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ORIGINAL PAPER

# The loss of global DNA methylation due to decreased DNMT expression in the postnatal mouse ovaries may associate with infertility emerging during ovarian aging

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**Abstract**
Ovarian aging is one of the main causes of female infertility, and its molecular background is still largely unknown. As DNA methylation regulates many oogenesis/folliculogenesis-related genes, the expression levels and cellular localizations of DNA methyltransferases (DNMTs) playing key roles in this process is important in the ovaries from early to aged terms. In the present study, we aimed to evaluate the spatial and temporal expression of the *Dnmt1*, *Dnmt3a*, *Dnmt3b*, and *Dnmt3l* genes as well as global DNA methylation levels in the mouse ovaries during aging. For this purpose, the following groups were created: young (1- and 2-week old; $n=3$ from each week), prepubertal (3- and 4-week-old; $n=3$ from each week), pubertal (5- and 6-week-old; $n=3$ from each week), postpubertal (16- and 18-week-old; $n=3$ from each week), and aged (52-, 60- and 72-week-old; $n=3$ from each week). We found here that *Dnmt1*, *Dnmt3a*, and *Dnmt3l* genes' expression at mRNA and protein levels as well as global DNA methylation profiles were gradually and significantly decreased in the postnatal ovaries from young to aged groups ($P<0.05$). In contrast, there was a remarkable increase of *Dnmt3b* expression in the pubertal, postpubertal and aged groups ($P<0.05$). Our findings suggest that the significantly altered DNMT expression and global DNA methylation levels during ovarian aging may contribute to female infertility development at the later terms of lifespan. Also, new researches are required to determine the molecular biological mechanism(s) that how altered DNMT expression and decreased DNA methylation lead to ovarian aging.

**Keywords** DNA methylation · DNA methyltransferase · Ovarian aging · Infertility

## Introduction

The female fertility progressively decreases during reproductive aging due to depletion of oocyte reserve and significant decrease of antral follicle count (Gosden et al. 1983). These losses mostly likely originate from dramatic changes in oocyte physiology (Tarin et al. 2001; Eichenlaub-Ritter et al. 2004; de Bruin et al. 2004), altered hypothalamic-pituitary ovarian axis (Nelson et al. 1981; Flurkey et al. 1982), impaired oocyte-granulosa cell cross talk and mitochondrial dysfunction during ovarian aging [reviewed in May-Panloup et al. (2016)]. These changes may also lead to increased rate of resorption (Holinka et al. 1979), fetal death (Cnattingius et al. 1992), low birth weight (Roberts et al. 1994; Berkowitz et al. 1990) and various types of chromosomal anomalies (Eichenlaub-Ritter and Boll 1989; Hunt and Hassold 2008). In women, the significant decrease in fertility begins to appear approximately 35 years old (Navot et al. 1994; Sauer 1998). To date, the underlying molecular background of fertility decline in females during ovarian aging is not fully addressed. In this study, we aimed to examine the potential relationship between DNA methylation and gradual loss of female fertility during ovarian aging in mice.

As is known, DNA methylation, one of the main epigenetic mechanisms, functions in transcriptional repression (Bird 1992; Bird et al. 1995) and activation (Zhang et al. 2006) of development-related genes. It is well known that the global DNA methylation increases during oocyte maturation from germinal vesicle (GV) to metaphase II (MII) stages, reaching the highest level in MII oocytes (Smallwood

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and Kelsey 2012). Also, granulosa cells undergo DNA methylation to regulate repression or activation of the genes required for proper follicular development (Huntriss et al. 2004). In the process of establishing DNA methylation, DNA methyltransferase (DNMT) enzymes add a methyl group to the fifth carbon atom of the cytosine residues using S-adenosyl-L-methionine (AdoMet) as a methyl donor (Turek-Plewa and Jagodzinski 2005). This mainly occurs in cytosine-phosphate-guanine (CpG) dinucleotide islands and rarely in the non-CpG sites including cytosine-phosphate-thymine (CpT), cytosine-phosphate-adenine (CpA), and cytosine-phosphate-cytosine (CpC). Two different DNA methylation mechanisms have been identified: de novo and maintenance methylation, both of which are catalyzed by DNMTs. Up to date, structurally and functionally six different DNMTs including DNMT1, DNMT2, DNMT3A, DNMT3B, DNMT3C, and DNMT3L have been characterized. DNMT1 fundamentally plays an important role in the maintenance methylation of hemi-methylated strands during DNA replication and also contributes to de novo methylation process (Fatemi et al. 2002). DNMT3A and DNMT3B are responsible for de novo methylation (Turek-Plewa and Jagodzinski 2005). Although DNMT3L does not have any catalytic domain, it is capable of inducing DNMT3A and DNMT3B activities (Deplus et al. 2002; Margot et al. 2003). Thus, DNMT3L indirectly participates in de novo methylation. On the other hand, DNMT2 methylates the cytosine 38 localized in the anticodon loop of aspartic acid transfer RNA instead of being involved in DNA methylation (Goll et al. 2006). DNMT3C is a recently defined DNMT type, and protects the genome of male germ cells from potential transposon activity (Barau et al. 2016).

It has been reported that reproductive aging may lead to decreased global DNA methylation in the mouse MII oocytes as well as in the 2-cell, 4-cell, 8-cell, and morula-stage embryos (Yue et al. 2012). Also, Paczkowskiet al. (2015) revealed that there is an eight-fold increase in the expression level of the imprinted gene, *Kcnq1* (potassium voltage-gated channel subfamily q member 1), possibly due to methylation defect in the MII oocytes obtained from 15 mounts old aged mice when compared to 4- to 5-week old counterparts (Paczkowski et al. 2015). The loss of DNA methylation most likely results from changed expression of DNMTs during aging. Also, it has been revealed by many studies in this field that, the loss of DNMTs may lead to female infertility, imprinting disorders, epigenetic anomalies, or embryonic lethality [reviewed in Uysal et al. (2015)]. To elicit the potential relationship between DNA methylation levels and DNMT expression from early to aged terms, we have evaluated the spatial and temporal expression of the *Dnmt1*, *Dnmt3a*, *Dnmt3b*, and *Dnmt3l* genes at mRNA and protein levels as well as global DNA methylation profiles in the young (1- and 2-week old), prepubertal (3- and 4-week old), pubertal (5- and 6-week old), postpubertal (16- and 18-week old) and aged (52-, 60-, and 72-week old) mouse ovaries.

## Material and methods

### Collection of postnatal mouse ovaries

The postnatal ovaries have been obtained from Balb/C female mice at the ages of 1-, 2-, 3-, 4-, 5-, 6-, 16-, 18-, 52-, 60-, and 72-week. All mice in the current work were housed with free access to food and water, and kept under a 12 h light–dark cycle in the Akdeniz University Animals Laboratory Application and Research Centre. All experimental protocols were approved by the Akdeniz University Institutional Animal Care and Use Committee (Protocol no. 2017.05.007). The ovaries of three female mice ($n=3$) from each week were dissected under sterile conditions following cervical dislocation immediately after ether inhalation. It is noteworthy that we have used one ovary from each week for quantitative real time polymerase chain reaction (qRT-PCR) analysis, and the other one was taken for routine paraffin embedding for the purpose of immunohistochemical staining. As described in our previous study (Ozturk et al. 2015), the five groups were created dependent on the similarities related to the number and type of ovarian follicles and stroma composition of the postnatal ovaries. These groups were as following: young (1- and 2-week-old; $n=3$ from each week), prepubertal (3- and 4-week-old; $n=3$ from each week), pubertal (5- and 6-week-old; $n=3$ from each week), postpubertal (16- and 18-week-old; $n=3$ from each week), and aged (52-, 60- and 72-week-old; $n=3$ from each week).

