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Research Article

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Overaccumulation of Fat Caused Rapid Reproductive Senescence but not Loss of Ovarian Reserve in *ob/ob* Mice

Mohammad Lalmoddin Mollah,[1] Hee-Seon Yang,[1] SoRa Jeon,[1] KilSoo Kim,[2] and Yong-Pil Cheon[1]

[1]Division of Development and Physiology, School of Bioscience and Chemistry, Sungshin Women University, Seoul 02844, South Korea; and [2]College of Veterinary Medicine, Kyungpook National University, Daegu 41566, Korea

ORCiD number: 0000-0002-8497-9257 (Y.-P. Cheon).

Abbreviations: AMH, anti-Müllerian hormone; E2, 17β-estradiol; GC, granulosa cell; P4, progesterone; PBS, phosphate-buffered saline; T, testosterone; WAT, white adipose tissue.

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Abstract

Ovarian reserve and fertility are reduced by aging and a poor energy balance. To date, the relationships of high energy accumulation and aging with the ovarian reserve have not been elucidated. Here, the effects of obesity on the aging ovarian reserve were evaluated in a leptin-deficient (*ob*/*ob*) mouse model. Abnormal estrous cyclicity appeared as early as 6 weeks and worsened with aging. The blood level patterns of 17β-estradiol (E2), testosterone (T), and progesterone (P4) with aging were similar between lean and *ob/ob* mice. The blood level of E2 but not P4 or T was similar at 24 weeks. Many more atretic follicles but fewer corpora lutea were observed in *ob/ob* mice than in lean mice within all age groups. Anti-Müllerian hormone (*Amh*) mRNA levels were similar between genotypes. *Dazl, Stra8, and ZP3* mRNAs were highly expressed in *ob/ob* mice after 12 weeks. *Sohlh1* and *Ybx2* mRNAs were highly expressed at 24 weeks in *ob/ob* compared with lean mice. In addition, SOHLH1-positive primordial follicle counts were significantly increased in *ob*/*ob* mice at 24 weeks. The proportions of AMH-positive secondary and small antral follicles were similar between genotypes. Together, these results show that the ovarian reserve lasts longer in *ob/ob* mice than in lean mice, suggesting that the loss of normal physiological or physical status causes decreased fertility at a young age in *ob/ob* mice and that an increase in adipocytes without leptin, as in *ob/ob* mice, can improve the ovarian reserve. Such knowledge can be applied to understanding reproductive dysfunction.

Key Words: *ob*/*ob*, fertility, ovarian reserve, sex steroid hormone, meiosis-stage marker

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Reproductive performance in females is known to decline with age. The ovaries, and particularly the follicles, are the primary targets of senescence effects. Recent evidence suggests that the dynamics of follicular growth undergo changes in mammals of advanced reproductive age. Health female have ~400 000 primordial follicles at puberty and ~1000 or fewer at 45 years. Age-dependent modifications in ovarian activity are closely connected with brain and endocrine changes, primarily variation in the circulating levels of gonadotropins and sex steroids [1, 2]. The decline in hypothalamic neuropeptides and neurochemicals, such as glutamate and norepinephrine, and the 17β-estradiol (E2)-mediated decline in gonadotropin-releasing hormone (GnRH) neuronal activity are among the reasons for ovarian aging involving the hypothalamus-pituitary-ovarian axis [1, 3]. As this decline progresses with age, preovulatory follicles grow more slowly and achieve a smaller diameter at ovulation [4]. In contrast, preovulatory follicles are larger and granulosa cell proliferative activity is higher in old rats than in young rats with 4-day estrous cycles [5].

Studies on energy balance have shown that chronic energy deficiency promotes compensatory mechanisms to conserve fuel for vital physiological functions with suppression of the hypothalamic-pituitary-ovarian axis and menstrual dysfunction [6]. On the other hand, studies on the effects of female obesity and fertility have shown that obese and overweight women are more likely than healthy-weight women to have reduced fertility [7]. Although the effects of obesity or energy deficiency on female reproduction and oocytes are emerging, the precise underlying mechanisms remain unclear. Inflammatory factors caused by obesity such as tumor necrosis factor-α and interleukins, are suggested as mediators of dysfunction in the ovary [8]. Additionally, various factors, including hormones and the hypothalamic neuropeptide Y4, are also known direct or indirect factors for the downregulation of ovarian function and impaired fertility [9, 10].

Leptin, a hormone secreted from adipose tissue and the placenta, is lacking in leptin-deficient (*ob*/*ob*) mice. These *ob*/*ob* female mice are morbidly obese, infertile, and hyperphasic and exhibit hyperglycemia and insulin resistance, and their sterility is usually permanent. However, when these animals are exogenously treated with leptin, they lose body weight and regain their fertility, and the follicles are normalized [11, 12]. Leptin induces the release of pituitary gonadotropins by inducing hypothalamic GnRH-independent effects of leptin on ovarian stimulation and follicular development [13]. Mutations causing infertility through obesity tend to be related to insufficient hypothalamo-pituitary-gonadal drive during the development of reproductive organs. The involvement of hypothalamic-pituitary dysfunction in obesity-related reproductive failure has been well described [14].

The hypothalamus-pituitary-ovarian axis is an important factor in ovarian senescence, and hypothalamic-pituitary dysfunction is observed in obesity. On the other hand, the age-associated decline in fertility depends on both the quality and quantity of follicles, and caloric restriction blocks aging-related increases in oocyte abnormalities [15]. However, thus far, it is not clear whether obesity is the cause of shortening or improving ovary aging. The present study investigated the ovarian morphology, follicular counts, hormone profile, and profiles of follicle-stage specific marker genes in lean and *ob*/*ob* mice to evaluate the effects of leptin-deficient obesity on the loss of fertility activity by aging.

Materials and Methods

Animals

Female leptin-deficient obese (B6.VLepob, '*ob*/*ob*') and lean mice, 3, 6, 9, 12, and 24 weeks of age and body weights 14.4 ± 3.79, 20.7 ± 3.27, 37.4 ± 4.90, 50.4 ± 3.63, 57.4 ± 4.43, and 72.8 ± 4.77g, respectively, were obtained from the Korean Research Institute of Bioscience and Biotechnology (Daejeon, Korea). Animal care and use were conducted according to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. Animals were housed in environmentally controlled conditions with a 14:10 light:dark schedule (light-on at 05:00) at a temperature of 22 ± 1 °C and relative humidity of 50% ± 5%. They were given free access to water and a standard rodent chow diet. Body weight was determined using an electronic balance (Mettler Toledo, Greifensee Switzerland), blood samples were collected via the ophthalmic venous plexus, and nonblood glucose levels were determined using a glucometer (Super Glucocard II, KDK Co, Japan). All animal procedures described were approved by the Institutional Animal Care and Use Committee at Sungshin University.

