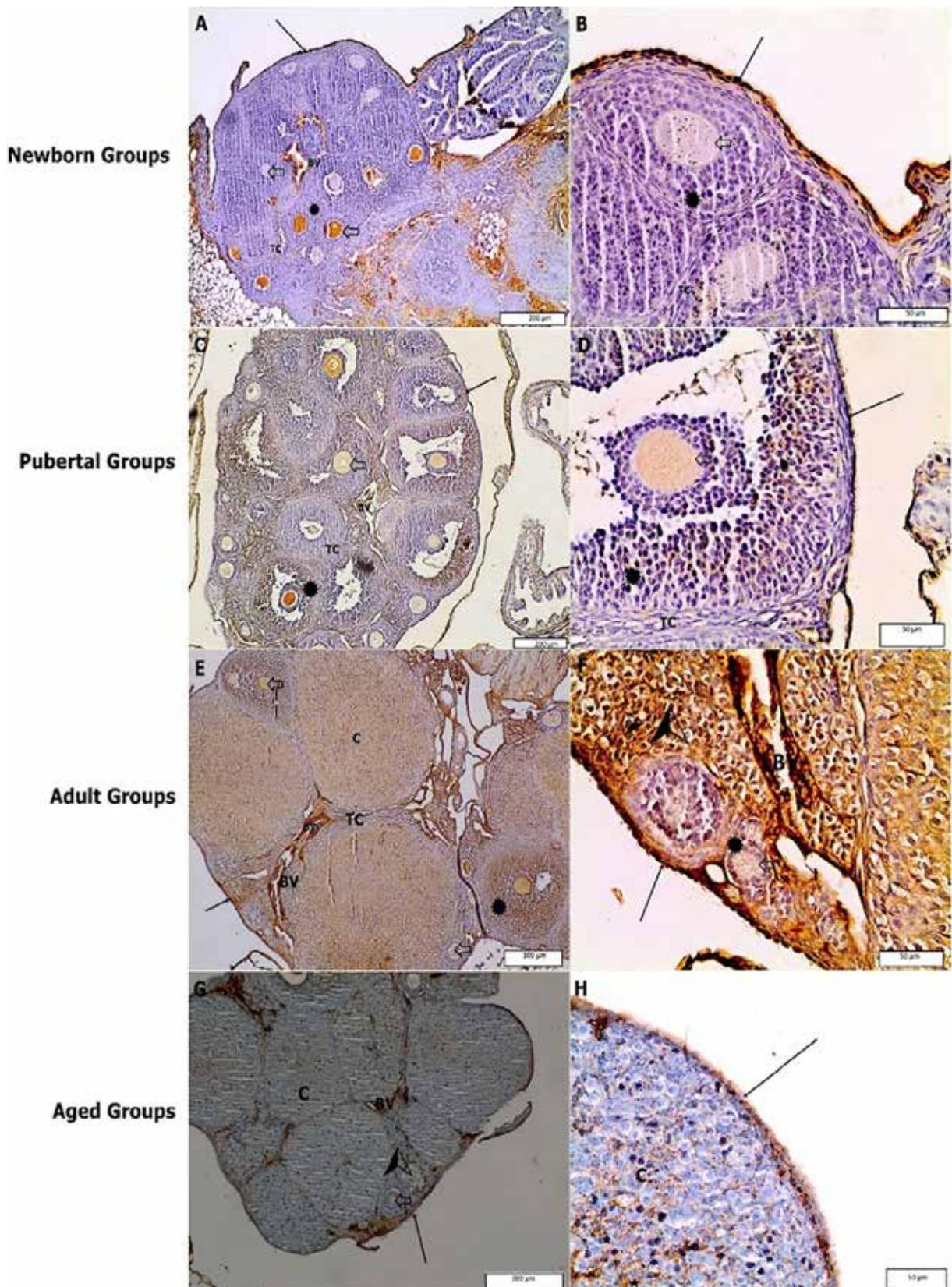
Newborn groups: It was interesting to observe that oocytes in some primordial and primary follicles had strong immunoreactions, whereas some of them were negative or weak positive. In addition, granulosa cells and OSE displayed negative staining (Fig 3 A, B).