### Gene expression analysis by qRT-PCR

Total RNA isolation from postnatal mouse ovaries was performed using Trizol reagent (Life Technologies, Darmstadt, Germany) according to the instructions of the manufacturer. The concentration of the extracted RNA was calculated by measuring the absorbance at 260 and 280 nm. The 10 µg of the total RNA was subjected to DNase I (Ambion, Austin, Texas, USA) to eliminate genomic DNA contamination. Then, we carried out reverse transcription reaction with the RETROscript kit (Ambion, Austin, TX, USA) in two steps based on the manufacturer's instructions: first, equal amounts of DNase-treated RNA (2 µg) and random decamers were incubated at 85 °C for 3 min to unwind any secondary structure therein. Second, the reverse transcription reaction components composed of 2 µl of 10X RT buffer, 4 µl of 1.25 mM dNTP mix, 1 µl of RNase inhibitor (10 units/µl), and 1 µl of MMLV-RT (100 units/µl) were added, and then incubated at 44 °C for 1 h. Finally, MMLV-RT enzyme

was inactivated at 92 °C for 10 min. The relative expression levels of the *Dnmt1*, *Dnmt3a*, *Dnmt3b*, and *Dnmt3l* mRNAs in the postnatal mouse ovaries at the ages of 1-, 2-, 3-, 4-, 5-, 6-, 16-, 18-, 52-, 60-, and 72-week were assessed in triplicate by qRT-PCR. The qRT-PCR reaction was set up in a reaction volume of 25 µl containing 12.5 µl of 2XSYBR green supermix (Qiagen, Valencia, CA, USA), 0.4 µM of each primer and 1 µl of cDNA template, and PCR cycling was performed on Rotor-Gene (Corbett Research, Sydney, Australia). The *β-Actin* expression was used as an internal control, against which *Dnmt1*, *Dnmt3a*, *Dnmt3b*, and *Dnmt3l* genes expression was normalized. After that the relative expression levels of the target genes were determined by $2^{-\Delta\Delta Ct}$ (cycle threshold) method and reported as fold changes in the postnatal ovaries. Note that the specificity of qRT-PCR products was confirmed by a melting curve analysis at the end of each reaction. It is important to note that the fold changes of the *Dnmt* genes for each week were subsequently classified into postnatal ovary groups (mentioned above) to further determine the differences from early to aged terms.

### Paraffin embedding

The postnatal mouse ovaries obtained from those ages were immersed in Bouin's solution (Sigma-Aldrich, St. Louis, MO, USA) at + 4 °C for 12 h, and then dehydrated through a graded ethanol series. Following that the ovaries were cleared in xylene and subsequently embedded in paraffin. Serial cross sections at 5 µm thickness were cut from the paraffin blocks using a rotary microtome (Leica, Nussloch, Germany), and mounted on the superfrost plus glass slides (Thermo Scientific, Rockford, IL, USA) which were used later for immunostaining.

### Immunohistochemistry

The immunoexpression of the DNMT1, DNMT3A, DNMT3B, and DNMT3L proteins as well as 5-methylcytosine (5mC) staining in the postnatal mouse ovaries were evaluated by immunohistochemistry. The paraffin sections at 5 µm thickness were deparaffinized in fresh xylene, and then rehydrated in a series of decreasing ethanol concentrations. After deparaffinization and rehydration processes, citrate buffer (0.01 M; pH 6.0) was treated for antigen retrieval using microwave (2 × 5 min at 750 W). The endogenous peroxidase activity in the sections was blocked by 3% hydrogen peroxide prepared in methanol for 30 min at room temperature (RT). Following several washes with 1X phosphate buffered saline (PBS), sections were blocked with blocking buffer (Thermo Scientific) at RT for 7 min to prevent non-specific binding. Then, sections were incubated with the following primary antibodies: DNMT1 (1:100; catalog no. ab87654, Abcam), DNMT3A (1:200; catalog no. ab23565, Abcam), DNMT3B (1:300; catalog no. ab2851, Abcam), DNMT3L (1:50; catalog no. 194094, Abcam) and 5mC (1:200; catalog no. MABE146, Millipore) at + 4 °C overnight. One of three sections of each slide was incubated with isotype IgG antibody (catalog no. ab172730, Abcam) for testing the specifity of primary and even secondary antibodies. After that, sections were washed three times in PBS for 15 min each, and subsequently incubated with anti-rabbit secondary antibody (1:500; catalog no. BA-1000, Vector Laboratories) for DNMT1, DNMT3A, DNMT3B, and DNMT3L expression, and incubated with anti-mouse secondary antibody (1:500; catalog no. BA-9200, Vector) for 5mC staining at RT for 1 h. Finally, sections were incubated with streptavidin–horseradish peroxidase (HRP) complex (catalog no. TS-125, Thermo Scientific) for 20 min at RT. We visualized the immune reactions using 3,3′-diaminobenzidine (DAB) chromogen (catalog no. D4168, Sigma-Aldrich) under a light microscope. Then, sections were washed in tap water and counterstained with Mayer's hematoxylin. The immunostaning of DNMT1, DNMT3A, DNMT3B, and DNMT3L proteins as well as 5mC staining in all groups were captured under Zeiss Scope.A1 Axio biology microscope attached with AxioCamMRc5 camera (Carl Zeiss, Oberkochen, Germany). The representative micrographs of young and prepubertal groups were captured at 400 ×, and of pubertal, postpubertal and aged groups at 200 × original magnifications. Their dimensions were at 1292 × 968 *(*width × height*)* pixels, and each of them has a depth of 24 bits. Also, all micrographs were evaluated for the related expression using the software, AxioVision (Axio Vs40 V.4.8.2.0), and presented a resolution of 300 dpi in the panels. The relative immunostaining intensity of the DNMT proteins and 5mC staining in all ages has been analyzed using ImageJ software (National Institutes of Health, Bethesda, Maryland, USA). It is worth noting that we have analyzed at least six images from each week.

### Statistical analysis

The data from all experiments were examined by one-way analysis of variance (one-way ANOVA) followed by Dunn's post hoc test. We conducted the statistical calculations using SigmaStat for Windows, version 3.5 (Jandel Scientific Corp). $P < 0.05$ was considered to be statistically significant.

## Results

In the current study, the spatial and temporal expression of the *Dnmt1*, *Dnmt3a*, *Dnmt3b*, and *Dnmt3l* genes at mRNA and protein levels were for the first time evaluated in the postnatal mouse ovaries from early to aged terms. In addition, the global DNA methylation levels were analyzed. We

found that most DNMT expression and global DNA methylation levels were significantly decreased in the postnatal ovaries from young to aged groups, suggesting that these changes may underlie female infertility emerging during ovarian aging.

### *Dnmt1*, *Dnmt3a*, *Dnmt3b*, and *Dnmt3l* genes expression in the postnatal mouse ovaries

The *Dnmt1* mRNA expression exhibited a decreasing trend from 1- to 72-week-old ovaries except for the expression in 5-week-old ovary that had the lowest expression (Fig. 1a; $P < 0.05$). It is noteworthy that there were no differences detected among certain ovaries at distinct ages. When we have analyzed the *Dnmt1* expression between postnatal ovary groups including young, prepubertal, pubertal, postpubertal and aged, it gradually decreased from young to pubertal groups having similar expression levels with the postpubertal and aged groups (Fig. 1b). Importantly, young group possessed significantly higher *Dnmt1* transcript level when compared with the remaining groups ($P < 0.05$). Also, the prepubertal group exhibited remarkably higher *Dnmt1* expression than those of in the postpubertal and aged groups (Fig. 1b; $P < 0.05$).

While the highest *Dnmt3a* expression was detected in 1-week ovary, the lowest expression was in 16-week old ovary (Fig. 2a; $P < 0.05$). And, expressional fluctuations exist among the remaining weeks ($P < 0.05$). In the postnatal ovary groups, the highest and lowest *Dnmt3a* expressions were detected in the young and postpubertal groups, respectively (Fig. 2b; $P < 0.05$). Also, the prepubertal, pubertal and aged groups showing similar expression levels possessed higher *Dnmt3a* expression when compared to the postpubertal group ($P < 0.05$), but lower than young group ($P < 0.05$).

We have also examined the *Dnmt3b* expression in all ages and postnatal ovary groups (Fig. 3). The *Dnmt3b* mRNA expression was in the highest level in 18-week, and lowest in 72-week ovaries (Fig. 3a). The 6- and 60-week ovaries had significantly higher *Dnmt3b* expression in comparison to the remaining ages ($P < 0.05$). Conversely, 2-, 4-, 16-, and 52-week ovaries possessed significantly lower *Dnmt3b* expression than in the remaining weeks except for 72-week-old ovary (Fig. 3a; $P < 0.05$). In the postnatal ovary groups, although the *Dnmt3a* gene was expressed in a gradually increasing manner from young to postpubertal groups, it slightly decreased in the aged group (Fig. 3b). It is worth noting that the young and prepubertal groups had significantly lower *Dnmt3b* expression when compared to the pubertal, postpubertal and aged groups ($P < 0.05$).