Adipose Tissue Collection and Analysis

At 24 weeks of age, animals were sacrificed by cervical dislocation. White adipose tissue (WAT) deposits in periovarian, retroperitoneal, and mesenteric tissues were dissected out and weighted.

Estimating Estrous Phase by Vaginal Smear

The estrous cycle was staged by examining vaginal smears obtained following standardized protocols in lean and *ob*/

ob mice at 6, 9, and 12 weeks of age (n = 10 per group). Briefly, 20 μL of normal saline solution was used for lavage for approximately 3 weeks to determine estrous cyclicity and smeared onto glass slides. The samples were stained with hematoxylin-eosin and examined under a light microscope. The numbers of epithelial cells and leukocytes were counted, and their relative ratio was used as a criterion.

Ovary Histological Evaluation, Morphological Classification, and Follicle Count

Histological analysis was performed based on the report of Rucker et al [16]. Ovaries were removed and fixed with Bouin's solution (10% paraformaldehyde, 0.16% picric acid in phosphate-buffered saline [PBS]) and embedded in Paraplast (Sigma, MO, USA). Paraffin block samples were sectioned completely at 8-μm thickness and stained with hematoxylin-eosin for histological observation. Follicle counts were performed on every tenth section. Follicles were classified as primordial follicles if they had an oocyte surrounded by flat granulosa cells (GCs). Primary follicles were defined as having an oocyte surrounded by a signal layer of cuboidal GCs. Follicles were classified as secondary if they possessed 2 or more GC layers with no visible antrum. Early tertiary follicles (early antral follicles) had several layers of GCs and 1 layer of theca cells with 1 or 2 small areas of follicular fluid (antrum). Preovulatory follicles had a rim of cumulus cells surrounding the oocytes. Atretic follicles had a condense section of nucleus in GC and deformation of oocytes. Corpora lutea were defined as follicles consisting of lutein cells and serving as temporary endocrine structures in mammals involved in the production of estrogen and progesterone (P4). The number of follicles was counted in at least 3 independent mice per group.

Hormonal Determination

Blood was collected with cardiac puncture just after euthanasia, kept 30 minutes at room temperature (RT), centrifuged (1500*g*) 10 minutes at 4 °C, and used for analysis. Serum concentrations of E2, P4, and testosterone (T) were measured by ELISA kits (Cusabio, Cat no. CSB-E05109m [17], CSB-E05104m [18], and CSB-E05101m [19], respectively, TX, USA). These kits have been validated for measurements of E2, P4, and T in the blood serum of mice according to the manufacturer's protocols. The average reportable range for E2 was 40 to 1000 pg/mL, P4 was 0.3 to 10 ng/mL, and T was 0.1 to 20 ng/mL. The minimum detection dose of mouse E2 is less than 40 pg/mL, P4 is less than 0.2 ng/mL, and T is less than 0.05 ng/mL. The intra-assay and interassay variability were both under 15%.

Total RNA Extraction and Real-Time Reverse Transcriptase–Polymerase Chain Reaction

Total RNA was extracted using TRIzol Reagent (Invitrogen, Cat #: TR118, San Diego, CA, USA) according to the manufacturer's instructions with modification. Briefly, the samples were homogenized with TRIzol Reagent (1mL/100 mg) and stored for 10 minutes at RT. Chloroform (200 μL/mL) was added to the homogenates and shaken vigorously for 15 seconds. Then, the mixture was stored for 15 minutes at RT and centrifuged at 12 000*g* for 15 minutes at 4°C. The RNA was precipitated by adding 0.5 mL isopropanol, mixing gently, incubating the samples for 10 minutes at RT, and centrifuging them at 12 000*g* for 8 minutes at 4 °C. Then, the supernatant was removed, and the RNA pellet was washed in 1 mL 75% ethyl alcohol, mixed, and centrifuged at 7500*g* for 5 minutes at 4 °C. The supernatant was discarded again, and the pellet was dried to remove ethyl alcohol and combined with 50 μL diethyl pyrocarbonate (DEPC)-treated water. Total RNA was assessed by a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Cat #: ND-2000, Wilmington, DE, USA) and kept at −80 °C until use. First-strand cDNA was synthesized using Accuscript High Fidelity Reverse Transcriptase (Agilent Technologies, Cat #: 600089, CA, USA) with 5 μg total RNA in accordance with the manufacturer's instructions. The reaction mixture was incubated at 42 °C for 1 hour and at 70 °C for 10 minutes to terminate cDNA synthesis. The cDNA was stored at -20 °C. Real-time PCR was performed using SYBR Premix Ex Taq (TaKaRa, Cat #: RR420A, Japan) and a Thermal Cycler Dice Real-Time System TP800 (TaKaRa, Cat #: TP800, Japan). Each reaction was run in triplicate. Dissociation curves were plotted for all reactions to confirm amplification of a single product with the appropriate melting temperature. The fold change in gene expression was calculated using the double delta Ct method with the housekeeping gene 36B4 as an internal control.

Immunofluorescence

The paraffin-embedded ovarian sections were deparaffinized in xylene, serially rehydrated through a graded ethyl alcohol series, and rinsed with tap water. Antigen retrieval was performed with 0.01M sodium citrate buffer (pH 6.0), washed with PBS containing 0.5% Triton X-100 (PBST) for 5 minutes and washed with PBS for 5 minutes. Sections were blocked with 1% normal blocking serum in PBS for 1 hour at RT and then incubated with conjugated antibodies against AMH (BIOSS, Cat #: bs-4687R-FITC, USA, dilution 1:100,) [20], SOHLH1 (BIOSS, Cat #:

bs-12278R-A594, USA, dilution 1:100) [21], and NOBOX (BIOSS, Cat #: bs-12273R-Cy5, USA, dilution 1:100) [22] for 1 hour at RT. After 3 washes using PBST, they were counterstained with DAPI (Sigma-Aldrich, Cat #: D9542, USA, dilution 1:500) for 30 minutes at RT and mounted with Fluoromount in PBS. Finally, samples were analyzed by confocal microscopy (ZEISS, LSM T-PMT HAL 100).

Statistical Analysis

The values are expressed as means ± SD. Significant differences between the lean and *ob/ob* mouse groups were determined by 1-way analysis of variance (ANOVA), and post hoc comparisons were performed by Student *t* test. *P* values less than 0.05 were considered significant in the experiments.

