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**Unique morpho-functional characteristics of
folliculogenesis found in the ovary of cotton rat
(*Sigmodon hispidus*)**

コットンラット (*Sigmodon hispidus*) の卵胞形成にみられる特異な形態機能

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Abbreviations

ART: Assisted reproductive technology

Bbs: Balbiani bodies

BL: Basal lamina

BLB: Basal lamina breaching

CR: Cotton rat

DAB: Diaminobenzidine tetrahydrochloride

DDX4: DEAD-box helicase 4

DNOs: Double nucleated oocytes

FECs: Follicular epithelial cells

FOXL2: Forkhead box L 2

GCs: Granulosa cells

GCI: Granulosa cell invasiveness

GTA: Glutaraldehyde

HE: Hematoxylin-eosin

H₂O₂: Hydrogen peroxide

ICs: Interstitial cells

IHC: Immunohistochemistry

IFC: Immunofluorescence

LD: Long distance

MCs: Mitochondrial clouds

MOFs: Multi-oocyte follicles

NBF: Neutral buffer formalin

NDS: Normal donkey serum

NGS: Normal goat serum

OsO₄: Osmium tetroxide

p63: Protein (Tumor) 63

PAS-H: Periodic acid Schiff-hematoxylin

PB: Phosphate buffer

PCNA: Proliferative cell nuclear antigen

PF: Primary Follicle

PFA: Paraformaldehyde

PGCs: Proliferating germ cells

PrF: Primordial follicle

PNDs: Post-natal days

SD: Short distance

SF: Secondary Follicle

SH: *Sigmodon hispidus*

SNOs: Single nucleated oocytes

SOFs: Single oocyte follicles

TB: Tris buffer

TF: Tertiary follicle

TEM: Transmission electron microscopy

TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling

ZP: Zona pellucida

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Preface

Ovary is an important organ in the female reproductive system and the role in production of oocyte and hormones. Also, the ovary is responsible for reproduction, and act as a reservoir of follicles. This reservoir serves as the source of developing follicles throughout the reproductive lifespan. Depending on the species, the reservoir of follicles is set up in the ovaries of fetuses or neonates, or a sharp reduction in their number, incompatible with fertility in adults (Monniaux, 2018). The developing follicles from a reserve of primordial follicles (PrFs) constituted early in life and gradually emptied by both follicle growth activation and ovulation and follicle degeneration (Monniaux et al., 2014). However, the importance of good quality and quantity of primordial germ cell (PGC) reserves during folliculogenesis implies to obtain maximum fertilizable oocytes that plays a pivotal role in veterinary field such as superovulation and assisted reproductive technologies (ART) in mammalian species including humans.

Even though folliculogenesis is considered a key component of reproductive physiology, but there remain many unanswered questions in the recent research on folliculogenesis in mammals. It was established several decades ago that the follicles contain only a single oocyte having a single nucleus. However, some unclear aspects were recently observed for the process of folliculogenesis in some species, such as the occurrence of multi-oocyte follicles (MOFs) in the same ovary as single-oocyte follicles (SOFs). The presence of MOFs has been described in several animal species, showing several species-related frequency differences such as in cattle (Ireland et al., 2008; Silva-Santos and Seneda, 2011), dogs (Payan-Carreiraa and Pires, 2008; Reynaud et al., 2009), pigs (Greenwald and Moor, 1989; Stankiewicz et al., 2009), and sheep (Hadek,

1958; Oliveira et al., 2017a). Moreover, binuclear oocytes are usually found in possum (Hartman, 1926). Also, the reported prevalence of "polynuclear" follicles was 58% in women under the age of 20 years, whereas similar follicles were found in 13% of women aged 20 to 39 years, and none in the ovaries of women 40 years or older (Dandekar et al., 1988). Double nucleated cells can also be observed in other cell types, such as hepatocytes or trophoblasts (Donne et al., 2020). However, the development and fate of MOFs and other disturbances are still unclear.

Rodents such as mice and rats are frequently used species for studying infertility related issues because of their frequent estrus cycle and short pregnancy duration. Cotton rat (CR) is one the experimental rat species that have been used recently as a model for many diseases to mimic that in mammals (Blanco et al., 2014). CRs are the rodents belonging to the family *Cricetidae* (with hamsters and gerbils) and are distributed over North and South America. There are seven species of cotton rats; of these, only *Sigmodon hispidus* (SH) and *Sigmodon fulviventer* are used as laboratory animals, and SH is more commonly used compared with *Sigmodon fulviventer*. SH is by far the most widely distributed of the CR species, ranging across the southern United States, from Virginia to southeastern California, southward through Mexico and Central America into the tropical regions of northern America (Prince, 1994). There are 25 subspecies of SH. This subspecies is well characterized and is commonly used in biomedical research (Faith et al., 1997).

In CRs, the first estrus cycle starts between 20 and 30 days of age (puberty onset is at 30-40 days of age) and the estrus cycle averages 9 days (4-20 days; not stable), gestation length is 27 days, and hair growth is present at birth (Faith et al., 1997). Laboratory-reared CR females also undergo first estrus between 20 and 30 days of age

and generally attain puberty (the time when an individual becomes sexually mature) between 30 and 40 days of age. Although CR is prolific in nature and produces nine litters per year, averaging five to six pups per litter (Faith et al., 1997). Recent studies reported unique characteristics of CR intestine (Chuluunbaatar et al., 2020), pharyngeal pouch remnants (Nakamura et al., 2018), metabolic disorders (Nakamura et al., 2019), as well as an experimental model for renal anemia (Ichii et al., 2016), and kidney diseases (Ichii et al., 2018). However, the reproductive characteristics or ovarian development and their relationship with CR ovarian morphology remains unclear.