The *Dnmt3l* gene was expressed at the highest level in 2-week and lowest level in 72-week-old ovaries (Fig. 4a; $P < 0.05$). The low *Dnmt3l* expression in 1-week reached to the high level in 2-week and gradually decreased from 2- to 5-week-old ovaries ($P < 0.05$). The remarkably increased *Dnmt3l* expression in 6-week ovary dramatically declined to low levels in 16-, 18-, 52-week old ovaries ($P < 0.05$). Eventually, its expression gradually decreased from 52- to 72-week-old ovaries (Fig. 4a; $P < 0.05$). In the postnatal ovary groups, the *Dnmt3l* expression progressively and

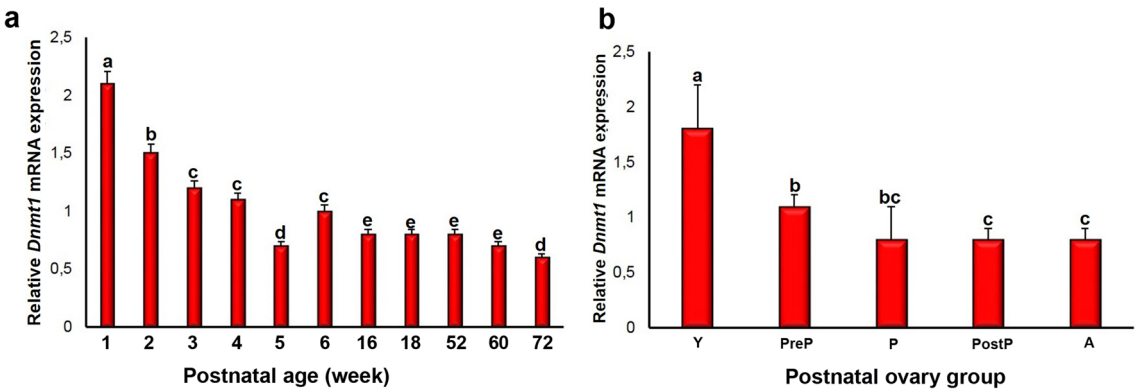


**Fig. 1** The *Dnmt1* mRNA expression levels in the postnatal mouse ovaries. The *Dnmt1* expression was normalized against to the *β-Actin* levels. **a** The *Dnmt1* expression in the postnatal ovaries at different ages ($n = 3$ from each week). It is noteworthy that the relative *Dnmt1* expression in the 6-week old ovary was set to 1. The relative *Dnmt1* levels exhibited decreasing trend from 1- to 72-week-old ovaries except for the predominant reduction in 5-week-old ovary ($P < 0.05$). **b** The *Dnmt1* expression in the postnatal ovary groups including young (1- and 2-week-old; $n = 6$), prepubertal (3- and 4-week-old; $n = 6$), pubertal (5- and 6-week-old; $n = 6$), postpubertal (16- and 18-week-old; $n = 6$), and aged (52-, 60-, and 72-week-old; $n = 9$). Herein, the relative *Dnmt1* expression in the prepubertal group was set to 1. In general, *Dnmt1* expression gradually reduced from young to aged groups ($P < 0.05$). The statistical significance was determined by one-way ANOVA followed by Dunn's post hoc test. The different letters of each column indicate statistical significance ($P < 0.05$). All data are presented as the mean ± standard deviation (SD). Y, young ovary group; PreP, prepubertal ovary group; P, pubertal ovary group; PostP: postpubertal ovary group; A, aged ovary group

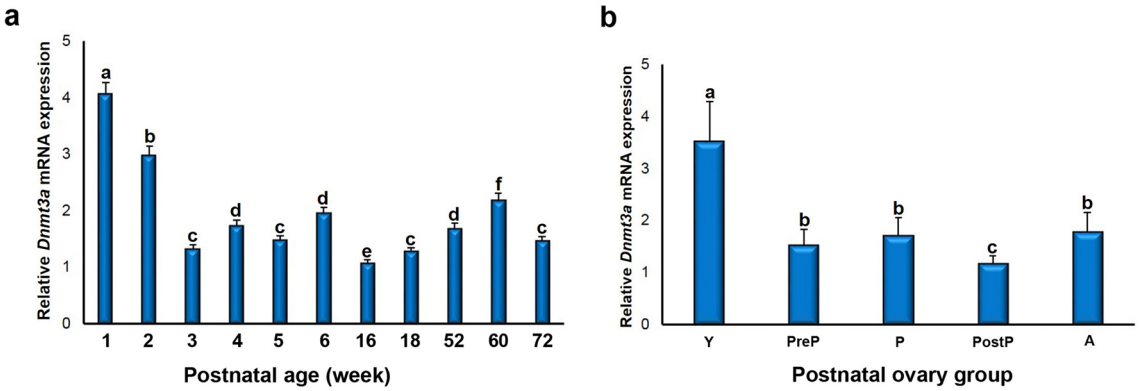


**Fig. 2** The *Dnmt3a* mRNA expression in the postnatal mouse ovaries. The *Dnmt3a* expression was normalized against to the *β-Actin* levels. **a** The *Dnmt3a* expression in the postnatal ovaries at different ages ($n=3$ from each week). It is noteworthy that the relative *Dnmt3a* expression in 16-week-old ovary was set to 1. Its relative levels exhibited fluctuations from 1- to 72-week-old ovaries ($P<0.05$). **b** The *Dnmt3a* expression in the postnatal ovary groups involving young (1- and 2-week-old; $n=6$), prepubertal (3- and 4-week-old; $n=6$), pubertal (5- and 6-week-old; $n=6$), postpubertal (16- and 18-week-old; $n=6$), and aged (52-, 60-, and 72-week-old; $n=9$). Herein, the relative *Dnmt3a* expression in the postpubertal group was set to 1. The highest and lowest *Dnmt3a* expressions were detected in the young and postpubertal groups, respectively ($P<0.05$). The statistical significance was determined by one-way ANOVA followed by Dunn's post hoc test. The different letters of each column indicate statistical significance ($P<0.05$). All data are presented as the mean ± standard deviation (SD). Y, young ovary group; PreP, prepubertal ovary group; P, pubertal ovary group; PostP, postpubertal ovary group; A, aged ovary group

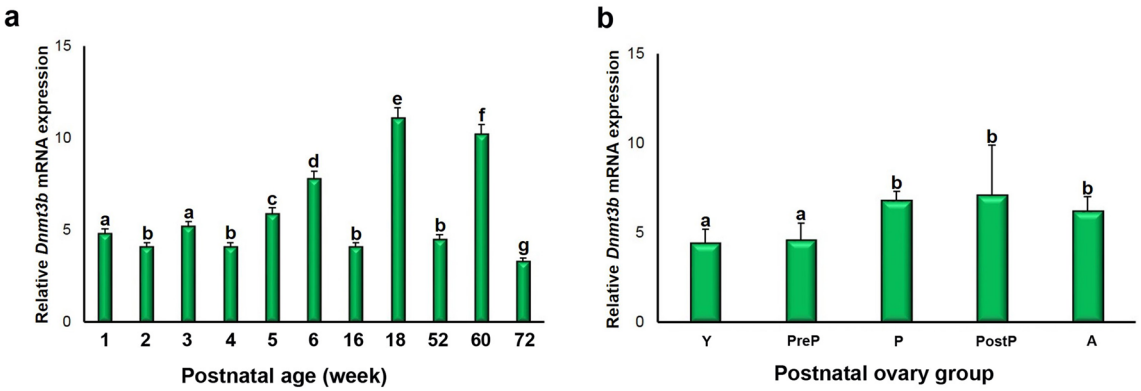


**Fig. 3** The *Dnmt3b* mRNA expression in the postnatal mouse ovaries. The *Dnmt3b* gene expression was normalized against to the *β-Actin* levels. **a** The *Dnmt3b* expression in the postnatal ovaries at different ages ($n=3$ from each week). It is noteworthy that the relative *Dnmt3b* expression in 72-week-old ovary was set to 1. We found the highest *Dnmt3b* expression in 18-week-old and lowest expression in 72-week-old ovaries. **b** The *Dnmt3b* expression in the postnatal ovary groups including young (1- and 2-week-old; $n=6$), prepubertal (3- and 4-week-old; $n=6$), pubertal (5- and 6-week-old; $n=6$), postpubertal (16- and 18-week-old; $n=6$), and aged (52-, 60- and 72-week-old; $n=9$). The relative *Dnmt3b* expression in the young group was set to 1. The *Dnmt3b* gene was expressed in an increasing trend from young to aged groups ($P<0.05$). The statistical significance was determined by one-way ANOVA followed by Dunn's post hoc test. The different letters of each column indicate statistical significance ($P<0.05$). All data are presented as the mean ± standard deviation (SD). Y, young ovary group; PreP, prepubertal ovary group; P, pubertal ovary group; PostP, postpubertal ovary group; A, aged ovary group

significantly decreased from young to aged groups (Fig. 4b; $P<0.05$).