Results

Body Weight, Blood Glucose Levels, and Abdominal White Adipose Tissue Weight

The mice had the typical feature of *ob/ob* mice. The body weight, blood glucose levels, and white adipose tissue (WAT) changes in the experimental groups are shown in Figure 1. The body weight of female *ob/ob* mice was significantly higher during the whole experiment compared with that of lean mice. From 3 weeks to 12 weeks, body weight increased rapidly, and from 12 weeks to 24 weeks, weight increased gradually (Fig. 1A). The blood glucose levels in lean mice did not significantly change during the whole experiment, but the blood glucose levels in *ob/ob* mice were markedly high, and the blood glucose level increased up to 9 weeks; subsequently, the blood glucose level decreased, with similar levels at 3 weeks and 24 weeks (Fig. 1B). The abdominal fat content in 24-week-old *ob/ob* mice was 8.8-times higher than that in lean mice (Fig. 1C).

Age-Dependent Estrous Cyclicity Defects

Vaginal smears were taken daily for a period of 2 weeks to determine the length and regularity of the estrous cycle in lean and *ob/ob* female mice. Lean mice had normal estrous cycles. On the other hand, the estrous cycles of *ob/ob* mice showed increasing irregularity as the mice aged (Fig. 2). For example, at 12 weeks of age, lean mice tested showed normal estrous cyclicity (Fig. 2A), but *ob/ob* mice showed

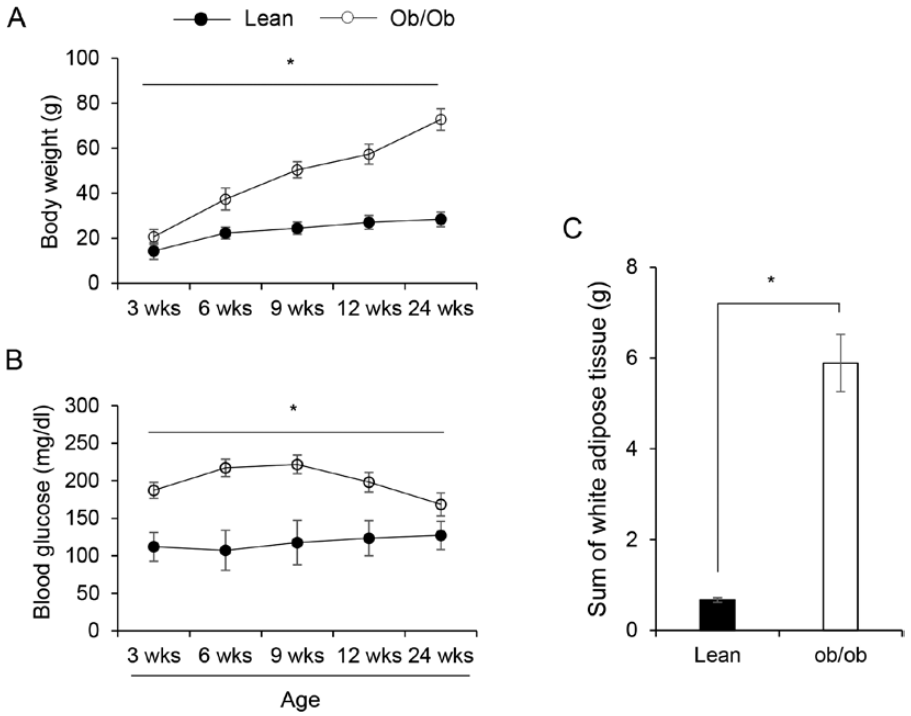

Figure 1. Characteristics of body weight, nonfasting blood glucose levels, and abdominal white adipose tissue weight in lean and *ob/ob* mice with aging. **A,** The body weights of female *ob/ob* mice were significantly higher during the whole experiment than those of lean mice. From 3 to 12 weeks, body weight increased rapidly, and from 12 to 24 weeks, weight increased gradually. **B,** The blood glucose levels in lean mice were not significantly changed during the whole experiment, but the blood glucose levels in *ob/ob* mice were markedly high, and the blood glucose level increased up to 9 weeks; subsequently, the blood glucose level decreased, with similar levels at 3 weeks and 24 weeks. **C,** The abdominal fat content in 24-week-old *ob/ob* mice was 8.8-times higher than that in lean mice. $P < 0.05$ lean vs control at the same age. *: $P < 0.05$ between lean and *ob/ob* mice in the same age groups (Student *t* test after ANOVA).

abnormal estrous cyclicity as early as 6 weeks of age (Fig. 2B) [23]. In 9-week-old *ob/ob* female mice, cyclicity also was similar to the 6-week-old mice (Fig. 2C) [23]. The percentage of epithelial cells was increased by aging, and most of the cells were epithelial cells at 12 weeks of age (Fig. 2D) [23]. The morphological characteristics of the epithelial cells were not different between lean and *ob/ob* mice (Fig. 2E). Thus, the incidence of abnormal cyclicity increased in *ob/ob* mice as obesity/insulin resistance became more severe.

Hormone Profiles in Lean and ob/ob Mice

To determine the possible reasons for cyclicity defects in *ob/ob* mice, the levels of blood steroid hormone were measured using serum samples collected from mice at 3, 6, 9, 12, and 24 weeks of age. The patterns of E2 serum level by age were similar between lean and *ob/ob* mice, although the level was significantly low in *ob/ob* mice except at 24 weeks. In 24-week-old *ob/ob* mice, the level was increased and became similar to that of lean mice of the same age. After sex maturation, the level was increased by aging until 24 weeks (Fig. 3A).

The patterns of P4 serum levels by age were similar between *ob/ob* and lean mice in all age groups. However, the levels were significantly lower in *ob/ob* mice than in lean mice of the same age. In 24-week-old lean mice, the P4 serum level (1.2 ± 0.06 μg/mL) was significantly lower than in 3-week-old lean mice (2.8 ± 0.03 μg/mL), but it was not different in *ob/ob* mice between 3-week-old (1.1 ± 0.11 μg/mL) and 24-week-old (1.2 ± 0.11) *ob/ob* mice. It was significantly higher at 24 weeks than at 6 weeks (1.2 ± 0.11 μg/mL). After sexual maturation, P4 serum levels increased significantly with age until 12 weeks and then decreased in both lean and *ob/ob* mice (Fig. 3B).