Therefore, this study, the ovarian morphology, especially folliculogenesis, of CR inbred strains, HIS/Hiph (from the Hokkaido Institute of Public Health) and HIS/Mz (from the University of Miyazaki), was intimately examined and their characteristics were clarified. In Chapter 1, the author revealed the developmental changes of the ovary in the neonatal HIS/Hiph, and found the appearance of MOFs and double nucleated oocytes (DNOs) “follicles with two nuclei”. In Chapter 2, the author examined the 4-week-old age as the time when the first estrus and puberty onset of CR are observed and clarified the CR strain-specific differences, especially focusing on MOF and DNO. In Chapter 3, the ovaries of CRs (HIS/Hiph, 6-8 weeks old) that had reached the fertile stage were scrutinized, and the author proposed the possible mechanisms and characteristics of abundance of MOFs and DNOs by using a histomorphology and morphometry and ultrastructure analysis.

Conclusively, follicular development and the formation of unique phenotypes implicate CR as a useful model animal to study folliculogenesis and oocytogenesis, as well as their follicular disturbances (MOFs/DNOs) in mammalian species including humans.

Contents of this thesis were published in the following articles.

1. Islam MR, Ichii O, Nakamura T, Irie T, Masum MA, Hosotani M, Otani Y, Namba T, Chuluunbaatar T, Elewa YHA, Kon Y. Developmental changes of the ovary in neonatal cotton rat (*Sigmodon hispidus*). *Front Physiol (Reproduction)* 2021, **11**: 601927.
2. Islam MR, Ichii O, Nakamura T, Irie T, Shinohara A, Masum MA, Otani Y, Namba T, Chuluunbaatar T, Elewa YHA, Kon Y. Comparison of ovarian morphology and follicular disturbances between two inbred strains of cotton rats (*Sigmodon hispidus*). *Animals (Animal Reproduction)* 2021, **11**: 1768.
3. Islam MR, Ichii O, Nakamura T, Irie T, Masum MA, Hosotani M, Otani Y, Elewa YHA, Kon Y. Unique morphological characteristics in the ovary of cotton rat (*Sigmodon hispidus*). *J Reprod Dev* 2020, **66**: 529-538.

Chapter 1

Developmental changes of the ovary in neonatal cotton rat

Introduction

Fertility and the entire reproductive lifespan of females depend on the limited and non-restorable reserves of oocytes in neonatal ovaries. Follicle assembly and development have been well documented in many mammalian species (Byskov and Lintern-Moore, 1973; Pepling and Spradling, 2001; Sawyer et al., 2002; Tingen et al., 2009). However, processes that occur at mid-gestation in humans (Hirshfield, 1991; Maheshwari and Fowler, 2008) and during the first postnatal days (PNDs) in rodents remain unclear (Pepling, 2012; Tingen et al., 2009). The germ cell nest is an important developmental stage in the formation of the germline. The nest is evolutionarily conserved in males and females of species ranging from higher insects to frogs, rodents, and other vertebrates (Pepling et al., 1999). In each of these organisms, most of the single PGCs divide synchronously with incomplete cytokinesis or nuclear divisions to form a cluster of cells connected by intercellular bridges (Gondos et al., 1971; Pepling and Spradling, 1998). Nest breakdown involves the degeneration of many germ cell nuclei and the invasion of the pre-granulosa cells (GCs) into the germ cell nests (Pepling and Spradling, 2001). After breakage, the majority of ovarian follicles enclose only a single oocyte, although reports have described two or more oocytes within a single follicle in the ovaries of several mammalian species (Balciuniene et al., 2006; Gondos et al., 1971; Hartman, 1926; Lucci et al., 1999; Payan-Carreira and Pires, 2008; Stankiewicz et al., 2009; Smits and Cortvrindt, 2002; Yang and Fortune, 2008). Furthermore, single oocytes or multi-oocytes are surrounded by a single layer of flattened squamous or multi-layer cuboidal somatic cells termed GCs. The theca cell layer starts to develop from secondary follicles (SFs). Less attention has been paid to

the role of theca cells in follicular function compared to that of GCs (Tajima et al., 2007). However, the communication between oocytes and GCs along with theca cell layers leads to the survival of normal follicles, and its disturbances.

In this study, the author investigated the morphological characteristics and the mechanisms of development of CR subspecies of neonatal SH ovary at PND0 to PND7 maintained at Hokkaido Institute of Public Health (HIS/Hiph) by using histological analysis. In this chapter, the author found that the general histology and folliculogenesis in HIS/Hiph ovaries were similar to those in other mammals. Moreover, HIS/Hiph have developed unique phenotypes such as DNOs, and MOFs. DNOs observed in all categories of ages, tended to be frequently observed in primordial ones whether MOFs were started to develop from PND4 with greater frequency in primary ones. These findings suggest that the appearance of DNOs and MOFs in the neonatal HIS/Hiph ovary serves as a useful model to better understand folliculogenesis and oocytogenesis as well as their follicular abnormalities in mammalian species including humans.

Materials and Methods

Ethical statements

This study was approved by the Institutional Animal Care and Use Committee of the Graduate School of Veterinary Medicine, Hokkaido University (approval no. 20-0012) and Hokkaido Institute of Public Health (Sapporo, Japan; approval no. K27-03). The authors adopted to *The Guide for the Care and Use of Laboratory Animals of Hokkaido University, Graduate School of Veterinary Medicine* (approved by the Association for Assessment and Accreditation of Laboratory Animal Care International) and Hokkaido Institute of Public Health.

Animals and tissue processing

SH were maintained as the HIS/Hiph strain through continuous inbreeding under conventional conditions at the Hokkaido Institute of Public Health. Another, outbred Jcl:ICR mice was purchased from Japan SLC Inc. (Hamamatsu, Japan). All mice were maintained under specific pathogen-free conditions. Food and water were provided *ad libitum*.

Twenty-four female HIS/Hiph (eight in each age group), and 15 female ICRs (five in each age group), and their ovaries were collected at PND0, PND4, and PND7. Each tissue was fixed in 10% neutral buffer formalin (NBF), 4% paraformaldehyde (PFA), or 2.5% glutaraldehyde (GTA) in 0.1 M phosphate buffer (PB) for histological analysis, immunohistochemistry and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays, and ultrastructural analysis.