## DNMT1, DNMT3A, DNMT3B, and DNMT3L expression in the postnatal ovaries

The DNMT1 expression was detected in the follicles at different developmental stages, stromal and luteal cells in the postnatal ovaries (Fig. 5a). In the follicles from primordial to antral stages, both oocytes and granulosa cells intensively expressed DNMT1 in their cytoplasmic and nuclear regions. Similarly, it was localized either in cytoplasm or nucleus of the theca, luteal and stromal cells (Fig. 5a). When we have further evaluated the relative DNMT1 expression levels in the postnatal ovaries at different ages, it exhibited gradually decreasing trend from 1- to 72-week-old ovaries except

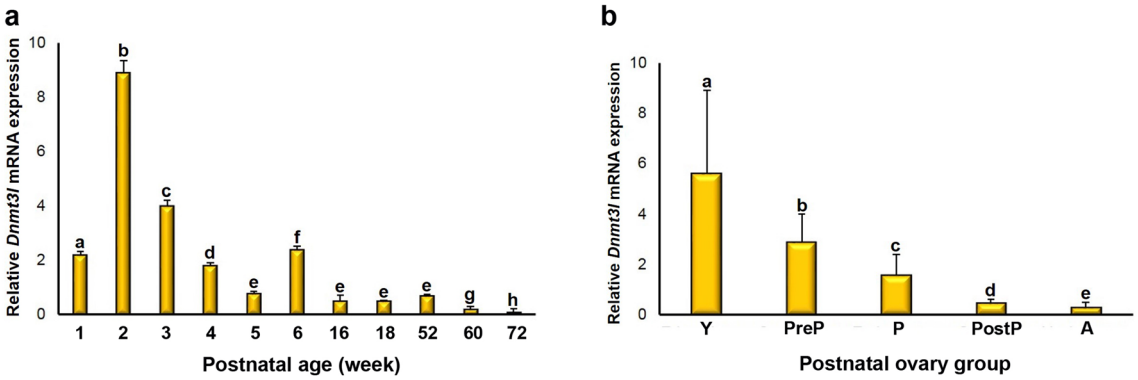


**Fig. 4** The *Dnmt3l* mRNA expression in the postnatal mouse ovaries. The *Dnmt3l* expression was normalized against to the *β-Actin* levels. **a** The *Dnmt3l* expression in the postnatal ovaries at different ages ($n=3$ from each week). It is noteworthy that the relative *Dnmt3l* expression in 5-week-old ovary was set to 1. The relative *Dnmt3l* expression was at the low levels from 18- to 72-week-old ovaries as well as in 5-week-old ovary ($P<0.05$). **b** The *Dnmt3l* expression in the postnatal ovary groups involving young (1- and 2-week-old; $n=6$), prepubertal (3- and 4-week-old; n=6), pubertal (5- and 6-week-old; $n=6$), postpubertal (16- and 18-week-old; $n=6$), and aged (52-, 60-, and 72-week-old; $n=9$). The relative *Dnmt3l* expression in the postpubertal group was set to 1. The *Dnmt3l* mRNA expression gradually decreased from young to aged ovaries ($P<0.05$). The statistical significance was determined by one-way ANOVA followed by Dunn's post hoc test. The different letters of each column indicate statistical significance ($P<0.05$). All data are presented as the mean ± standard deviation (SD). Y, young ovary group; PreP, prepubertal ovary group; P, pubertal ovary group; PostP, postpubertal ovary group; A, aged ovary group

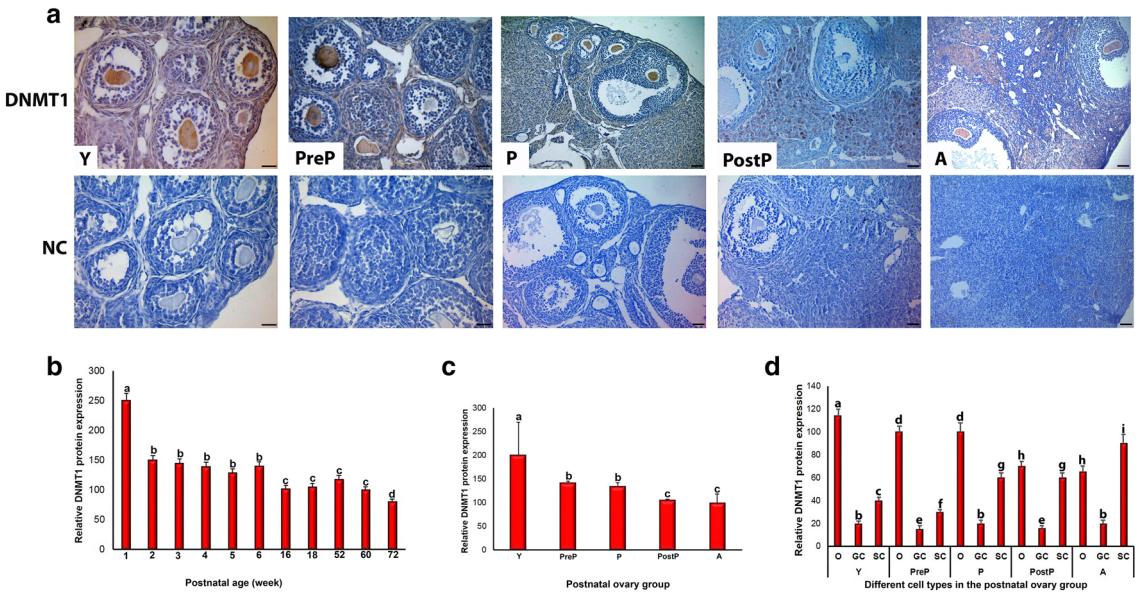


**Fig. 5** The immunoexpression of DNMT1 in the postnatal mouse ovaries. **a** DNMT1 was localized in the cytoplasmic and nuclear regions of the oocytes and granulosa cells of the follicles at different developmental stages and stromal cells as well in the young, prepubertal, pubertal, postpubertal and aged ovary groups. It is important to note that the negative control (NC) section involving the isotype immunoglobulin G antibody at the same concentration with the DNMT1 primary antibody were used instead of the primary antibody in each immunohistochemistry application to detect presence of any non-specific staining of primary or secondary antibody. Furthermore, the relative DNMT1 expression levels were measured using ImageJ software in the postnatal ovaries. **b** The relative DNMT1 expression in the postnatal ovaries exhibited decreasing tendency from 1- to 72-week-old ($P<0.05$). **c** In the postnatal ovary groups, DNMT1 expression gradually declined from young to aged groups ($P<0.05$). **d** The relative DNMT1 expression in the postnatal ovary groups progressively decreased in the oocytes from young to aged groups ($P<0.05$), but exhibited fluctuations in the granulosa and stromal cells ($P<0.05$). The statistical significance was determined by one-way ANOVA followed by Dunn's post hoc test. The $P<0.05$ was considered statistically significant, shown by different letters on the columns. The representative micrographs of young and prepubertal groups were captured at × 400, and of pubertal, postpubertal and aged groups at × 200 original magnifications. All data are presented as the mean ± standard deviation (SD). Scale bar = 50 µm. Y, young ovary group; PreP, prepubertal ovary group; P, pubertal ovary group; PostP, postpubertal ovary group; A, aged ovary group; O, oocytes; GC, granulosa cells; SC, stromal cells

for small fluctuations in certain weeks (Fig. 5b). While the highest DNMT1 expression was present in 1-week ovary, the lowest expression was detected in 72-week-old ovaries ($P<0.05$). The ovaries from 2- to 6-week-old had significantly higher DNMT1 expression than those of in 16-, 18-, 52-, 60- and 72-week-old ovaries ($P<0.05$). Also, the 72-week ovary possessed lower DNMT1 expression in comparison to 16-, 18-, 52-, and 60-week-old ovaries (Fig. 5b; $P<0.05$). When DNMT1 expression was compared among the postnatal ovary groups, we observed the highest expression in the young group and lowest expression in the aged group (Fig. 5c; $P<0.05$). Importantly, the DNMT1 expression gradually declined from young to aged groups. It has been documented that the prepubertal and pubertal groups exhibited significantly higher DNMT1 levels than those of in the postpubertal and aged groups (Fig. 5c; $P<0.05$). In the DNMT1 expression analysis of each cell type including oocytes and granulosa cells of the follicles at different stages as well as in the stromal cells of the postnatal ovary groups, we observed the highest expression level in the oocytes of young group and lowest expressions in the granulosa cells of prepubertal and postpubertal groups (Fig. 5d; $P<0.05$). The DNMT1 expression showed gradual decrease from young to aged groups in the oocytes, ($P<0.05$). For the granulosa cells, the young, pubertal and aged groups possessed significantly higher DNMT1 levels when compared to the prepubertal and postpubertal ones ($P<0.05$). The DNMT1 expression in the stromal cells remarkably declined from young to prepubertal groups, increased in the pubertal and postpubertal groups, and further enhanced in the aged group ($P<0.05$).