Pubertal groups: Negative and poor reactions were observed in oocytes in primary follicles; however, oocytes in secondary and tertiary follicles demonstrated strong positive reactions. Besides, we noticed that granulosa cells of primary and secondary follicles showed negative reaction but tertiary follicles consisted of cells showing both positive and negative reactions. In pubertal groups, OSE, theca cells and stroma exhibited negative reaction for Oct 3/4 (Fig 3 C, D).

Adult groups: In this group, not only negative but also positive staining oocytes were noted. While negative immunostaining was found in primordial granulosa cells, positive immunostaining was detected in primary, secondary and tertiary follicles' granulosa cells. Cytoplasmic positive staining for Oct 3/4 was observed in corpus luteal cells. Positive staining for Oct 3/4 which gradually changes from weak to strong

Table 2: Expression and distribution of Oct3/4 in developing mouse ovary

Oct 3/4 expression	Newborn groups	Pubertal groups	Adult groups	Aged groups
Surface epithelium	-	-	+++	+++
Oocytes of primordial follicles	-	+	+	
Granulosa cells of primordial follicles	-	-	-	
Oocytes of primary follicles	++	++	++	-
Granulosa cells of primary follicles	-	-	-	-
Oocytes of secondary follicles	Not avaliable	++	++	Not avaliable
Granulosa cells of secondary follicles	Not avaliable	+	++	Not avaliable
Oocytes of tertiary follicles	Not avaliable	+	++	Not avaliable
Granulosa cells of tertiary follicles	Not avaliable	+	-	-
Corpus Luteum	Not avaliable	-	+++	-
Theca cells	Not avaliable	-	-	-
Blood vessels	-	+++	+++	+++
Ovarian stroma	+	-	+	+++
OSE	-	-	+++	+++