Histological analysis

The paraffin blocks of 5 ovaries from each age group of HIS/Hiph were cut to a thickness of 2 μm to obtain whole ovarian sections. The sections were stained with hematoxylin-eosin (HE) or periodic acid Schiff-hematoxylin (PAS-H) to examine ovarian morphology in neonatal HIS/Hiph. The stained sections mounted on glass slides were scanned using a NanoZoomer 2.0 RS virtual slide scanner (Hamamatsu Photonics, Shizuoka, Japan). The data were used for histoplanimetry. Microscopy examination was done using a model BZ-X710 microscope (Keyence, Osaka, Japan) to obtain the histological images.

For histoplanimetry using HE-stained sections, ovarian follicles were classified, either in nest as cluster form or based on their stages, as PrF, primary (PF), SF, and tertiary follicles (TF), according to previously reported studies in rabbits (Al-Mufti et al., 1988). Their numbers were counted every five sections. The number of various developing follicles was multiplied by five to determine the total number of follicles per ovary (Gaytán et al., 2014; Su et al., 2013). Only follicles containing visible oocytes were counted to avoid double counting. The number and percentages of developing follicles were counted from the total number of follicles, excluding nests. PrFs had an oocyte surrounded by a single layer of flattened follicular epithelial cells (FECs). PFs were identified as oocytes enclosed by a layer of GCs. SFs contained two or six layers of GCs surrounding the oocyte. TFs with small follicular spaces between FECs began to form, and these follicles were composed of more than six GC layers.

The developmental stage of MOFs and DNOs were enumerated. The percentage of MOFs or DNOs among all follicles was also calculated.

Immunofluorescence

The oocyte marker DEAD-box helicase 4 (DDX4) and the FEC marker Forkhead box L2 (FOXL2) were analyzed to determine whether the oocytes of HIS/Hiph, in particular those in MOFs or DNOs. Staining conditions are listed in Table 1-1. Briefly, DDX4 and FOXL2 were expressed in germ cells and FECs from the time of their first appearance to adulthood in mice (Yamashita et al., 2015). Deparaffinized sections were treated with 10 mM citrate buffer for 20 min at 105°C for antigen retrieval. These sections were then blocked with 5% normal donkey serum (NDS) (Sigma-Aldrich, St. Louis, MO, USA). They were incubated overnight with rabbit anti-DDX4 (1:500 dilution; ab13840; Abcam, Cambridge, UK) and goat anti-rabbit FOXL2 antibody (1:1000; ab5096; Abcam) at 4°C. The sections were incubated for 1 h at room temperature with Alexa Fluor-488 labeled donkey anti-rabbit IgG (1:500, Life Technologies, Carlsbad, CA, USA) and Alexa Fluor-564 labeled donkey anti-goat IgG (1:500, Life Technologies) for DDX4 and FOXL2 double immunofluorescence. The sections were counterstained with Hoechst 33342 (1:200; Dojindo, Kumamoto, Japan). The stained sections were examined using a BZ-X710 microscope (Keyence).

Immunohistochemistry

For immunohistochemical staining of Ki67 and conditions are listed in Table 1-1, deparaffinized sections were treated with 10 mM tris buffer for 10 min at 121°C for antigen retrieval, and incubated in 0.3% hydrogen peroxide (H₂O₂)/methanol solution for 10 min to quench endogenous peroxidase activity. The sections were then blocked with 10% normal goat serum (NGS) using the SABPO kit (Nichirei Bioscience, Tokyo, Japan) for Ki67 staining. Sections were incubated overnight with rabbit anti-Ki67

antibody (1:800; ab15580; Abcam) at 4°C. This antibody has been reported to show positive immunoreactivity in rat GCs (Urata et al., 2020). Negative controls were run with normal rabbit IgG (sc-2027; Santa Cruz Biotechnology, California, USA). The sections were then treated with biotinylated goat anti-rabbit IgG using the SABPO kit (Nichirei Bioscience) for 30 min at room temperature. This was followed by incubation with streptavidin-horseradish peroxidase using the SABPO kit (Nichirei Bioscience) for 30 min, followed by incubation with 3,3-diaminobenzidine tetrahydrochloride (DAB) in H₂O₂. Finally, the sections were counterstained with hematoxylin and dehydrated using ascending grades of alcohol. The stained sections were examined using a BZ-X710 microscope (Keyence).

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

Apoptosis was assessed by TUNEL assay using the *In Situ* Apoptosis Detection Kit (ab206386; Abcam). Neonatal ovaries (five in each age group) were fixed in 4% PFA, embedded in paraffin, and sectioned to a thickness of 3 µm. The five largest HIS/Hiph and ICR cross sections (fifteen µm interval) were placed in a slide from five ovaries of each age group at PND0, PND4, and PND7. The staining procedure was performed according to the manufacturer's instructions. Briefly, after deparaffinization, the sections were incubated with proteinase K at room temperature for 20 min. Endogenous peroxidase was inactivated by 3% H₂O₂/methanol for 5 min. The slides were incubated with TUNEL reaction mixture in a humidified chamber at 37°C for 90 min, followed by washing with PBS. The slides were incubated with a converter anti-fluorescein antibody conjugated with anti-fluorescein isothiocyanate horseradish peroxidase at 37°C for 30 min. After washing with PBS, the immunoreaction was visualized by incubating with

DAB in H₂O₂. Sections were counterstained with methylene green and incubated for 1-3 min. The stained-glass slides were scanned using a NanoZoomer 2.0 RS virtual slide scanner (Hamamatsu Photonics). A model BZ-X710 microscope (Keyence) was used to obtain the images. For TUNEL histoplanimetry, the number of positively stained oocytes was counted and averaged using five sections of different stages of each ovary of HIS/Hiph and ICR mice.