Similar to the DNMT1 expression, DNMT3A was expressed in the follicles at different stages, stromal and luteal cells in the postnatal ovaries (Fig. 6a). In the follicles, it was resided in the cytoplasmic and nuclear regions of the oocytes and granulosa cells as was in the theca, luteal and stromal cells. When we have evaluated the relative DNMT3A expression levels in the postnatal ovaries, the highest DNMT3A expression was present in 1-week ovary and lowest expressions in 3-, 4-, 16-, 18-, 60-, 72-week-old

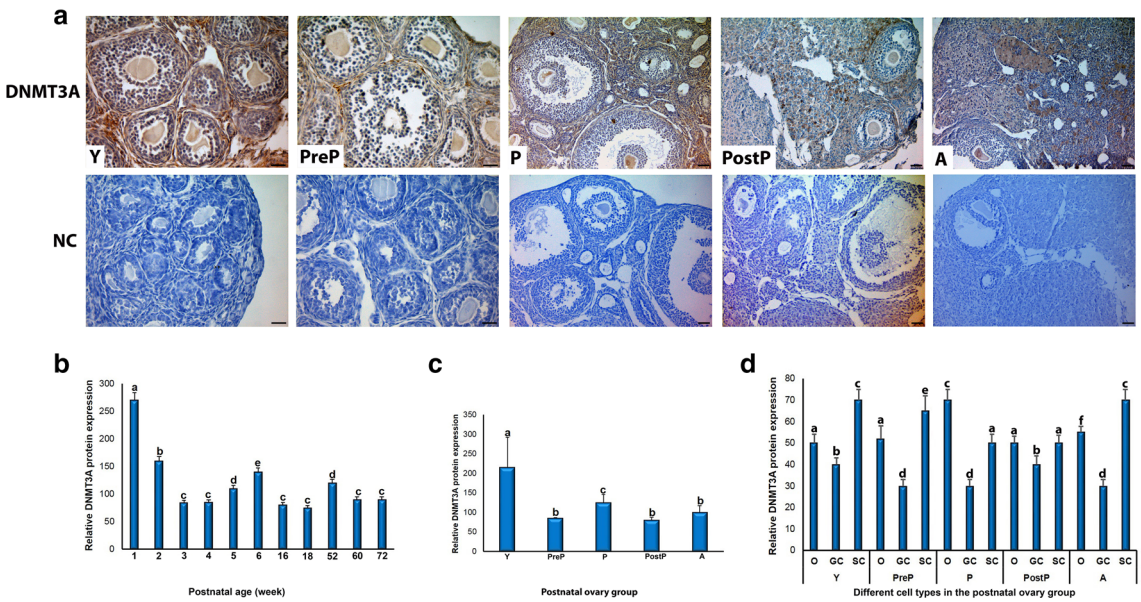


**Fig. 6** The immunoexpression of DNMT3A in the postnatal mouse ovaries. **a** The DNMT3A expression in the young, prepubertal, pubertal, postpubertal and aged ovary groups. We observed DNMT3A expression in the nuclear and cytoplasmic regions of the oocytes and granulosa cells at different follicular stages as well as in the stromal cells. It is important to note that the negative control (NC) section involving the isotype immunoglobulin G antibody at the same concentration with the DNMT3A primary antibody were used instead of the primary antibody in each immunohistochemistry application to detect presence of any non-specific staining of primary or secondary antibody. Furthermore, the relative DNMT3A expression levels were measured using ImageJ software in the postnatal ovaries. **b** The relative DNMT3A expression in the postnatal ovaries. We have detected expressional fluctuations from 1-week to 72-week-old ovaries ($P<0.05$). **c** In the postnatal ovary groups, the young and pubertal groups had significantly higher DNMT3A expression when compared to the remaining ones ($P<0.05$). **d** The relative DNMT3A expression in oocytes and granulosa cells of the follicles at different stages as well as in the stromal cells of postnatal ovary groups. We have observed significant differences in these cell types among groups ($P<0.05$). The statistical significance was determined by one-way ANOVA followed by Dunn's post hoc test. The $P<0.05$ was considered statistically significant, shown by different letters on the columns. The representative micrographs of young and prepubertal groups were captured at × 400, and of pubertal, postpubertal and aged groups at × 200 original magnifications. All data are presented as the mean ± standard deviation (SD). Scale bar = 50 µm. Y, young ovary group; PreP, prepubertal ovary group; P, pubertal ovary group; PostP, postpubertal ovary group; A, aged ovary group; O, oocytes; GC, granulosa cells; SC, stromal cells

ovaries (Fig. 6b; $P<0.05$). Also, the 5-, 6-, 52-week ovaries possessed remarkably higher DNMT3A expression in comparison to 72-week-old ovaries, but lower than 2-week ovary ($P<0.05$). In the postnatal ovary groups, the highest DNMT3A expression was in the young group and lowest expressions in the prepubertal, postpubertal and aged groups (Fig. 6c; $P<0.05$). It is noteworthy that the pubertal group exhibited significantly higher DNMT3A expression than those of in the prepubertal, postpubertal and aged groups ($P<0.05$). The DNMT3A expression was also analyzed in each cell type including oocytes and granulosa cells of the follicles at different stages as well as in the stromal cells of the postnatal ovary groups (Fig. 6d). Its expression in the oocytes of the young and pubertal groups significantly increased in the pubertal group, remarkably declined in the postpubertal group and increased again in the aged group ($P<0.05$). In the granulosa cells, while the highest DNMT3A expression was found in the young and postpubertal groups, lowest expressions were determined in the prepubertal, pubertal and aged groups ($P<0.05$). For the stromal cells, we have detected DNMT3A expression at the high level in the young group, which showed gradual decreasing trend toward postpubertal group, and increased in the aged group to the level in the young group ($P<0.05$).

DNMT3B was localized in the cytoplasm and nucleus of oocytes and granulosa cells of the follicles at different developmental stages as well as in the stromal and luteal cells in the postnatal ovaries (Fig. 7a). When we have further evaluated the relative DNMT3B expression levels in the postnatal ovaries, highest DNMT3B expressions were detected in 6-, 18- and 60-week-old ovaries, but there were lowest expressions in 4- and 72-week-old ovaries (Fig. 7b; $P<0.05$). It is worth noting that the DNMT3B expression exhibited significant fluctuations from 1- to 72-week-old ovaries ($P<0.05$). In the postnatal ovary groups, we observed an increasing trend of DNMT3B expression towards later terms of postnatal ovaries (Fig. 7c; $P<0.05$). The young group had significantly higher DNMT3B expression level than in