Fig 2: The localization and distrubution of Nanog expression in developing mouse ovary. **(A-B)**: Newborn groups, **(C-D)**: Pubertal groups, **(E-F)**: Adult groups, **(G-H)**: Aged groups; Ovarian surface epithelium: (arrow), oocyte: (thick arrow), granulosa cells: (asterix), theca cells: (TC), corpus luteum: (C), stromal cells: (arrow head), blood vessels: (BV).
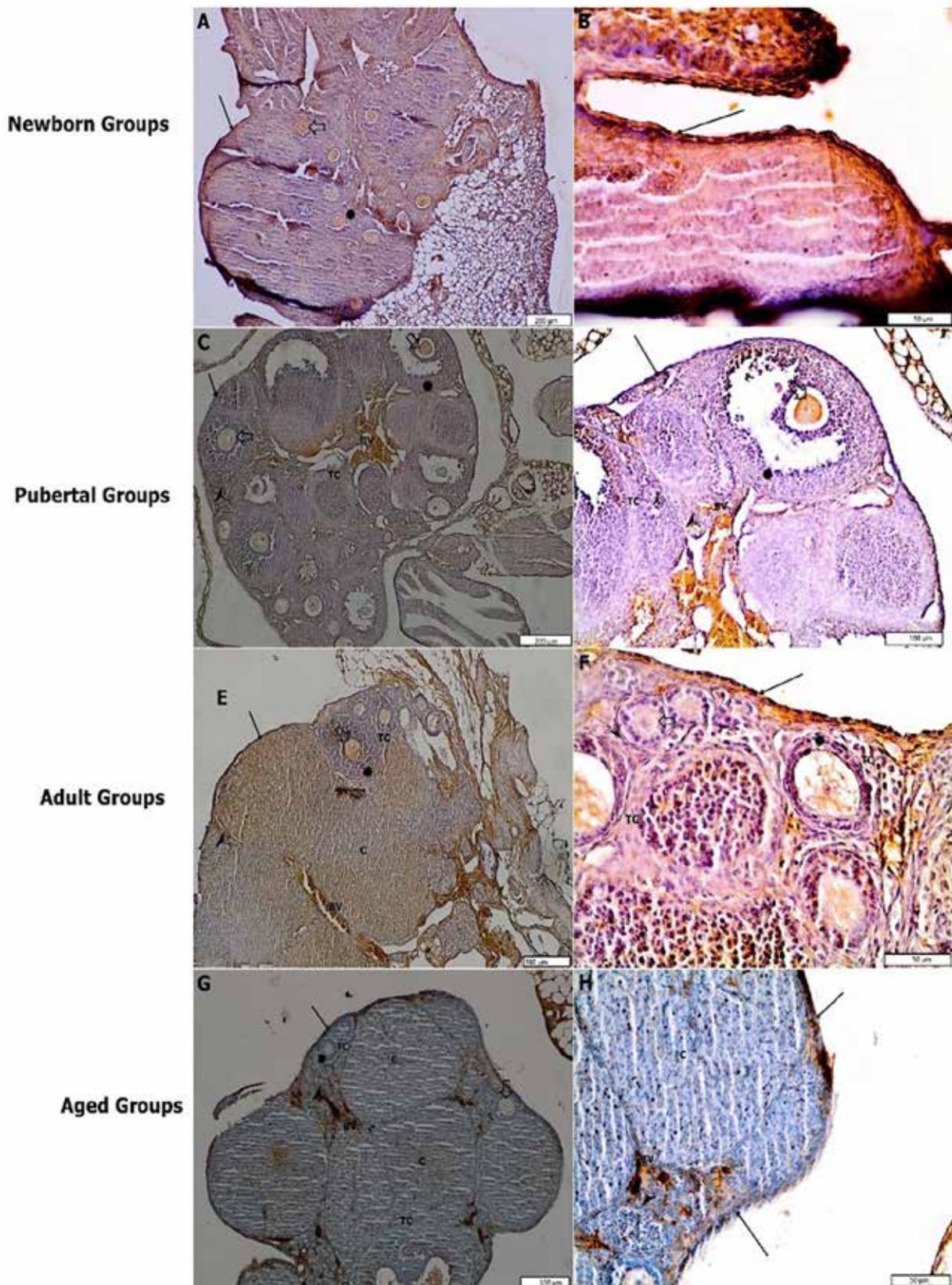
Fig 3: The localization and distrubution of Oct3/4 expression in developing mouse ovary. **(A-B)**: Newborn groups, **(C-D)**: Pubertal groups, **(E-F)**: Adult groups, **(G-H)**: Aged groups, ovarian surface epithelium; (arrow), oocyte; (thick arrow), granulosa cells; (asterix), theca cells; (TC), corpus luteum; (C), stromal cells; (arrow head), blood vessels; (BV).

Table 3: Expression and distribution of c-Kit in developing mouse ovary

c-Kit expression	Newborn groups	Pubertal groups	Adult groups	Aged groups
Surface epithelium	+	+++	+++	+++
Oocytes of primordial follicles	-	++	+	-
Granulosa of primordial follicles	-	+	-	-
Oocytes of primary follicles	++	++	+++	-
Granulosa cells of primary follicles	-	+	+	-
Oocytes of secondary follicles	Not avaliable	+++	+++	Not avaliable
Granulosa cells of secondary follicles	Not avaliable	+++	++	Not avaliable
Oocytes of tertiary follicles	Not avaliable	+++	+++	Not avaliable
Granulosa cells of tertiary follicles	Not avaliable	+++	++	Not avaliable
Corpus Luteum	Not avaliable	++	+++	-
Theca cells	Not avaliable	++	+++	-
Blood vessels	-	+++	+++	+++
Ovarian stroma	+	+++	+++	+++
OSE	+++	+++	+++	+++

was observed on the surface epithelium in adult mice. (Fig 3 E, F). In this group, theca cells were observed negative while blood vessels observed strong positive staining.

Aged groups

In aged groups, the OSE layer consists of cells showing positive and negative immunreactions. Negative immunreaction was demonstrated in oocytes and granulosa cells. While luteal cells had negative reactions, stromal cells and atretic follicles showed positive reactions (Fig 3 G, H).