The patterns of serum T levels were similar between *ob/ob* and lean mice in all age groups. However, compared with the levels of other sex steroid hormones, T levels were significantly low in all age groups except for the 6-week group (16.2 ± 0.53ng/ml in lean and 16.0 ± 0.84ng/ml in *ob/ob*). The serum T level was significantly lower at 24 weeks than at 3 weeks or 6 weeks in both *ob/ob* and lean mice. After sexual maturation, its level increased with age until 24 weeks (Fig. 3C). These results showed that the total levels of sex steroid hormones decreased from the immature stage until 24 weeks.

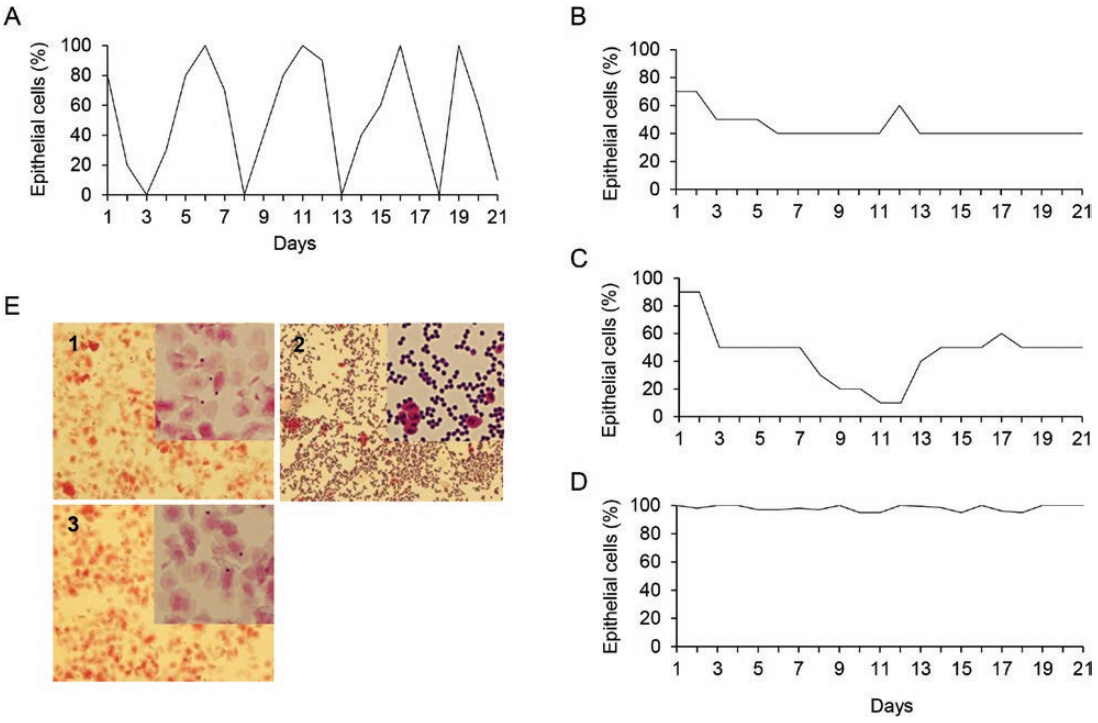

Figure 2. Disturbed estrous cycle in *ob/ob* mice. The lean and *ob/ob* mice were subjected to vaginal smearing to analyze their estrous cycles at the ages of 6 weeks, 9 weeks, and 12 weeks (n = 10 per group). The cell suspension was smeared on slides and stained with hematoxylin-eosin. **A,** Twelve-week-old lean mice had a normal estrous cycle (4-5 days). **B-D,** Percentages of epithelial cells in 6-week-old (B), 9-week-old (C), and 12-week-old (D) female *ob/ob* mice. The percentage of epithelial cells increased with aging. **E,** Photomicrographs of epithelial cells and leukocytes in vaginal fluid during the estrous cycle. **E1-E2,** cells from lean mice in the estrous and diestrous stages; **E3,** epithelial cells in the vaginal fluid of 12-week-old *ob/ob* mice.

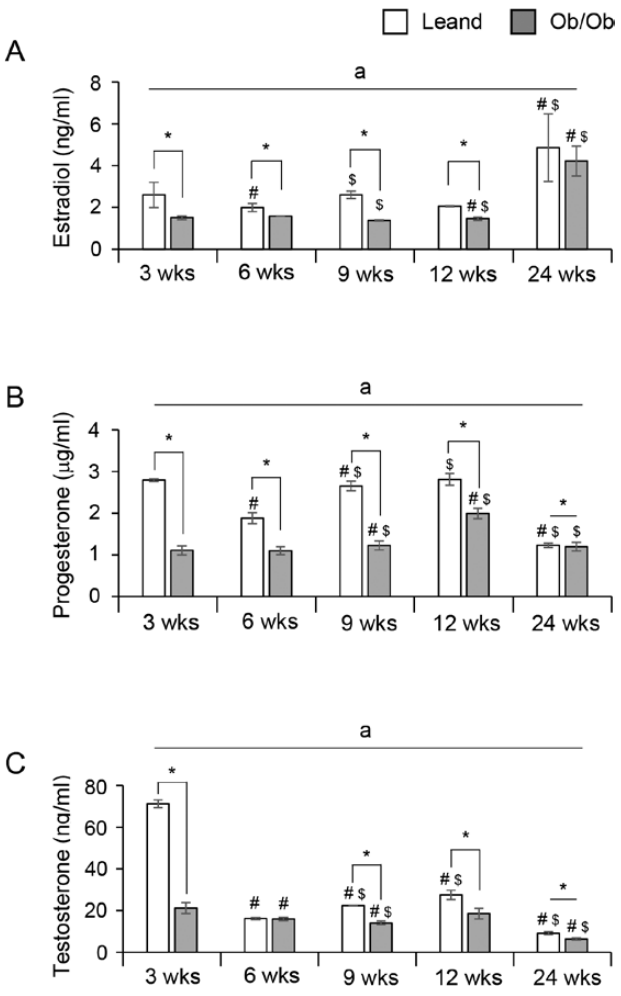

Figure 3. Chronological changes in serum levels of steroid hormones (mean ± SD) in lean and *ob/ob* mice at a series of ages representing the immature and mature periods (n = 10 per group). Sera were subjected to enzyme-linked immunosorbent assays to measure (A) 17β-estradiol, (B) progesterone, and (C) testosterone. Graphs represent the means ± standard deviations from 10 females per group. A letter above the line indicates a significant difference in the groups (ANOVA, $P < 0.05$). $^{\#}P < 0.05$ for comparison of 3-week-old mice versus other age groups, $^{\$}P < 0.05$ comparing 6-week-old mice with the other age groups.