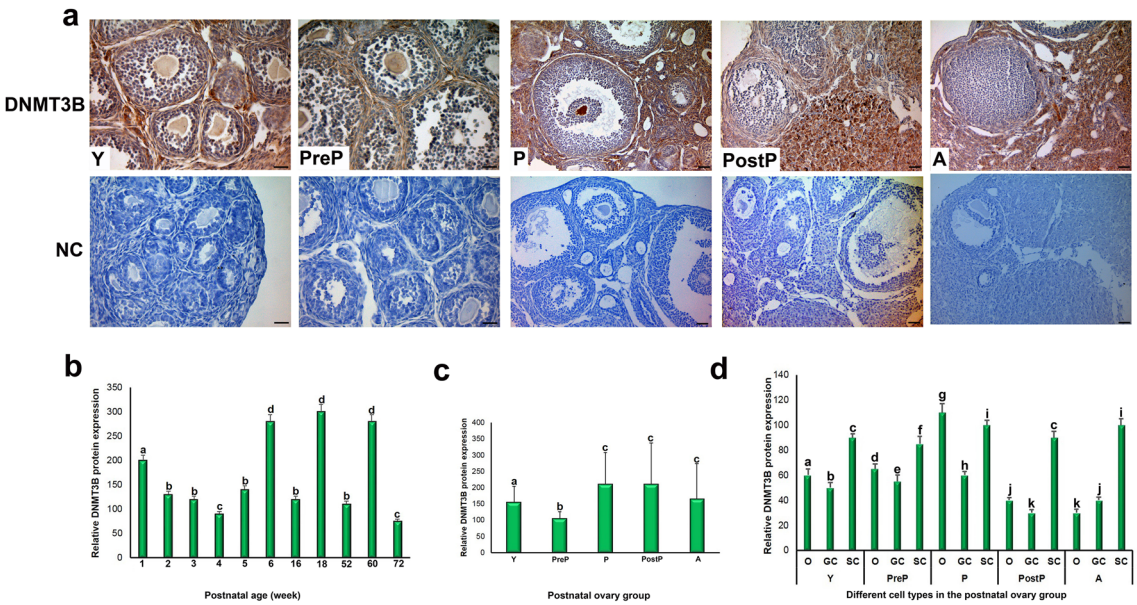


**Fig. 7** The immunoexpression of DNMT3B in the postnatal mouse ovaries. **a** The cellular localization of DNMT3B in the young, prepubertal, pubertal, postpubertal and aged ovary groups. The oocytes and granulosa cells of the follicles at the different stages localized DNMT3B in their cytoplasmic and nuclear regions as was in the stromal cells. It is important to note that the negative control (NC) section involving the isotype immunoglobulin G antibody at the same concentration with the DNMT3B primary antibody were used instead of the primary antibody in each immunohistochemistry application to detect presence of any non-specific staining of primary or secondary antibody. Furthermore, the relative expression levels of DNMT3B were measured using ImageJ software in the postnatal ovaries. **b** The relative DNMT3B expression in the postnatal ovaries from 1-week to 72-week-old. It reached the high levels in 6-, 18- and 60-week-old ovaries when compared with the remaining ones ($P<0.05$). **c** In the postnatal ovary groups, the pubertal, postpubertal and aged groups showed remarkably higher DNMT3B expression than in the young and prepubertal groups ($P<0.05$). **d** The relative DNMT3B expression in the oocytes and granulosa cells of the follicles at different stages as well as in the stromal cells of postnatal ovary groups. There were significant fluctuations of the DNMT3B expression in these cells from young to aged groups ($P<0.05$). The statistical significance was determined by one-way ANOVA followed by Dunn's post hoc test. The $P<0.05$ was considered statistically significant, shown by different letters on the columns. The representative micrographs of young and prepubertal groups were captured at × 400, and of pubertal, postpubertal and aged groups at × 200 original magnifications. All data are presented as the mean ± standard deviation (SD). Scale bar = 50 µm. Y, young ovary group; PreP, prepubertal ovary group; P, pubertal ovary group; PostP, postpubertal ovary group; A, aged ovary group; O, oocytes; GC, granulosa cells; SC, stromal cells

the prepubertal group ($P < 0.05$), and the pubertal, postpubertal and aged groups possessed higher expression when compared to early groups (Fig. 7c; $P < 0.05$). The DNMT3B expression was further evaluated in each cell type including oocytes and granulosa cells of follicles at different stages as well as in the stromal cells of postnatal ovary groups (Fig. 7d). The DNMT3B expression in the oocytes progressively increased from young to pubertal groups, and then decreased towards postpubertal and aged groups ($P < 0.05$). In the granulosa cells, the progressively increasing trend of DNMT3B expression from young to pubertal groups occurred, and it was at the low levels in the postpubertal and aged groups ($P < 0.05$). On the other hand, there were fluctuations of DNMT3B expression in the stromal cells from young to aged groups (Fig. 7d; $P < 0.05$).

We also analyzed the DNMT3L expression in the postnatal ovaries, and it was expressed in the follicles at different stages, stroma and corpus luteum (Fig. 8a). In the follicles, both oocytes and granulosa cells localized DNMT3L either in their cytoplasmic or nuclear regions as was in the theca, luteal and stromal cells. When we have further evaluated relative DNMT3L expression levels in the postnatal ovaries, the highest DNMT3L expression was present in 2-week ovary, but the lowest expression was in 72-week-old ovary (Fig. 8b; $P < 0.05$). The DNMT3L expression gradually decreased from 1- to 5-week ovaries except for 2-week ovary, and also it was gradually reduced from 6-week to 72-week ovaries ($P < 0.05$). When DNMT3L expression was compared among the postnatal ovary groups, it was gradually and significantly decreased from young to aged groups (Fig. 8c; $P < 0.05$). The DNMT3L expression in the oocytes of follicles at different stages progressively decreased from young to aged groups (Fig. 8d; $P < 0.05$). In the granulosa cells, its expression in the young group remarkably enhanced in the pubertal group, and then gradually decreased toward aged group ($P < 0.05$). Although DNMT3L expression levels exhibited gradually decreasing trend from young to postpubertal groups, it significantly increased in the aged group ($P < 0.05$).

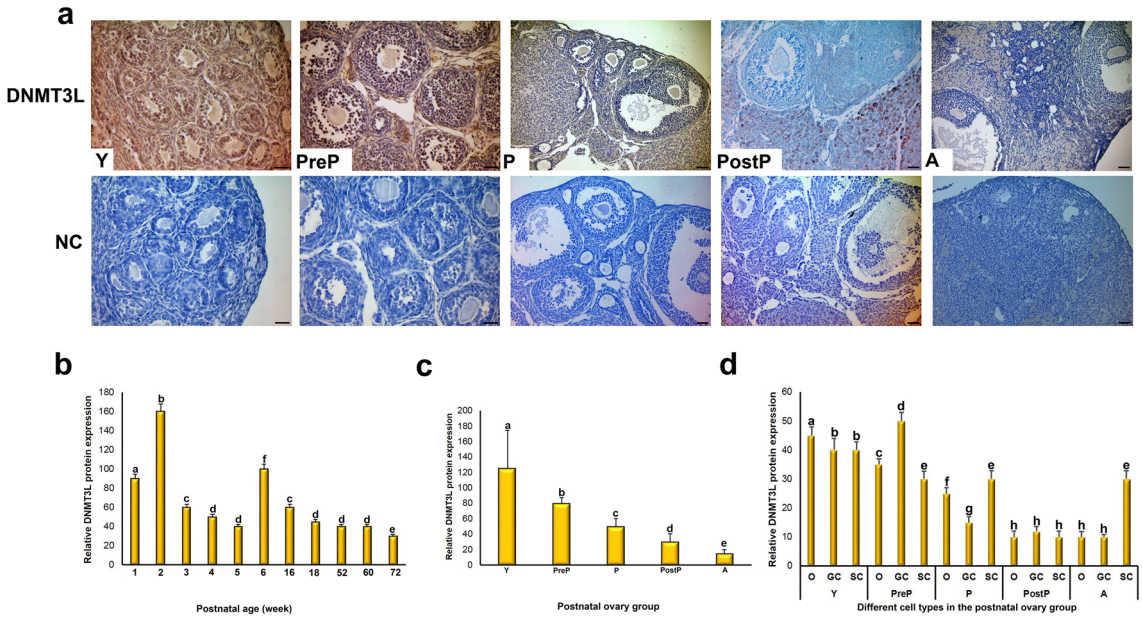


**Fig. 8** The immunoexpression of DNMT3L in the postnatal mouse ovaries. **a** The cellular localization of DNMT3L in the young, prepubertal, pubertal, postpubertal and aged ovary groups. We detected DNMT3L expression in the cytoplasm and nuclear regions of the oocytes and granulosa cells of the follicles at different stages as was in the stromal cells. It is important to note that the negative control (NC) section involving the isotype immunoglobulin G antibody at the same concentration with the DNMT3L primary antibody were used instead of the primary antibody in each immunohistochemistry application to detect presence of any non-specific staining of primary or secondary antibody. Furthermore, the relative expression levels of DNMT3L were measured using ImageJ software in the postnatal ovaries. **b** The relative DNMT3L expression in the postnatal ovaries from 1-week to 72-week-old. It exhibited gradual decreases either from 2- to 5-week or 6- to 72-week-old ovaries ($P < 0.05$). **c** The relative DNMT3L expression in the postnatal ovaries. There was a decreasing tendency of the DNMT3L expression from young to aged groups ($P < 0.05$). **d** The relative DNMT3L expression exhibited decreasing trend toward aged group either in the oocytes or granulosa cells of the follicles at different stages or stromal cells ($P < 0.05$). The statistical significance was determined by one-way ANOVA followed by Dunn's post hoc test. The $P < 0.05$ was considered statistically significant, shown by different letters on the columns. The representative micrographs of young and prepubertal groups were captured at × 400, and of pubertal, postpubertal and aged groups at × 200 original magnifications. All data are presented as the mean ± standard deviation (SD). Scale bar = 50 µm. Y, young ovary group; PreP, prepubertal ovary group; P, pubertal ovary group; PostP, postpubertal ovary group; A, aged ovary group; O, oocytes; GC, granulosa cells; SC, stromal cells