Electron microscopy

HIS/Hiph ovaries (three ovaries from each age group) were collected and immediately fixed with 2.5% GTA in 0.1 M PB for 4 h at 4°C, followed by post-fixation with 1% osmium tetroxide (OsO₄) in 0.1 M PB for 2 h. The specimens were dehydrated with ascending grades of alcohol and embedded in epoxy resin (Quetol 812 Mixture; Nisshin EM, Tokyo, Japan). The epoxy blocks were cut to a thickness of 60 nm. Ultrathin sections were mounted on grids and stained with uranyl acetate and lead citrate for 15 min and 10 min, respectively. Transmission electron microscopy (TEM) of the stained sections was done using a model JEM-1210 transmission electron microscope (JEOL, Tokyo, Japan).

Statistical analyses

The results are expressed as mean $\pm$ standard error. Results were analyzed using nonparametric methods. Two groups were compared using the Mann-Whitney *U*-test ($P < 0.05$ and $P < 0.01$). The Kruskal-Wallis test was used to compare the developmental stages of three or more follicle groups with MOFs or DNOs, and multiple comparisons were performed using the Scheffé's method ($P < 0.05$ and $P < 0.01$).

Results

Developing follicles in the neonatal HIS/Hiph ovary

Figure 1-1 presents results of the histological observation of the neonatal HIS/Hiph ovary at PND0, PND4, and PND7. At PND0, the developing follicles were dispersed throughout the ovary as PrF and PF, with most residing at the surface (Fig. 1-1a). At PND4, some follicles were surrounded by squamous FECs and were classified as PrF. A few of them were surrounded by a single layer of round cuboidal FECs and were classified as PF (Fig. 1-1b). With age, HIS/Hiph displayed increased numbers of PrF and PF (Fig. 1-1b and 1-1c). At PND7, the follicles had progressed to developmental stages PrF and PF, and several SFs surrounded by two or more layers of cuboidal GCs appeared in the deep cortex area (Fig. 1-1c).

Next, the author quantified the ovarian follicles at each developmental stage according to the morphology of the FEC and GC layers as mentioned in the Materials and Methods section and described elsewhere (Al-Mufti et al., 1988). Developing follicles up to SF were observed, but TF was not observed for any age. Histoplanimetry revealed a significantly higher total number of oocytes at PND0 (3159.8 ± 28.3), including those in nests, compared with the other ages ($P < 0.01$). Upon reaching adulthood, the ovaries of common mouse strains contain follicles of varying stages of development in the order of 3,000 to 5,000 (Tilly, 2003). These numbers significantly decreased with age (Fig. 1-1d). For the number and percentage of follicles at each developmental stage, PrF showed significantly higher values ($P < 0.01$) than PF and SF at all examined ages (Fig. 1-1e and 1-1f). For PrF, the number of follicles was

significantly higher ($P < 0.01$) at PND4 than at other ages (Fig. 1-1e). Furthermore, the number of PF and SF significantly increased ($P < 0.01$) with age (Fig. 1-1e). The percentage of PrF was significantly higher at PND4 and PND7 than at PND0 ($P < 0.01$), and that of PF was significantly higher at PND7 than at PND0 and PND4 ($P < 0.01$), but there was no significant difference in SF (Fig. 1-1f).

Development of DNOs in neonatal HIS/Hiph ovary

The author found unique characteristics as DNOs along with the developing follicles of neonatal HIS/Hiph ovary. The appearance of DNOs varies among developing follicles. At PND0 and PND4, DNOs were observed in the PrF (Fig. 1-2a and 1-2b). With age, at PND7, the author observed DNOs in PrF, PF, and SF (Fig. 1-2c). Most of the PrF-containing DNOs appeared in the periphery of the ovary, with others distributed throughout the ovary. In histomorphology, PrF DNOs tended to be frequently observed rather than PF, and SF DNOs. The PrF DNOs might be the result of fusion of two individual oogonia during nest breaking, and PF, and SF DNOs might be the result of nuclear division of oocytes without cytoplasmic cleavage during folliculogenesis.

The histoplanimetric data for DNO are shown in Figure 1-2d and 1-2e. Most DNOs appeared in the early developmental stages of folliculogenesis. At PND7, DNOs appeared in the PrF, PF, and SF. The percentage of DNOs in PrF at PND0 to PND7 tended to be higher than that in other developing follicles. A significant difference in the number of DNOs was observed between PrF and both PF and SF at PND7 ($P < 0.05$).

Development of MOFs in neonatal HIS/Hiph ovary

MOF was another important unique feature of the neonatal HIS/Hiph ovary. At

PND0, oocyte clusters and follicles dispersed throughout the ovary were evident. Therefore, the author did not consider MOFs at PND0. Subsequently, MOF, defined as a single follicle containing two or more oocytes, appeared at PND4 and PND7 in PrF, PF, and SF (Fig. 1-3a and 1-3b), respectively.

For histoplanimetry, MOFs classified as PF were most numerous in both PND4 and PND7 (Fig. 1-3c). A significant difference was observed in PF MOFs compared with PrF and SF MOFs at PND7 ($P < 0.01$). SF MOFs showed significantly higher percentages than PrF MOFs at PND7 ($P < 0.01$; Fig. 1-3d). The MOF number and percentages showed significant difference within follicle class at PND7 compared with that in PND4 ($P < 0.01$; Fig. 1-3c and 1-3d).