Expression of c-Kit: The distribution and localization of c-Kit expression in newborn, pubertal, adult and aged ovarian tissues are summerized in Table 3.

Newborn groups: In this group, negative reaction was observed in folicular cells; however, some oocytes showed strong positive reactions and some of them had no reaction. In addition, ovarian stromal cells demostrated a weak positive staining for c-Kit. Poor positive c-Kit immunreaction was observed on the surface epithelium in newborn groups (Fig 4 A, B).

Pubertal groups

Poor positive reaction was detected in granulosa cells of primordial and primary follicles. Moderate positive reaction was observed in the granulosa cells of secondary follicles which are close to the lumen, and in tertiary follicles positive staining intensity gradually increased. While oocytes in primordial and primary follicles showed a moderate positive staining, secondary and tertiary follicles' oocytes showed a strong positive staining for c-Kit. In addition, corpus luteal cells, theca cells and stromal cells demonstrated strong immunoreactivity for c-Kit. In the pubertal ovary, c-Kit expression was higher compared to OCT3/4 and NANOG expression.

The OSE showed strong positive staining in pubertal groups (Fig 4 C, D).

Adult groups

Moderate and strong staining was determined in granulosa cells of primordial follicles, whereas oocytes of primordial follicles were negative for c-Kit. We noticed that oocytes and basal granulosa cells in secondary and tertiary follicles exhibited strong positive reactions for c-Kit. Strong positive c-Kit staining was found on OSE and corpus luteum in 12-week adult groups (Fig. 4: E, F). Theca cells, stroma and blood vessels were also strong positive in these groups.
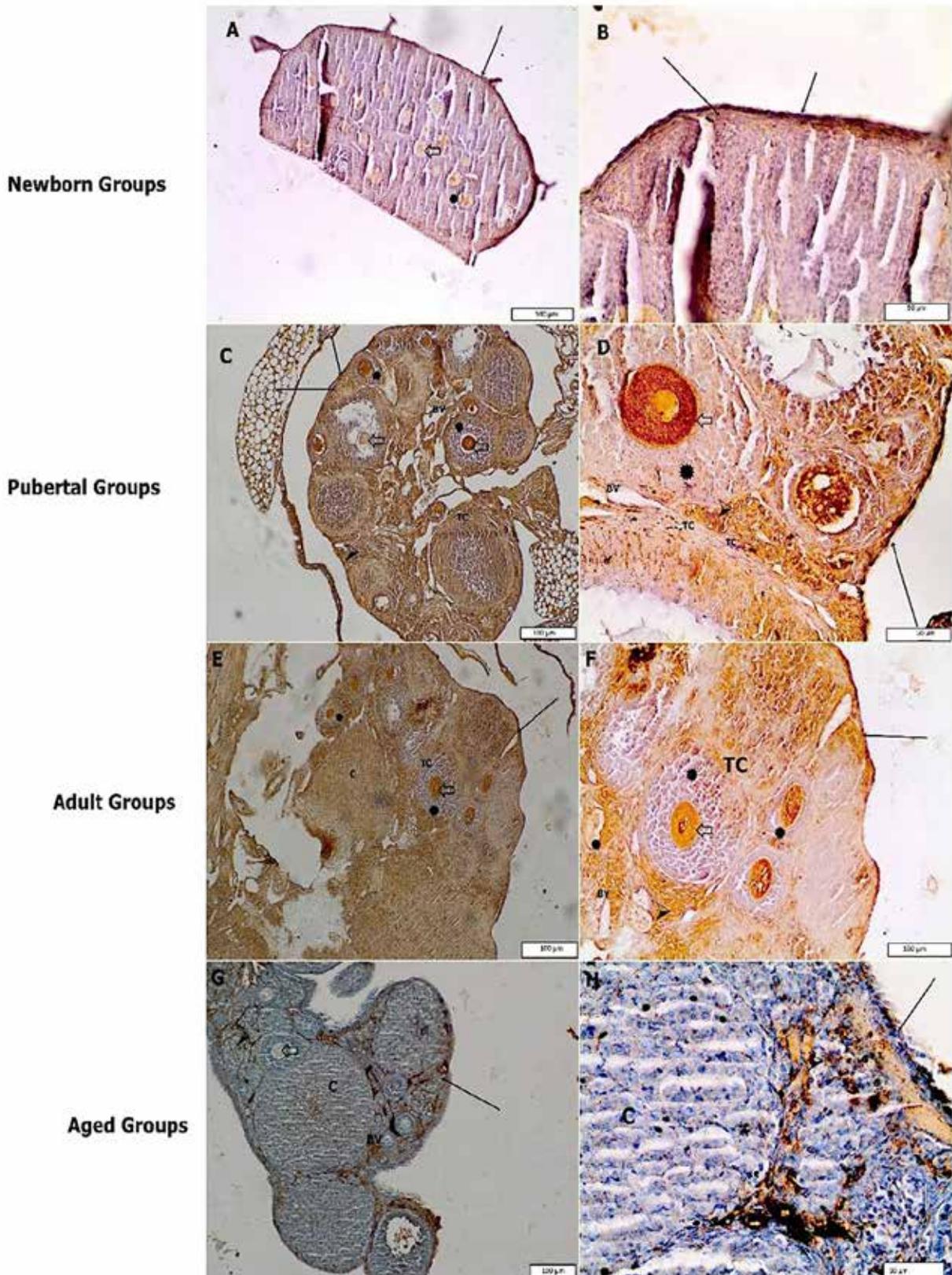
Aged groups