### 5-Methylcytosine (5mC) staining in the postnatal ovaries

The global DNA methylation measured by 5mC staining was evaluated in the follicles at different development stages, stromal and luteal cells within corpus luteum in the postnatal ovaries (Fig. 9a). As expected, both oocytes and granulosa cells showed 5mC staining in the nucleus as was in the theca, luteal and stromal cells.

The relative levels of 5mC staining in the postnatal ovaries was highest in 1-week and lowest in 72-week-old ovaries (Fig. 9b; $P < 0.05$). The intensity of 5mC staining gradually decreased from 1- to 3-week ovaries, and remained at the same levels from 3- to 5-week ovaries ($P < 0.05$). The increased 5mC level in 6-week ovary significantly decreased to low levels in the ovaries from 16- to 60-week-old, and further reduced in 72-week-old ovary ($P < 0.05$). When the intensity of 5mC staining was compared among the postnatal ovary groups, we observed the highest and lowest levels in the young and aged groups, respectively (Fig. 9c; $P < 0.05$). Also, the pubertal and postpubertal groups exhibited significantly higher 5mC levels than those of in the prepubertal and aged groups ($P < 0.05$). Moreover, the relative intensity of 5mC staining was evaluated in each cell type including oocytes and granulosa cells of the follicles at different stages as well as in the stromal cells of postnatal ovary groups (Fig. 9d). The relative 5mC staining in the oocytes decreased from young to prepubertal group, increased in the pubertal group and gradually declined toward aged group ($P < 0.05$). In the granulosa cells, the gradual decrease of 5mC staining intensity from young to pubertal group increased in the postpubertal group and decreased again in the aged group ($P < 0.05$). Although the relative intensity of 5mC staining in the stromal cells gradually decreased from young to postpubertal groups, it significantly enhanced in the aged group ($P < 0.05$).

## Discussion

In the present study, we have revealed for the first time that *Dnmt1*, *Dnmt3a*, *Dnmt3b*, and *Dnmt3l* genes expression at mRNA and protein levels as well as global DNA methylation profiles exhibited significant changes in the postnatal mouse ovaries from early to aged terms. Consistent with the decreasing global DNA methylation levels, DNMT1, DNMT3A, and DNMT3L expression remarkably reduced with ovary aging compared to the early terms. On the other hand, DNMT3B was at the high levels in the pubertal, postpubertal and aged groups when compared to the early groups. Based on the data documented in our previous study (Ozturk et al. 2015), the gradual decrease of number and type of follicles and oocytes reserve from early to aged terms may be one of the major reasons for the altered DNMT expression and global DNA methylation as well.

It has been reported that the *Dnmt* genes including *Dnmt1* isoforms (*Dnmt1o* and *Dnmt1s*), Dnmt-associated protein-1 (*Dmap1*), *Dnmt3a* and *Dnmt3l* are significantly down-regulated in the aged MII oocytes collected from 42–45 weeks old mice, whereas *Dnmt3b* is up-regulated compared to the MII oocytes from young mice at the age of 5–6 weeks old using q-PCR (Hamatani et al. 2004). In parallel with this study, we found that the *Dnmt1, Dnmt3a,* and *Dnmt3l* genes expression gradually decreased in the aged ovaries when compared to the early periods although there were slight fluctuations in the *Dnmt3a* expression. Also, *Dnmt3b* gene was expressed at the high levels from pubertal to aged terms as is reported in the former study. Another study by Yue et al. (2012) found that DNMT1, DNMT3A, DNMT3B, and DNMT3L expression levels significantly decrease in the aged MII oocytes obtained from 35–40 weeks old mice compared with MII oocytes collected from young mice at the ages of 6–8 weeks old by Western blotting (Yue et al. 2012). The researchers also showed that there is a reduced global DNA methylation in the aged MII oocytes compared to counterparts using 5mC staining. Analogously, DNMT1, DNMT3A, DNMT3L and global DNA methylation levels significantly declined in accompanying with ovarian aging; however, DNMT3B expression in the aged ovaries was compatible with the pubertal and postpubertal groups in the current study. The difference for the DNMT3B expression in both studies may result from analyzing distinct samples and using different protein analysis techniques. Furthermore, the high expression of DNMT3B in the aged group suggests that it may have different roles in the follicles and somatic cells out of its primary role in maintaining DNA methylation. The finding from human embryonic stem cells lacking *Dnmt3b* gene (Liao et al. 2015) partially support our suggestion because several different phenotypes such ICF (immunodeficiency, centromeric instability, and facial dysmorphism) syndrome, inherited diseases and cancer could emerge in the loss of DNMT3B (Gagliardi et al. 2018). It is also documented that DNMT3B is involved in hematopoietic stem cells self-renewal and differentiation (Challen et al. 2014), and osteoarthritis suppression (Zhou et al. 2019). The additional role(s) of DNMT3B in oocytes and granulosa cells seem to be the subject of future studies.

As is known, DNA methylation is one of the epigenetic mechanisms and plays important roles in the expressional regulation of the genes in the oocytes and granulosa cells during folliculogenesis as well as in other ovarian cells. Consistently, global DNA methylation gradually increases during oocyte growth, and reaches the highest level in MII oocytes (Smallwood and Kelsey 2012). During oocyte maturation from GV to MII stages, genomic imprints are also established (Yang et al. 2007). It is noteworthy that