Ovarian Morphology and the Number of Growing Follicles in ob/ob and Lean Mice

The ovarian growth morphology of the *ob/ob* and lean mice at 5 different ages did not differ by group (data not shown). At 3 weeks of age, the ovaries of lean and *ob/ob* mice contained similar numbers of early antral and preovulatory follicles, but atretic follicle numbers were increased in *ob/ob* mice compared with lean mice (Fig. 4A, 4B). At 6 weeks of age, the ovaries of lean mice had many corpora lutea but fewer follicles in other stages (Fig. 4C, 4D). At 9 and 12 weeks of age, the ovaries of *ob/ob* mice contained significantly more atretic follicles than those of lean mice, and a few corpora lutea were seen in ovaries from *ob/ob* mice (Fig. 4E-4H). At 24 weeks of age, the ovaries of lean mice contained significantly larger numbers of preovulatory follicles than those of *ob/ob* mice, and the ovaries of the lean mice had well developed corpora lutea. In ovaries from *ob/ob* mice, a large number of atretic follicles and many large empty follicles were observed, and no properly developed corpora lutea were present (Fig. 4I, 4J).

The number of follicles in each stage dramatically changed with age. At 3 weeks of age, the ovaries of lean and *ob/ob* mice contained similar numbers of early tertiary follicles and preovulatory follicles, but atretic follicle numbers were increased in *ob/ob* mice compared with lean mice. As expected, corpora lutea were not observed in either *ob/ob* or lean mice (Fig. 4). At 6 weeks of age, the ovaries of lean and *ob/ob* mice had very different numbers of follicles at each stage. The numbers of early tertiary follicles, preovulatory follicles, and corpus luteum were significantly lower in the ovaries of *ob/ob* mice than in those of lean mice. However, the number of atretic follicles in the ovaries of *ob/ob* mice was significantly greater than the number in the follicles of lean mice (Fig. 4).

At 9 weeks of age, the numbers of early tertiary follicles and corpora lutea were similar between *ob/ob* and lean mice. The number of preovulatory follicles was significantly lower in *ob/ob* mice than in lean mice. On the other hand, the number of atretic follicles was significantly higher in *ob/ob* mice than in lean mice (Fig. 4). At 12 weeks of age, the numbers of preovulatory follicles and corpora lutea were significantly lower in *ob/ob* mice than in lean mice, as shown in 9-week-old mice. The number of early tertiary follicles was similar between *ob/ob* and lean mice. However, the number of atretic follicles was significantly higher in *ob/ob* mice than in lean mice (Fig. 4). At 24 weeks of age, the numbers of preovulatory follicles and corpora lutea were significantly lower in *ob/ob* mice than in lean mice. The number of early tertiary follicles was similar between *ob/ob* and lean mice. However, the number of atretic follicles was significantly higher in *ob/ob* mice than in lean mice.

Expression of Markers Associated With Primordial, Primary, and Secondary Follicles as Well as Fertility Reserve

The evaluated ovarian morphology data and the number of follicles showed that the number of early tertiary follicles was not different between *ob/ob* and lean mice after sex maturation. Therefore, we investigated the expression profiles of germline-specific meiosis-commitment genes (deleted in azoospermia-like [*Dazl*], stimulated by retinoic acid gene 8 [*Stra8*]), primordial oocyte formation marker genes (spermatogenesis and oogenesis specific basic

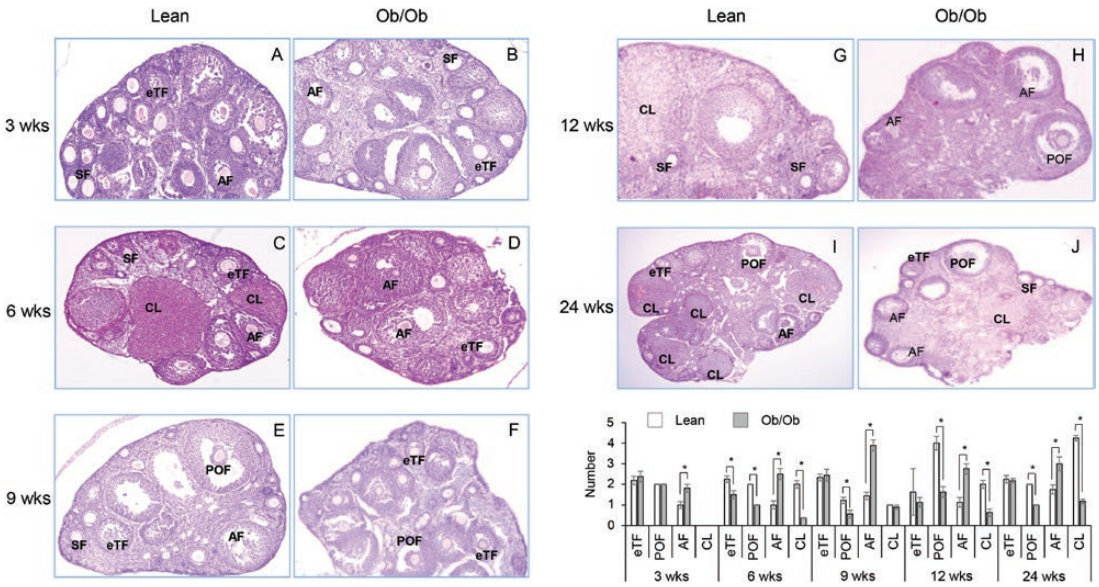

Figure 4. Photomicrography of ovaries and the numbers of tertiary follicles, preovulatory follicles, atretic follicles, and corpora lutea in the lean and *ob/ob* mice by aging. Ovaries fixed in Bouin's solution were subjected to hematoxylin-eosin staining (n = 3 mice per group). Every tenth section in the series was used to count the number of follicles (mean ± SD). Abbreviations: AF, atretic follicle; CL, corpus luteum; eTF, early tertiary follicle, POF, preovulatory follicle; SF, secondary follicle. * $P < 0.05$ between lean and *ob/ob* in the same age groups (Student *t* test).

helix-loop-helix 1 [*Sohlh1*], NOBOX oogenesis homeobox [*Nobox*]), a meiotically-arrested oocyte marker gene (Y box protein-2 [*Ybx2*]), and a growing oocyte marker gene (zona pellucida glycoprotein 3 [*Zp3*]). Additionally, the expression patterns of the generally accepted ovarian reserve marker anti-Müllerian hormone (*Amh*) were also analyzed in the ovaries of the experimental groups. The expression levels of *Dazl* mRNA were significantly lower in 3-week-old *ob/ob* mice than in lean mice. However, its levels were significantly increased in 6-week-old and 12-week-old *ob/ob* mice and were similar to those of lean mice (Fig. 5A). Another meiosis-commitment gene, *Stra8*, was expressed at significantly high levels from 6 weeks to 24 weeks of age (Fig. 5B).