In the aged group, staining pattern is similar to other groups. Negative and positive cells for c-Kit were determined on surface epithelium. Atretic follicles and stromal cells stained strongly but negative reaction was observed in luteal cells (Fig 4 G, H).

DISCUSSION

The present study provides a comprehensive evaluation of pluripotent stem cell expression in developmental ovary using immunohistochemistry and PCR technique. Importantly, the study demonstrates the expression of multiple stem cells markers shown collectively in ovary tissues from newborn to aged period. Information from this study describes the expression of multiple embryonic/pluripotent stem cell markers in granulosa cells, oocytes and OSE cells during ovary development to help evaluate the pluripotent nature of these cells.

Oct 4 (Oct 3/4, Oct 3) is the most important transcription factor and it is expressed in embryonic stem cells, primordial germ cells, germ cells and also in germ cell tumors[20,21]. Oct 4 inhibits differentiation of embryonic cells[22,23]. Decreasing expression of Oct

Fig 4: The localization and distrubution of c-Kit expression in developing mouse ovary. **(A-B)**: Newborn groups, **(C-D)**: Pubertal groups, **(E-F)**: Adult groups, **(G-H)**: Aged groups, ovarian surface epithelium; (arrow), oocyte; (thick arrow), granulosa cells; (asterix), theca cells; (TC), corpus luteum; (C), stromal cells; (arrow head), blood vessels; (BV).

3/4 initiates differentiation in embryonic cells and with the beginning of differentiation the synthesis of Oct 4 decreases. In a study by Gota *et al*, it was established that Oct 3/4 was expressed in germ cells at intrauterine 17th and 24th weeks and its expression decreased in later periods[24]. After birth, Oct 3/4 is not expressed by germ cells. Contrary to Gota's findings, our study showed a strong positive Oct 3/4 expression in oocytes of newborn, pubertal and adult ovary. Also, granulosa cells in pubertal ovary were positive. In adult mouse ovary, granulosa cells of secondary and tertiary follicles were positive while theca cells were negative. The surface epithelium showed positive staining in newborn, adult and aged ovary. In addition, stromal cells were positive in adult groups. In aged ovary, only the surface epithelium observed positive staining for Oct 3/4.