Most MOFs had two oocytes in a single follicle. However, some displayed three or more oocytes in a single follicle (Fig. 1-4). The sizes and shapes of MOFs varied compared to SOF as well as the developing follicles containing MOFs due to the increased number of oocytes and FECs/GCs. Moreover, morphological variation was observed in different developing follicles during folliculogenesis. Two or more oocytes were observed attached or joined to each other as PF to PF (Fig. 1-4a: PND4), PF to SF (Fig. 1-4d: PND7) by GCs expansion. Some MOFs showed the follicle spheres linked by a bridge of GCs (Fig. 1-4c: PND4 and 1-4e-f: PND7). Examination of semi-serial sections at PND7 revealed that the apparently single follicles or MOF follicles (Fig. 1-4g) in one section were connected to those of the next sections (Fig. 1-4h and i) by GCs. Further, some MOFs (Fig. 1-4j-l) and SOFs (Fig. 1-4j) at PND7 had irregular outlines showing bulging of the follicle surface within the basal lamina (BL).

Characterization of SOFs, MOFs, and DNOs in neonatal HIS/Hiph ovary

The author examined the oocyte and follicular development stages in neonatal HIS/Hiph ovaries at PND0, PND4, and PND7 using double immunofluorescence of the DDX4 oocyte marker and FOXL2 FEC marker (Fig. 1-5a-c). Immunoreactivities of DDX4 and FOXL2 were detected in the cytoplasm of oocytes and nuclei of FECs, respectively, throughout the observation period.

At PND0, numerous DDX4-positive oocytes were observed in the periphery of the cortices (Fig. 1-5a). Additionally, PrF and a few PFs surrounded by squamous and cuboidal FOXL2-positive FECs were observed in the superficial and deep cortices. The PF and very few SFs surrounded by cuboidal FECs observed at the inner cortices and MOFs were first detected. They shared a common follicular area (Fig. 1-5b) in the PND4 HIS/Hiph ovary. Unlike PND4, SFs with two or more layers of cuboidal GCs and MOFs were observed at PND7 (Fig. 1-5c).

However, Ki67 staining demonstrated the proliferative activity of GCs, but not of oocytes, in the SOFs and MOFs (Fig. 1-6a-c). During the development of follicles, proliferation increased when flattened squamous GCs were converted to cuboidal GCs. As the majority of follicles were surrounded by flattened squamous GCs at PND0, we hypothesized that the formation of MOF was absent. With age, proliferation increased in the developing follicles, combined with rapid folliculogenesis, and formation of MOFs was observed at PND4. However, proliferative activity was absent in oocytes, so the formation of DNOs was unclear.

Apoptosis in neonatal HIS/Hiph ovary compared with other rodents (mice)

The author also observed the developing oocytes at PND0, PND4, and PND7 by the TUNEL assay. This experiment was done with the hypothesis that oocyte apoptosis is

related to the formation of unique characteristics of the ovary in HIS/Hiph. A few TUNEL-positive apoptotic oocytes in neonatal HIS/Hiph were observed at PND0. They were scarce in PND4 and PND7 (Fig. 1-7a). Conversely, ICR mice had numerous TUNEL-positive apoptotic oocytes at PND0, which decreased with age (Fig. 1-7a). The proportion of apoptotic oocytes in HIS/Hiph compared with that in ICRs could play a role in the formation of MOFs.

The number of TUNEL-positive apoptotic oocytes decreased with age in both HIS/Hiph and ICR mice, and was significantly higher in neonatal ICR mice than in neonatal HIS/Hiph ($P < 0.01$; Fig. 1-7b). Within age, the number was also significantly higher in ICR mice compared with that in HIS/Hiph ($P < 0.01$).

Ultrastructural features of neonatal HIS/Hiph ovaries

Results of TEM analysis of follicles and oocytes in the neonatal HIS/Hiph ovary are presented in Fig. 1-8. SOF containing single nucleated oocytes (SNOs) are shown in Fig. 1-8a-c. SOFs classified as PrF had SNO-containing mitochondrial clouds (MCs) dispersed throughout the cytoplasm that were surrounded by squamous FECs at PND0 (Fig. 1-8a). In SOFs classified as PF, SNOs were surrounded by cuboidal FEC (or GCs) at PND4 and PND7 (Fig. 1-8b and 1-8c). A clear zona pellucida (ZP) was observed with larger PFs at PND7 (Fig. 1-8c). These SNOs contained MCs and occasionally showed polarity in their cytoplasm at PND4 (Fig. 1-8b).

MOF SNOs classified as PrF at PND4 (Fig. 1-8d) and PF at PND7 (Fig. 1-8e) were surrounded and separated by squamous and cuboidal FECs, respectively. The author also determined the GC expansion characteristics, which were the most important factors in the formation of MOFs at PND7 (Fig. 1-8f).

In PrF, double nuclei were separated by rough endoplasmic reticulum (RER) at PND0 (Fig. 1-8g). In PF, MCs were localized between double nuclei at PND7 (Fig. 1-8h). No DNOs were detected at PND4 by TEM.

Discussion

Ovarian development and function depend on somatic cell-oocyte communication, which is also important for follicle integrity. The breaking of germline cysts and establishment of the PrF pool for future recruitment are coordinated by the oocyte and somatic cell identity and their interactions (Matzuk et al., 2002; Tingen et al., 2009). The mechanisms by which cell–cell communication networks are established between pre-GCs and oocytes within new PrFs remain unknown. Two distinct populations of PrFs (medullary and cortical) exist in the postnatal ovary. The medullary PrFs begin to grow as soon as they are formed, whereas the cortical PrFs mature gradually over the reproductive lifespan of the animal (Hirshfield, 1992; Zheng et al., 2014). In this study, the author observed the neonatal HIS/Hiph ovaries at PND0 to PND7, where the most characteristic features of DNOs, MOFs, and fewer apoptotic oocytes were found along with the development of typical follicles. The presence of MOFs in adult ovaries has been reported in several mammals (Ireland et al., 2008; Lucci et al., 1999; Payan-Carreira and Pires, 2008). The development of neonatal HIS/Hiph-specific DNOs and the abundance of MOFs have been clarified as unique features during nest-breaking and folliculogenesis, compared with other mammalian species.