The next step of meiosis commitment in oogenesis is meiosis initiation; *Sohlh1*, a marker of this step, was scarcely detectable at the mRNA level. Nonetheless, its mRNA expression was detected from 6 weeks of age and was significantly higher in lean mice than in *ob/ob* mice, and such expression patterns were detected until 12 weeks. However, its expression levels were increased in *ob/ob* mice at 24 weeks of age (Fig. 5C). Another marker of meiosis initiation, *Nobox* was expressed in 3-week-old and 6-week-old lean and *ob/ob* mice at similar levels (Fig. 5D).

Ybx2, a meiosis arrest marker, was detected at 3 weeks, and its mRNA expression level was significantly higher in 3-week-old *ob/ob* mice than in same-age lean mice. From 6 to 12 weeks of age, its expression levels were similar between *ob/ob* and lean mice. On the other hand, its expression was increased in *ob/ob* mice at 24 weeks of age compared with lean mice of the same age (Fig. 5E).

The mRNA expression level of *Zp3*, a marker of growing oocytes, was significantly lower in 3-week-old and 6-week-old *ob/ob* mice than in lean mice of the same ages. Its mRNA expression levels at 9 weeks of age were similar between *ob/ob* and lean mice. However, its mRNA expression levels from 12 weeks to 24 weeks were significantly higher in *ob/ob* mice than in lean mice (Fig. 5F).

Amh, a generally accepted ovarian reserve marker expressed in GC, had similar mRNA expression levels between *ob/ob* and lean mice from 3 weeks to 9 weeks. Its mRNA expression level was significantly higher in 12-week-old *ob/ob* mice than in lean mice of the same age, but the groups had equalized again by 24 weeks of age (Fig. 5G).

These results showed that the expression levels of meiosis commitment, meiosis initiation, meiosis arrest, growing oocytes, and ovarian reserve markers were similar or higher at 24 weeks of age in *ob/ob* mice than lean mice. These markers represent the number of primordial or primary follicles. Therefore, we performed immunofluorescence with AMH, SOHLH1, and NOBOX in ovarian sections from 6-week-old and 24-week-old *ob/ob* and lean mice (Fig. 6A). Their expression patterns were similar to those of their mRNA (Fig. 5 and 6). The number of SOHLH1-positive primordial follicles was similar at 6 weeks but significantly higher at 24 weeks in *ob/ob* mice than in lean mice of the same age (Fig. 6B). The numbers of NOBOX-positive primary and secondary follicles were

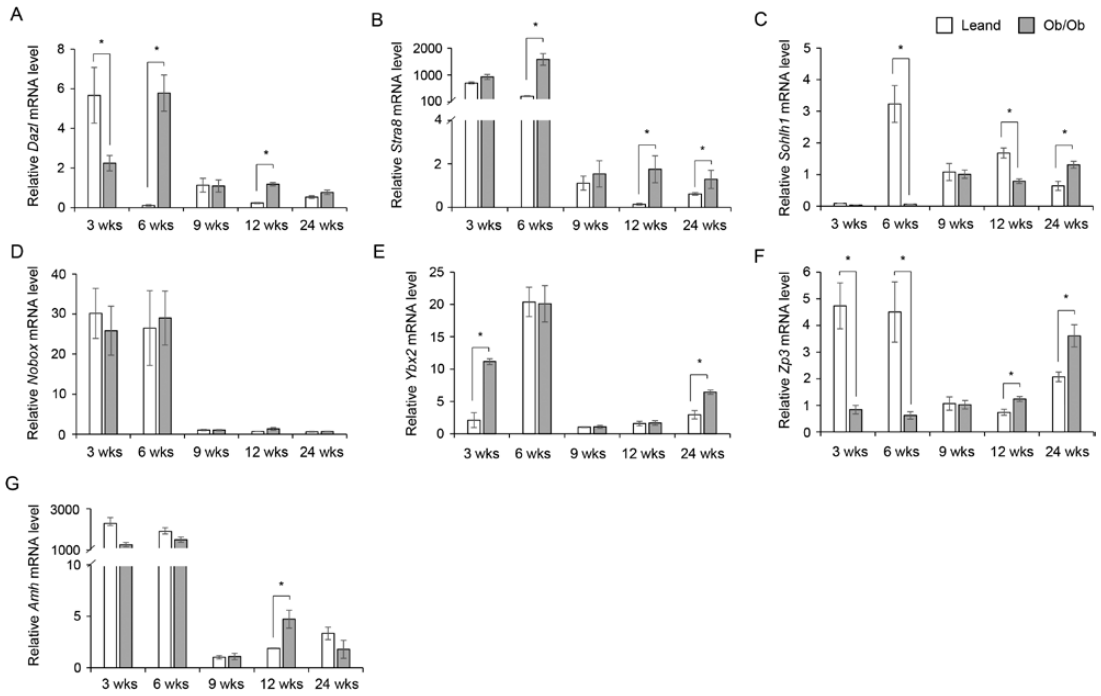

Figure 5. The expression profiles of meiosis-committed, meiosis-initiated, meiosis-arrested, growing oocytes and ovarian reservation markers by age. The ovaries of each age group were collected and kept at –80 °C until use. Total RNA was isolated with TRIzol Reagent according to the manufacturer's instructions, and then first-strand cDNA was synthesized. Real-time PCR analysis was performed with SYBR premix Taq. The data are mean (± SD) of 3 replicates. **P* < 0.05 (Student *t* test).

significantly higher at 6 weeks of age in *ob/ob* mice than in lean mice. At 24 weeks old, the number of primary follicles was significantly lower in ob/ob mice, but the number of secondary follicles was similar between *ob/ob* and lean mice (Fig. 6B). In the case of AMH-positive secondary and early tertiary follicles, their numbers were significantly higher in 6-week-old *ob/ob* mice than in lean mice. On the other hand, the numbers of secondary and early tertiary follicles at 24 weeks of age did not differ between *ob/ob* and lean mice.