Another stem cell marker Nanog was discovered in 2003. Pre-implantation embryos, embryonic stem cells, embryonic germ cells and embryonic carcinoma cells were positive for Nanog using immunohistochemical techniques and RT-PCR studies. It was also reported that unfertilized oocytes were negative for Nanog[25-27]. In another study in 2005 by Yamaguchia *et al*, negative expression for Nanog in ovarian follicles of eight weeks mice was found[28]. In contrast to these studies, we found positive reactions in oocytes of newborn, pubertal and adult ovary. We also found a moderate staining for Nanog in granulosa cells of pubertal and adult groups and a negative staining in stroma of newborn ovary. A moderate staining in stroma cells was seen in pubertal groups while theca cells were negative. In aged groups, a strong staining in stroma cells and corpus luteii were observed. Nanog expression was negative in adult ovary as in the pubertal groups. In aged ovarian tissue, only OSE cells and stromal cells showed positive expression.

C-Kit receptor is a type III receptor tyrosine kinase and it plays a role in oocyte-granulosa interaction[29]. Robinson *et al* showed c-Kit mRNA in oogonia which produced mitotic activity[30]. Yoshida and colleagues (1997) reported that when the primary follicles were developed, the expression of c-Kit was blocked[29]. Driancourt (2000) showed that c-Kit expression was positive on 8 - 14 pc days and also oocytes of primordial follicles and growing follicles had strong positive in postnatal period[31]. Human primordial germ cells showed positive c-Kit in between the 7th and 13th week during 21 weeks of gestation. Stoop (2005) reported that ovarian tissue had positive reaction for c-Kit during intrauterine life[32].

Kang (2003) notified oocytes of primordial and primary follicles were positive in postnatal seven days and also granulosa cells had positive reaction for c-Kit

in 21 days[33]. Our results supported these findings, we also found positive c-Kit expression in oocytes of primordial and primary, secondary, tertiary follicles in newborn, pubertal and adult groups. However in adults, pubertal and neonatal period, granulosa cells of primordial and primary follicles did not demostrate positive staining for c-Kit. Contrary to granulosa cells of primordial, primary, secondary and tertiary follicle' granulosa cells, the theca cells showed moderate staining in pubertal groups and strong staining in adult groups for c-Kit.

OSE has an important role in the physiology of the ovary and ovarian cancers. 85 - 90% of ovarian cancer originated from OSE and these cancers are lethal[34]. Very little differentiated OSE, epithelial tissue, unlike many, suffered both at the change of epithelial and mesenchymal markers.

OSE exhibited epithelial and mesenchymal markers apart from many epithelial tissues[35]. In recent years, several studies have shown the presence of mature OSE stem cells[35]. Parte and his colleagues showed that the cultured stem cells are derived from OSE expressed Oct 3/4 and SSEA-4. Virant-Klun reported that stem cells derived from OSE are similar to embryonic stem cells and they showed Sox-2, Oct 4 and Nanog expression[17]. Virant-Klun isolated OSE cells from postmenopausal woman who had naturally no follicles and from premature ovarian failure patients and these epithelial cells were transformed to oocytes by them. In 2008, Zhang *et al* charactarerized cells expressing germ cell markers Oct 3/4, MVH, the SCF-R (the c-Kit-CD117) and SSEA-1 in nonfollicle structures in ovary[36]. In our study, it was interesting to observe that all OSE groups were positive for Nanog and for c-Kit. On the other hand, OSE was negative for Oct in newborn and pubertal period but had positive reaction in aged and adult groups. These results may be effective in strengthening the understanding of postnatal oogenesis and development of ovarian cancer.

CONCLUSION

In this study, oocytes in newborn groups were positive for all the three stem cells markers but granulosa cells were negative. In pubertal and adult stage, oocytes and granulosa cells of secondary and tertiary follicles were positive while primary follicles' granulosa cells were negative for Oct 3/4, c-Kit and Nanog by using immunohistochemical and RT-PCR tecniques (Fig 1-4). Hormones or paracrine effects in puberty can cause the negative expression in granulosa cells of secondary and tertiary follicles to change to positive expression. These findings suggest that Nanog, Oct 3/4 and c-Kit may be very effective on

folliculogenesis. Theca cells were positive only for c-Kit and also in the pubertal ovary, c-Kit expression was higher compared to Oct 3/4 and Nanog expression. As a result of these findings, we assumed that the staining of c-Kit may be related to estrogen hormone.