Four different hypothetical mechanisms may explain the formation of DNOs and MOFs. The first involves DNOs, via fusion of two individual follicles during nest breaking or nuclear division of oocytes without cytoplasmic cleavage occurring during folliculogenesis. Secondly, concerning MOFs, encasement of two or more oocytes into PrF, PF, and SF may occur during follicle assembly. Thirdly, and also for MOFs, invasiveness or expansion of GCs within the follicles may occur. Finally, for MOFs,

oogonia transformation to oocytes may occur or the number of apoptotic oocytes may be less.

The author found DNOs in all developing stages of the follicle, but tended to be numerous in PrF. Double nucleated cells were also observed in other tissues, such as hepatocytes or trophoblasts. These double nuclei indicated their active transcriptional activity (Donne et al., 2020). The volume of hepatocytes in human and mouse livers is approximately doubled with the doubling of DNA content, resulting from cytokinesis failure that occurs progressively during the course of postnatal development (Martin et al., 2002). DNO formation may be attributed to the fusion of two individual oogonia during nest-breaking, nuclear division without cytoplasmic cleavage during folliculogenesis, or other unknown factors. Moreover, proliferative activity was absent in the oocytes of neonatal HIS/Hiph, as indicated by Ki67 staining. There have been no reports of DNOs in other mammals, except polynucleated oocytes in humans (Dandekar et al., 1988). However, binuclear oocytes are usually found in opossums (Hartman, 1926). Based on these findings, the author hypothesizes that DNOs in HIS/Hiph form due to fusion of two individual oogonia during nest breaking or nuclear division of oocytes without cytoplasmic cleavage occurring during folliculogenesis.

The author found another unique characteristics as MOFs in neonates at PND4 and PND7. PND0 was excluded because follicles that were clustered or dispersed throughout the ovary appeared to be MOFs. The presence of MOFs in adult ovaries has been reported in mammals (Ireland et al., 2008; Lucci et al., 1999; Payan-Carreira and Pires, 2008). The present data show that the number and percentages of MOFs increased from PND4 to PND7. This observation strongly supports the hypothesis that the vast majority of MOFs found in peripubertal HIS/Hiph are generated by connection or

fusion of adjacent growing follicles. Oogonia can proliferate in fetal mammals, and most of develop into oocytes by the initiation of the first meiotic division. Oogonia divide synchronously with intercellular bridges due to incomplete cytokinesis (Pepling et al., 2007). The bridges generally disappear in oocytes. Therefore, dysregulated cell division during oocytogenesis may also contribute to the formation of MOFs. It has been proposed that MOFs may be derived from the failure of oocyte nest breakdown during the early stages of folliculogenesis (Lucci et al., 1999; Oktem and Urman, 2010; Oliveira et al., 2017a; Safran et al., 1998; Zeilmaker et al., 1983). However, in HIS/Hiph at PND4, nest-breaking appeared to be almost complete, implying the separation of the nest to each follicle dispersed throughout the ovary. Therefore, the dysregulation of this process might also contribute to the production of MOFs in HIS/Hiph at PND4 and PND7.

Another factor may be the encasement of multiple oocytes in PrF during folliculogenesis due to their more rapid developmental rate compared to the differentiation of the surrounding somatic cells (Balciuniene et al., 2006; Kennedy, 1924; Lucci et al., 1999; Telfer and Gosden, 1987). Furthermore, the presence of irregularly shaped follicles with bulges and projections of the follicle surface, follicles with breaches of the follicle wall with apparent migration of GC into the ovarian stroma, and linked follicles sharing GCs is suggestive of follicle joining (Fig. 1-4g-l). Moreover, irregular outlines of MOF showed the bulging of the follicle surface within the BL (Fig. 1-4j-l). Oocytes, GCs, and theca cells form follicle units within the ovary with BL separating the granulosa and theca cells compartments. Signaling between all three of these cell types is essential to support follicle development, oocyte development, and ovulation (Christensen et al, 2017). The FOXL2-positive FECs showed that the

projection of cells started at PND4. These observations strongly suggest a sequence of events responsible for follicle fusion. According to such morphologic indices, it can be hypothesized that the formation of bulges on the surface of some growing follicles and the eventual breaching of the follicle wall, which allows GCs to invade the perifollicular stroma, constitute the first steps for MOF generation. However, although the proliferative activity of MOFs has been described in mammals (Oliveira et al., 2017b), Ki67 staining presently demonstrated proliferative activity of GCs, but not of oocytes, in the MOFs of HIS/Hiph. A recent study suggested the invasive capacity of GCs, which could contribute to the connection of the follicles and the generation of the MOFs in the peripubertal rat ovary (Gaytán et al., 2014). Based on these considerations, it is likely that MOFs form either by fusion or GC expansion/invasiveness (GCI) during the development of follicles.

Morphologically, apoptosis is found in ovarian follicles throughout fetal and adult life (Arends et al., 1990). Apoptosis is an essential component of ovarian function and development. However, in neonatal HIS/Hiph, the number of TUNEL-positive apoptotic oocytes was comparatively lower than that in other rodents (e.g., ICR mice). The proportion of apoptotic oocytes in HIS/Hiph compared to that in ICR could play a role in the formation of MOFs. Cells with a high regeneration and division rate, and cells under endocrine control are particularly susceptible to apoptosis (Wyllie, 1987). Therefore, germ cells, especially oogonia, are prone to be involved in apoptosis (Qi et al., 2008). Germ cell proliferation and apoptosis during the period of development leads to PrF formation (Fulton et al., 2005), but a lower number of apoptotic cells in the ovary may stimulate the formation of MOFs.