Discussion

The progressive loss of the primordial follicle pool causes ovarian aging, and the size of the pool of remaining oocytes is defined as the ovarian reserve. The known adverse factors that contribute to reproductive decline include aging, oxidative stress, abnormal energy amounts, and physiological stress [24]. Advanced female age is an obligate factor in ovarian reserve or ovarian aging and is related to energy homeostasis in the ovarian microenvironment, such as oxidative stress and advanced glycation end products [25]. An association between infertility and energy imbalances in the body has been demonstrated; however, most of these studies have primarily focused on the relationship between obesity in females and reduced fertility [7]. Obesity induced by diet or leptin mutation impairs nuclear maturation [26, 27]. The effects of obesity on ovarian aging are controversial and remain incompletely understood. In this study, we evaluated the possible effects of leptin-dependent obesity on the preservation of primordial follicles.

A sufficient mass of adipose tissue is needed for proper onset of puberty and maintenance of fertility, indicating an important link between energy homeostasis and reproductive function. However, high-fat model mice, including both diet-induced and mutation-induced models, show impaired reproduction and reduced fertility. In *ob/ob* mice, the mutation of an energy balance regulator, hypothalamic neuropeptide Y receptor 4, or transplantation of adipose tissue from wild-type restores fertility [10, 13]. This means that energy balance is important to the recovery of fertility in *ob/ob* female mice. Interestingly, as shown in the results, by 6 weeks of age estrous cyclicity was abnormal in obese mice, and the incidence of abnormal estrous cyclicity gradually increased at 12 weeks of age without morphological changes in epithelial cells in lean and *ob/ob* mice, similar to the study of Chakraborty et al [28] and Ng et al [29] cornified epithelial cells observed continuously in 14 to 16 weeks of age with *ob/ob* mice. On the other hand, the blood level of E2 was lower after 9 to 12 weeks but not 24 weeks compared with that of 6 weeks in *ob/ob* mice, as seen in the results. This means that the loss of cyclicity does not depend only on estrogen or leptin in mice, as suggested by Ng et al, although food intake, leptin, and estrogen

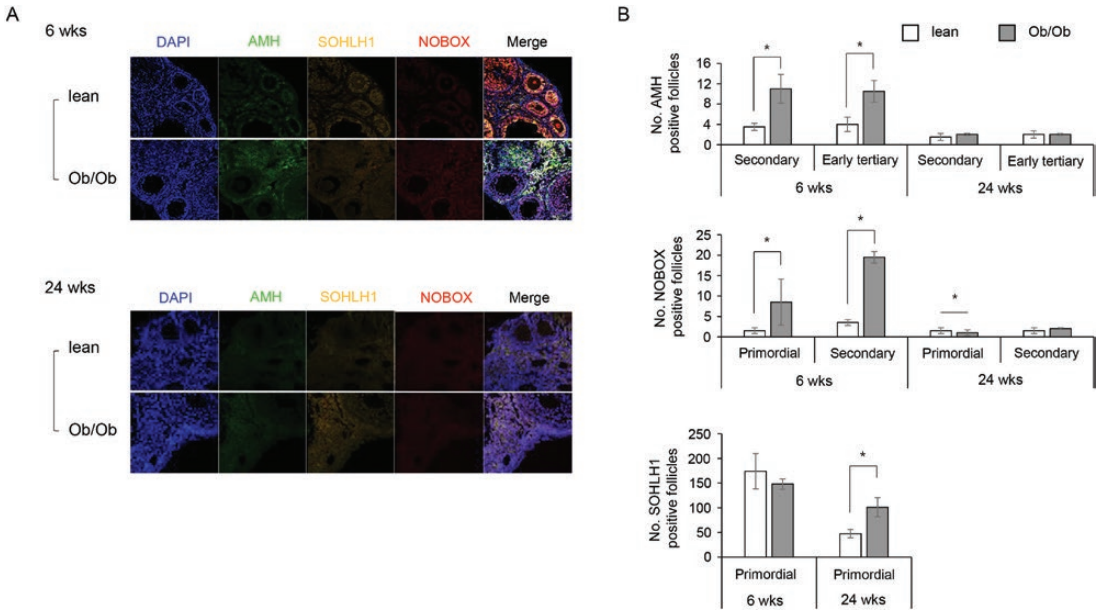

Figure 6. The localization of AMH, SOHLH1, and NOBOX, and the number of follicles in the various age groups of *ob/ob* and lean mice. Immunofluorescent staining was performed with antibodies against AMH (green), SOHLH1 (yellow), and NOBOX (red). The nuclei were stained with DAPI (blue). **A,** Photomicrography of immunofluorescence staining for AMH, SOHLH1, and NOBOX in 6-week-old and 24-week-old *ob/ob* and lean mice. **B,** The numbers of follicles specifically positive for AMH, NOBOX, and SOHLH1. The data are the mean (± SD) of 3 replicates (3 animals per group). $*P < 0.05$ (Student *t* test).

impact cyclicity [29]. Taken together, these results suggest that there are alterations in physiological status, but they respond to the cellular-level regulatory signals of reproductive organs by changing physiological properties in *ob/ob* obese mice.

One of the major findings of the current study is the deleterious effect of obesity by leptin deficiency on folliculogenesis. The changes in the number of atretic follicles are not known, although the increased number in atretic follicles is more prevalent in untreated or gonadotropin-stimulated *ob/ob* mice [30, 31]. In this study, age-dependent patterns of atretic follicles were revealed. The number of atretic follicles was significantly higher in the *ob/ob* group than in the control group in all examined age groups (by more than 2-fold except in the 3- and 24-week age groups). The number of follicles present in the ovaries of *ob/ob* mice was reduced, and there was a distinct increase in apoptotic GCs and atretic follicles in these animals. Nevertheless, quantitative analysis of the various stages of follicular increases in leptin-lacking animals is critical. These findings suggest that folliculogenesis is impaired beyond the preantral multilaminar follicle phase.

The mechanism of obesity in declined fertility and in the follicular morphological variation described in our results must be associated with alterations in ovarian steroidogenesis. It has been suggested that leptin may have a bimodal effect on the ovary that is dependent on the hormone concentration. At physiological concentrations, leptin enhanced estrogen production from human luteinized GCs and both E2 and P4 production from in vitro cultures of preantral mouse follicles [32]. Leptin has also been shown to influence ovulation through a luteinizing hormone (LH)-independent pathway [13]. Leptin-deficient mice are known to be hypogonadotropic. The excessive storage of lipids in *ob/ob* mice induces steroidogenic defects in the ovary by decreasing the expression of steroidogenic acute regulatory enzyme (StAR) [31]. The present study demonstrated that both serum E2 and P4 levels declined in *ob/ob* mice compared with lean control mice in an age-dependent manner. In lean mice, E2 levels continuously increased with age from 3 to 24 weeks. In *ob/ob* animals, by contrast, E2 levels continuously declined from 6 weeks to 9 weeks. The levels of E2 and P4 were significantly higher in lean mice than in *ob/ob* mice of the same age, except in the 24-week age group. T levels were also decreased in *ob/ob* mice compared with lean mice of the same age. Collectively, our findings suggest that the reduction in ovarian steroidogenesis is related to the changes in ovarian morphology seen in *ob/ob* animals, including the reductions in the numbers of healthy follicles. This decline may also result from the changing levels of metabolic regulators the absence of leptin.