Adult ovarian tissues showed a stronger Oct 3/4, c-Kit and Nanog staining as compared to newborn, pubertal groups. Particularly granulosa cells and oocytes of secondary and tertiary follicles in adult groups had strong immune reactions for Oct 3/4, c-Kit and Nanog. Moreover, granulosa cells of both secondary and tertiary follicles showed nuclear and cytoplasmic staining. This type of staining pattern was observed in different studies and it was reported that these molecules may be related to both nuclear DNA and mitochondrial DNA in cytoplasm. Particular granulosa cells were positive for Nanog, Oct 3/4 and c-Kit which may play a role in normal folliculogenesis.

In our study, aged groups showed positive reaction in the stromal and surface epithelial cells for all three markers and they may also be transformed into oncogenic stem cells.

Regarding the results, stromal cells, OSE cells and ovarian follicles may be a reference for further research for normal and pathological processes. According to our findings, each of the three stem cell markers may play an important role in these processes. However, c-Kit may be more effective than others, because stromal cells were positive in adult and pubertal ovaries as well as in aged ovary.

ACKNOWLEDGMENT

REFERENCES

1. Waldeyer W, Eierstock und Ei. Engleman, Leipzig 1870.
2. Zuckerman S. The number of oocytes in the mature ovary. Recent Prog Horm Res 1951; 6:63-109.
3. Allen E. Ovogenesis during sexual maturity. Am J Anat 1923; 31:439-481.
4. Simkins C. Development of the human ovary from birth to sexual maturity. Am J Anat 1932; 51:465-505.
5. Johnson JJ, Canning J, Kaneko T, Pru JK, Tilly JL. Germline stem cells and follicular renewal in the postnatal mammalian ovary. Nature 2004; 428:145-150.
6. Johnson J Bagley, Skaznik-Wikiel M, *et al*. Oocyte generation in adult mammalian ovaries by putative germ cells in bone marrow and peripheral blood. Cell 2005; 122:303-315.
7. Dyce PW, Li J. From skin cells to ovarian follicles? Cell Cycle 2006; 5:1371-1375.
8. Wang F, Wang L, Yao X, Lai D, Guo L. Human amniotic epithelial cells can differentiate into granulosa cells and restore folliculogenesis in a mouse model of chemotherapy-induced premature ovarian failure. Stem Cell Res and Ther 2013; 4:124.
9. Zou K, Yuan Z, Yang Z, *et al*. Production of offspring from a germline stem cell line derived from neonatal ovaries. Nat Cell Biol 2009; 11:631–636.
10. White YA, Woods DC, Takai Y, Ishihara O, Seki H, Tilly JL. Oocyte formation by mitotically active germ cells purified from ovaries of reproductive-age women. Nat Med 2012; 18 :413–421.
11. Zhou L, Wang, Kang JX, *et al*. Production of fat-1 transgenic rats using a postnatal female germline stem cell line. Mol Hum Reprod 2014; 20:271–281
12. Dunlop CE, Bayne RA, McLaughlin M, Telfer EE, Anderson RA. Isolation, purification and culture of oogonial stem cells from adult human and bovineovarian cortex. Lancet 2014; S45.
13. Bukovsky A, Svetlikova M, Caudle MR. Oogenesis in cultures derived from adult human ovaries. Reprod Biol Endocrinol 2005; 3:17.
14. Virant-Klun I, Zech N, Rozman P, *et al*. Putative stem cells with an embryonic character isolated from the ovarian surface epithelium of women with no naturally present follicles and oocytes. Differentiation 2008; 76:843-856.
15. Virant-Klun I, Rozman P, Cvjeticanin B, *et al*. Parthenogenetic embryo-like structures in the human ovarian surface epithelium cell culture in postmenopausal women with no naturally present follicles and oocytes. Stem Cells Dev 2009; 18:137-149.
16. Dunlop CE, Telfer EE, Anderson RA. Ovarian stem cells potential roles in infertility treatment and fertility preservation. Maturitas 2013; 76:279-283.
17. Virant-Klun I, Skutella T. Stem cells in aged mammalian ovaries. Aging 2010; 2:3–6.
18. Dyall S, Gayther SA, Dafou D. Cancer stem cells and epithelial ovarian cancer. J Oncol 2010; 2010:105269.
19. Ko SY, Lengyel E, Naora H. The Müllerian HOXA10 gene promotes growth of ovarian surface epithelial cells by stimulating epithelial-stromal interactions. Mol Cell Endocrinol 2010; 12:112-119.
20. Pesce M, Scholer HR. Oct-4: Control of totipotency and germline determination. Mol Reprod Dev 2000; 55:452- 457.
21. Chew JL, Loh YH, Zhang W, *et al*. Reciprocal transcriptional regulation of Pou5f1 and Sox2 via the Oct4/Sox2 complex in embryonic stem cells. Mol Cell Biol 2005; 25:6031-6046.
22. Avilion AA, Nicolis SK, Pevny LH, Perez LN, Vivian R, Lovell-Badge. Multipotent cell lineages in early mouse development depend on SOX2 function. Genes Dev 2003; 17:126–140.
23. Rosner MH, Vigano MA, Ozato K, *et al*. A POU-domain transcription factor in early stem cells and germ cells of the mammalian embryo. Nature 1990; 21:686-692.
24. Goto T, Adjaye J, Rodeck CH, Monk M. Identification of genes expressed in human primordial germ cells at the time of entry of the female germ line into meiosis. Mol Hum Reprod 1999; 5:851–860.