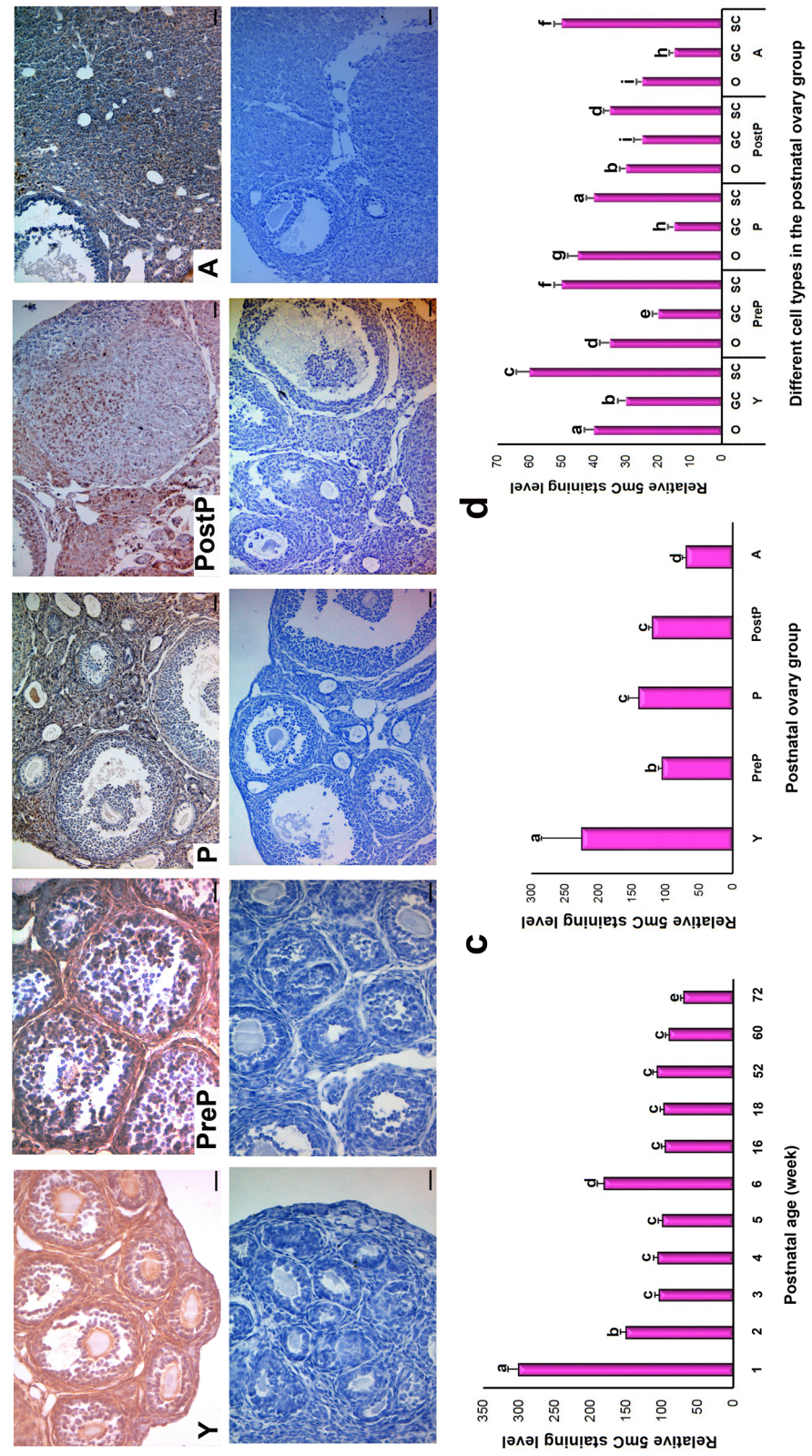


**Fig. 9** The 5-methylcytosine (5mC) staining in the postnatal mouse ovaries. **a** The immunostaining of 5mC in the young, prepubertal, pubertal, postpubertal and aged ovary groups. As expected, we have observed the 5mC staining in the nucleus of oocytes, granulosa cells and stromal cells. It is noteworthy that there was a weak background staining in the 5mC micrographs possibly due to non-specific binding of primary antibody to certain regions. It is important to note that the negative control (NC) section involving the isotype immunoglobulin G antibody at the same concentration with the 5mC primary antibody were used instead of the primary antibody in each immunohistochemistry application to detect presence of any non-specific staining of primary or secondary antibody. Furthermore, the relative levels of the 5mC staining were measured using ImageJ software in the postnatal ovaries. **b** The relative 5mC staining in the postnatal ovaries from 1- to 72-week old. We detected the highest and lowest intensity of 5mC staining in 1- and 72-week old ovaries ($P < 0.05$). **c** In the postnatal ovary groups, the relative intensity of 5mC staining gradually decreased from young to aged groups except for the prepubertal group ($P < 0.05$). **d** The relative intensity of 5mC staining in the oocytes and granulosa cells of the follicles at different stages as well as in the stromal cells of the postnatal ovary groups. In these cell types, there were significant fluctuations from young to aged groups ($P < 0.05$). The statistical significance was determined by one-way ANOVA followed by Dunn's post hoc test. The $P < 0.05$ was considered statistically significant, shown by different letters on the columns. The representative micrographs of young and prepubertal groups were captured at × 400, and of pubertal, postpubertal and aged groups at × 200 original magnifications. All data are presented as the mean ± standard deviation (SD). Scale bar = 50 µm. Y, young ovary group; PreP, prepubertal ovary group; P, pubertal ovary group; PostP, postpubertal ovary group; A, aged ovary group; O, oocytes; GC, granulosa cells; SC, stromal cells

granulosa cells undergo dynamic DNA methylation changes to regulate repression or activation of the genes required for their proliferation and differentiation during follicular development (Huntriss et al. 2004). The DNMTs including DNMT1, DNMT3A, DNMT3B, and DNMT3L contribute to maintenance and de novo methylation processes of these genes regulation during oogenesis and establishing genomic imprints as well. Therefore, the lack of DNMT results in abnormal global methylation or defects in the imprinting genes, which may lead to female infertility [reviewed in Uysal and Ozturk (2017)]. The significant changes in the expression of DNMT1, DNMT3A, and DNMT3L from early to aged terms in the present study most likely result in progressively decreased global DNA methylation in the later terms of mouse ovaries. The gradual decrease of the DNMT expression in accompanying with ovarian aging may originate from altered intraovarian factors and/or reproductive hormones such as estrogen, decreasing at the later lifespan. Also, decreasing mean numbers of follicles from primordial to antral stages as well as oocyte quality with ovarian aging (Ozturk et al. 2015; Ben-Or 1963) may be another reasons of the reduced DNMT1, DNMT3A, and DNMT3L expression since the oocytes and granulosa cells intensively expressed these proteins. Thus, the transcriptional and translational control of *Dnmt1, Dnmt3a, and Dnmt3l* genes by various types of ovarian factors should be analyzed in the oocytes and granulosa cells obtained from adult and aged mice.

As is known, female fertility decreases during biological aging due to depleted oocyte and follicle reserve, reduced oocyte quality, chromosomal anomalies, and mitochondria dysfunction [reviewed in May-Panloup et al. (2016)]. In humans, it has been reported that reproductive capacity dramatically declines after 35 years of age (Navot et al. 1994; Sauer 1998) that may result from increased embryo resorption (Holinka et al. 1979), fetal death (Cnattingius et al. 1992), and low birth weight (Roberts et al. 1994) emerged during aging. These adverse effects could originate from impaired regulation of the genes playing roles in oogenesis and folliculogenesis during ovarian aging.

To the best of our knowledge, there are a limited number of studies aimed to illuminate the molecular background of ovarian aging. In a recently published study on the ovaries of rats at the ages of 6-, 12- and 24-month-old, although *Dnmt1* and *Dnmt2* genes expression at mRNA and protein levels significantly decrease with aging, the expression of *Dnmt3a* and *Dnmt3b* genes gradually increase with aging from 6- to 24-month old (Li et al. 2020). These findings seem to be a large extent compatible with our results despite of being used two different rodent species. In line with these findings, Paczkowski et al. (2015) found that maternally (*Cdkn1c* and *Gnas*) and paternally expressed genes (*Igf2, Mest,* and *Peg3*) are highly methylated in the aged mouse ovary (15-month old) when compared to young one (4–5 weeks old) (Paczkowski et al. 2015). As DNMT3A and DNMT3B implicate in establishing paternal and maternal genomic imprints (Kaneda et al. 2004; Yamaguchi et al. 2013), increase of their expressions along with ovarian aging may lead to enhanced methylation in the target genes. On the other hand, the *Kcnq1* expression, a maternally imprinted gene, is up-regulated in the MII oocytes from aged group compared to young ones (Paczkowski et al. 2015) most likely due to dysregulation of DNA methylation mechanism. In more detailed study by Xi et al. (2019) examined the genome-wide DNA methylation and transcriptome-wide RNA expression in the pig ovaries obtained from young (180 days old, puberty stage of first ovulation) and aged ones (eight years old, reproductive exhaustion stage) (Xi et al. 2019). There are many differences between young and old pigs related to methylation regions, expression of mRNAs, microRNAs, long noncoding RNAs and circular RNAs, implicating in apoptosis, embryonic development, fertilization, ovarian cumulus expansion, and the ovulation cycle.

In the light of these studies, the altered DNMT expression even their intracellular localization during ovarian aging may underlie the expressional changes of the development-related and imprinting genes in the oocytes and granulosa cells as well as in other ovarian cells. Also, it is important to mention that researchers should look at ovarian aging in a wide perspective because many intracellular mechanisms directly or indirectly related to DNA methylation and expressional regulation of DNMTs can be influenced in the aged ovaries.

## Conclusion

In the present study, we have for the first time characterized the relative expressions and subcellular localizations of the *Dnmt1*, *Dnmt3a*, *Dnmt3b*, and *Dnmt3l* genes as wells as global DNA methylation levels in the postnatal mouse ovaries from early to aged terms. Our results indicated that the altered DNMT expression may underlie the changed global DNA methylation levels at the later term of lifespan. These changes may be one of the causes of ovarian aging leading to female infertility. The potential relationship between altered DNMT expression/global methylation and female infertility emerging with ovarian aging should be analyzed in the mouse and human samples to illuminate the related molecular biological mechanisms.

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**Author contributions** SO and FU have designed the study. FU created the data and wrote the article. SO evaluated the findings and critically read the article.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that there is no conflict of interest.

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