An interesting phenomenon in leptin-deficient *ob/ob* mice is that leptin treatment increases the total number of follicles. The numbers of follicles in all stages, especially primary and tertiary, are increased by leptin in *ob/ob* mice [11]. It is recognized that gonadotropin response

does not differ between *ob/ob* and wild-type mice at 13 weeks of age [33]. Until 16 weeks, the number of oocytes released upon induction was no lower in *ob/ob* mice than in control mice [34]. Interestingly, our results revealed that the number of primordial follicles at 24 weeks of age was much higher in *ob/ob* mice than in lean mice. In addition, the mRNA expression levels of meiosis-committed marker genes (*Dazl* and *Stra8*), meiosis-initiated marker genes (*Sohlh1* and *Ybx2*), and a growing oocyte marker gene (*Zp3*) at 24 weeks of age were significantly higher in *ob/ob* mice than in lean mice. In addition, the expression levels of AMH, a generally accepted marker of ovarian reserve versus ovarian aging, were significantly elevated in *ob/ob* mice at 12 weeks and similar at 24 weeks compared with the levels in age-matched lean mice. To date, the effects of obesity on ovarian reservation are very controversial. Some groups suggest that obesity has negative effects, but others propose the opposite. According to prior results, the patterns of meiotic-specific markers differed between aged *ob/ob* and wild-type mice [35]. According to Niikura et al [35], there was no expression of primordial oocyte markers in wild-type mice at 24 weeks of age, but in *ob/ob* mice of the same age, these markers were expressed. Based on these results, it is clear that obesity in *ob/ob* mice helps maintain ovarian reservation.

The mechanisms underlying the substantially increased number of primordial follicles in *ob/ob* mice compared with wild-type mice are not clear and require further study. However, we can obtain some clues from previous reports and our results. In the case of obesity induced by excess calorie consumption, the number of primordial follicle is lower than that in controls due to NAD-dependent protein deacetylase sirtuin-1 (SIRT1) and serine/threonine-protein kinase mTOR (mTOR) signaling [36]. SIRT1 and mTOR expression activity has been found to be under the control of leptin [37, 38]. On the other hand, neonatal overfeeding reduces the primordial follicle pool, while administration of a leptin antagonist rescues the size of the primordial follicle pool [39]. In lean rats, leptin administration dramatically reduced (0.307-times) the number of primordial follicles compared with obese rats (0.16-times vs 0.37-times) [40]. In addition, subfertility and compromised ovarian function have been suggested to be independent from obesity caused by a high-fat diet [41]. Improving the bioenergetics of GCs has been found to improve ovarian reserve along with Hbp1. The null of Hbp1 activates mitochondrial biogenesis without altered glycolysis [42]. Hbp1 represses follicle growth in a gene dosage-dependent manner [42]. In the follicles of *ob/ob* mice, defective mitochondria and lipid droplets are found primarily in oocytes but not GCs, and lipid accumulation depends on the follicle stage [31]. In addition, leptin has recently been revealed to be involved mitochondrial functions through its receptor [43], indicating that circulating leptin may be responsible to ovarian reserve. On the other hand, the levels of steroid hormone may be one of the reasons for the compromised ovarian reserve in *ob/ob* mice. Sex steroid hormones have various effects on follicle development according to the developmental stage, follicle stage, blood levels, etc. Androgen and estrogen have variable effects on primordial GCs. Estrogen along with estrogen receptor beta is suggested to be a modulator of primordial follicle activation [44]. Moreover, sex steroid hormones can regulate mitochondrial function. Estrogen upregulates the expression of nuclear respiratory factor-1 in mitochondria and protects against mitochondrial oxidative damage through its receptors [45]. Interestingly, users of hormone contraception, especially high-dose estrogen (>50 μg, ≥3 years), enter menopause at a slightly younger age than women who do not use contraceptives [46]. In some reports in humans, increased body mass index and energy intake are linked with a later onset of menopause [47]. According to this study, estrogen levels in *ob/ob* mice were lower than those in lean mice. Taken together, the results suggest that the null of leptin in *ob/ob* mice is the cause of the substantially higher number of primordial follicles compared with controls along with steroid hormone patterns. In addition, mitochondrial stability in inactive primordial follicles in *ob/ob* mice may also be involved in ovarian reserve.

In superovulated and in vivo fertilized oocytes in obese ICR mice, the rate of development to blastocysts was lower than for noninduced ovulation [48]. However, in vitro fertilized oocytes in *ob/ob* obesity mice have developmental competence [34]. In addition, it is known that some experimental treatments or in vitro fertilization can overcome the defects that obesity causes in oocyte quality (for example, supplementation with coenzyme Q10 in a mouse model) [49]. Regarding the infertility of leptin-deficient *ob/ob* mice, a series of new findings, elucidating relationships among reproductive aging, abnormal estrous cyclicity, ovarian morphology, follicle counts, and hormone profiles, showed significant differences between lean and *ob/ob* mice. In the present study, the blood hormonal levels of *ob/ob* mice were low compared with those of lean mice. The abnormalities in estrous cyclicity, ovarian morphology, follicular counts, and hormonal profiles differed substantially between *ob/ob* and lean mice, and the reproductive abnormalities seen in obesity gradually worsened with age. As obesity/insulin resistance became more severe in the obese mice, hormonal levels declined and follicular atresia accelerated. At 24 weeks of age, *ob/ob* mice had no properly developed corpora

lutea; many empty follicles were present, and the number of atretic follicle was increased. Compared to those of studies linking energy balance and ovarian reserve at systemic levels, these results suggest that the ovarian reserve capacity of *ob/ob* mice is greater than that of lean mice, and that these differences emerge with age. The results of this study may be useful for overcoming the subfertility of obese women.

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Additional Information

***Correspondence*:** Yong-Pil Cheon Ph.D., Division of Developmental Biology and Physiology, CDPR, Department of Biotechnology, Sungshin University, 147 Miadong, Kangbukgu, Seoul, 142–732, Korea. E-mail: ypcheon@sungshin.ac.kr.

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