25. Mitsui K, Tokuzawa Y, Itoh H, *et al*. The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. Cell 2003; 113:631–642.
26. Wang SH, Tsai MS, Chiang F, Li H. A novel NK-type homeobox gene, ENK (early embryo specific NK), preferentially expressed in embryonic stem cells. Gene Expr Patterns 2003; 3:99–103.
27. Hart AH, Hartley L, Ibrahim M, Robb L. Identification, cloning and expression analysis of the pluripotency promoting Nanog genes in mouse and human. Dev Dyn 2004; 230:187–198.
28. Yamaguchia S, Kimuraa H, Tadaa M, Nakatsuji N, Tadaa T. Nanog expression in mouse germ cell development. Gene Expr Patterns 2005; 5:639–646.
29. Yoshida H, Takakura N, Kataoka H, Kunisada T, Okamura H, Nishikawa SI. Stepwise requirement of c-kit tyrosine kinase in mouse ovarian follicle development. Dev Biol 1997; 184:122-137.
30. Robinson LL, Gaskell TL, Saunders PT, Anderson RA. Germ cell specific expression of c-kit in the human fetal gonad. Mol Hum Reprod 2001; 7:845–852.
31. Driancourt MA, Reynaud K, Cortvrindt R, Smitz J. Roles of kit and kit ligand in ovarian function. Rev Reprod 2000; 5:143–152.
32. Stoop H, Honecker F, Cools M, Krijger R, Bokemeyer C, Looijenga LHJ. Differentiation and development of human female germ cells during prenatal gonadogenesis: an immunohistochemical study. Hum Reprod 2005; 20:1466–1476.
33. Kang JS, Lee CJJ, Lee M, Rha JY, Song KW, Park MH. Follicular expression of c-Kit/SCF and inhibin-alpha in mouse ovary during development. J Histochem Cytochem 2003; 51:1447-1458.
34. Jemal A, Thomas T, Murray T, Thun M. Cancer statistics CA. Cancer J Clin 2002; 52:23–47.
35. Auersperg N, Wong AST, Choi KC, Kang SK, Leung PCK. Ovarian surface epithelium: biology, endocrinology and pathology. Endocr Rev 2001; 22:288.
36. Zhang D, Fouad H, Zoma WD, Salama SA, Wentz MJ, Al-Hendy A. Expression of stem and germ cell markers within nonfollicle structures in adult mouse ovary. Reprod Sci 2008; 15:139-146.