The thickness of the ZP in mammals is usually similar among oocytes

(Payan-Carreira and Pires, 2008). MCs tended to localize throughout the cytoplasm of SOFs, MOFs, and DNOs. These MCs have been termed Balbiani bodies (Bbs). Bbs contain large RNA-protein granules and are universally conserved in the oocytes of insects (Jaglarz et al., 2003), fish (Marlow and Mullins, 2008), rodents (Pepling et al., 2007), and humans (Albamonte et al., 2013). Bbs are transient structures, as they only exist in the dormant oocytes and are dispersed once the oocyte is activated (Pepling et al., 2007). Although the functional significance of Bbs in CRs is unclear, the polarity of Bbs in *Xenopus* oocytes is associated with germinal granules responsible for the determination of germ cell fate (Kloc et al., 2004). Light microscopy revealed nuclear polarity in MOF oocytes. Ultrastructurally, the polarity of MOF oocytes appeared to be caused by the aggregation of mitochondria in the cytoplasm.

The author summarized the findings of neonatal HIS/Hiph ovaries in Fig. 1-9. Clusters of PGCs and apoptosis both decreases, but the proportion of MOFs increases with age. The follicles develop up to the secondary stage, with the formation of DNOs and MOFs. A lower number of apoptotic cells indicates rapid folliculogenesis. The rate of MOFs increases with age, resulting in the mechanism of fusion or GC expansion. On the other hand, DNOs decrease, but are observed in developing follicles with age, because of nuclear division without any cytoplasmic cleavage.

Summary

Perinatally, the follicle population resides either in the nest or in the form of PrFs. After birth, within few days, the cyst breaking is completed, and the oocytes are lost through cell death including apoptosis. Therefore, the author investigated follicular development and its disturbances during folliculogenesis in the neonatal ovary of HIS/Hiph up to 7 days of age using light microscopy and TEM.

The neonatal HIS/Hiph ovaries showed most oocytes in the form of PrFs at PND0, after nest breaking almost completed at PND4, then follicles developed up to secondary stages at PND7. The number of follicle populations decreased with ages by cell death. Also, the reserve of developing follicles was more in neonate HIS/Hiph compared with other rodents owing to fewer apoptotic oocytes. The author found oocyte with two nuclei, two or more than two oocytes in a SOFs, and described as DNOs and MOFs during histological observation. The DNOs were observed at days of birth and were found in all stages of developing follicles, but the frequency was decreased with ages. The MOFs started to develop at PND4, and were found in all stages of developing follicles, and the frequency was increased with ages. Moreover, the proliferative activities were also observed only in GCs SOFs, DNOs, and MOFs. In TEM analysis, SOFs, MOFs, and DNOs revealed the MCs distributed throughout the cytoplasm of oocytes.

Therefore, these results revealed that the changes of follicular development depend on ages, and thus changes found DNOs and MOFs were the unique characteristics of neonatal HIS/Hiph.

Tables and Figures

Table 1-1: Summary of immunostaining conditions used for the analysis of ovarian follicles.

Target	Marker	Antigen retrieval	Blocking	Primary antibody	Secondary antibody
Oocyte	DDX4	CB 115°C, 15 min	5% NDS 10% NGS	Rabbit anti-DDX4 antibodies (Abcam, Cambridge, UK) 1: 500	Alexa Fluor 546 donkey anti-mouse IgG antibodies (1:500; life Technologies) for IFC Goat anti-rabbit (SABPO kit, Nichirei) 1:100 (Biotinylated) for IHC
Granulosa cells	FOXL2	CB 115°C, 15 min	5% NDS	Goat anti-FOXL2 antibodies (Abcam, Cambridge, UK) 1:1000	Alexa Fluor 546 donkey anti-goat IgG antibodies (1:500; life Technologies) for IFC Donkey anti-goat IgG antibody (1:400; Santa Cruz, Dallas, TX, USA) (Biotinylated) for IHC
Proliferative cells	Ki67	TB 121°C, 10 min	10% NGS	Rabbit anti-Ki67 antibodies (Abcam, Cambridge, UK) 1: 800	Goat anti-rabbit (SABPO kit, Nichirei) 1:100 (Biotinylated) for IHC

DDX4: DEAD- box helicase 4, FOXL2: Forkhead box L2, CB: Citrate buffer (pH 6.0), TB: Tris buffer (pH 9.0), NGS: Normal goat serum, NDS: Normal donkey serum, IFC: Immunofluorescence and IHC: Immunohistochemistry

Figure 1-1. Follicular development in the neonatal ovary of HIS/Hiph

(a-c) Histological observation of hematoxylin and eosin-stained sections in the ovary of neonatal HIS/Hiph at postnatal day (PND)0 (a), PND4 (b), and PND7 (c). The inset figure indicates the primordial follicles (PrF) (arrow), primary follicles (PF) (arrowhead), and secondary follicles (star). The higher magnification indicates the same square area in panels a, b and c. Bars = 100 and 50 μm (inset). (d) The total number of developing follicles, including clusters or nests, in the neonatal HIS/Hiph ovary at PND0, PND4, and PND7. Values are presented as the mean $\pm$ standard error. * indicates significant difference in the total number of developmental follicles at PND0, PND4, and PND7 (Kruskal-Wallis test followed by the Scheffé method, $*P < 0.05$ and $**P < 0.01$). (e and f) Total number and percentage of each developing follicle classified as PrF, PF, and SF in the neonatal HIS/Hiph ovary at PND0, PND4, and PND7. Values indicate the mean $\pm$ standard error. * indicates significance over time within a follicle type at PND0, PND4, and PND7 (Kruskal-Wallis test followed by the Scheffé method, $*P < 0.05$ and $**P < 0.01$). Pr, P, and S denote the significance compared with PrF, PF, and SF, respectively, at the same ages (Kruskal-Wallis test followed by the Scheffé method, $P < 0.05$). Apostrophes indicate a highly significant difference ($P < 0.01$). ND: not detected. N = 5 for each age.

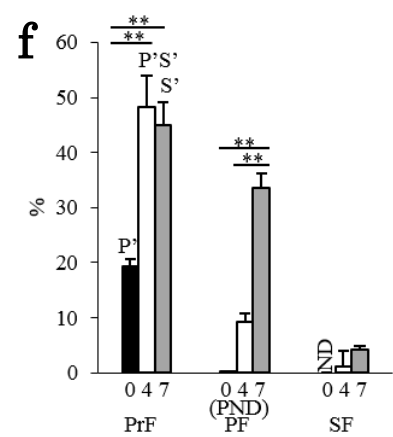
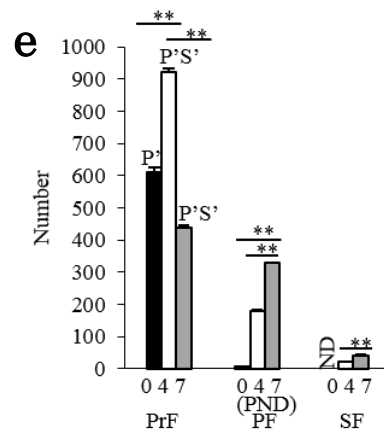
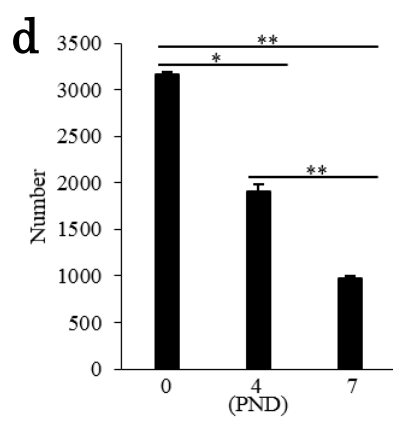
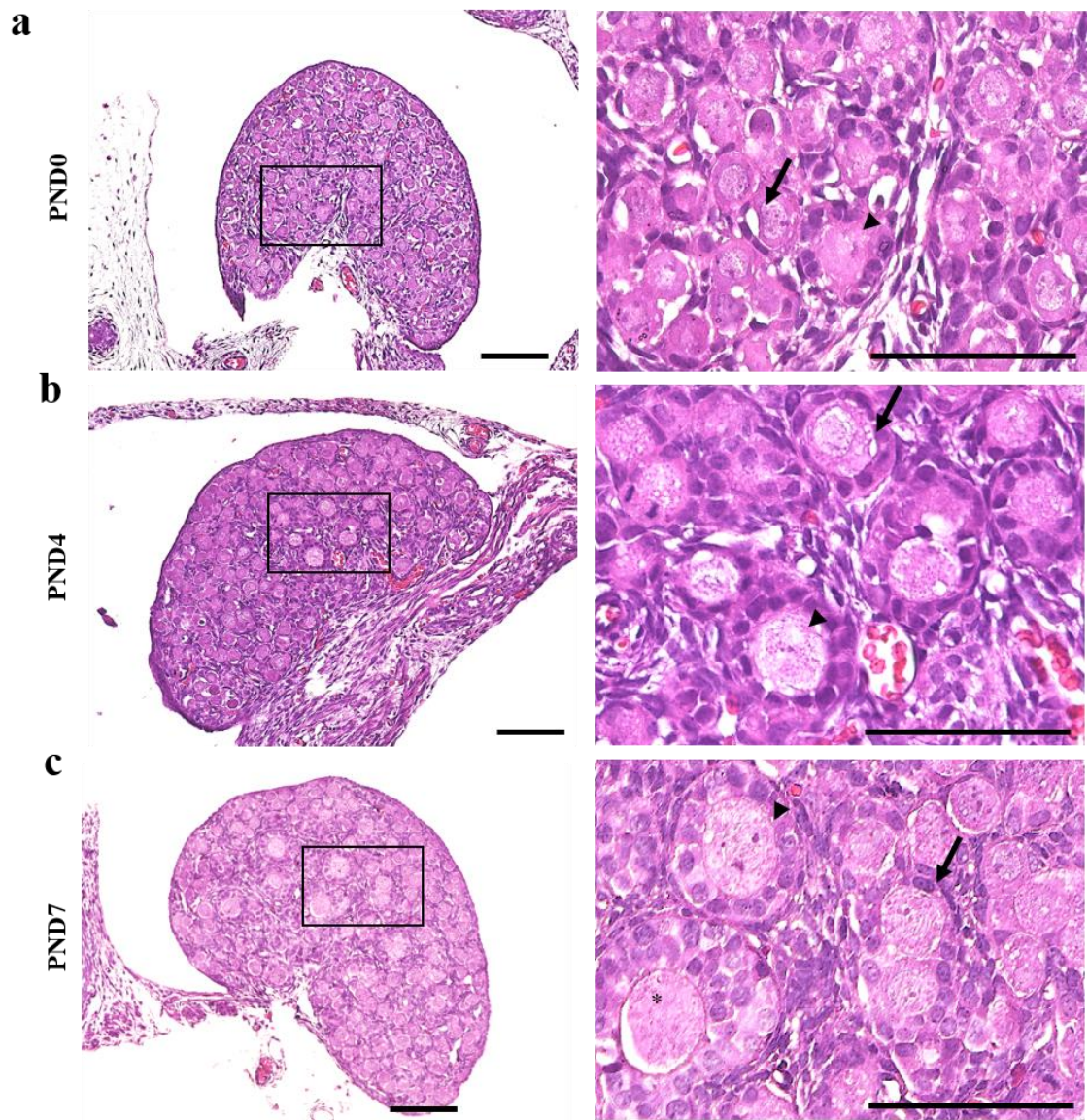


Figure 1-2. Development of double nucleated oocytes in the ovary of HIS/Hiph

(**a-c**) Histological observation of hematoxylin and eosin-stained sections in the neonatal ovary of HIS/Hiph at postnatal day (PND)0 (a), PND4 (b), and PND7 (c). Two nuclei are observed within a single oocyte follicle (SOF) classified as primordial follicle (PrF) (Panels a: PND0; b: PND4), primary follicle (PF), and secondary follicle (SF) (panel c: PND7), respectively. Each nucleus (asterisk) shares the same cytoplasm. Bar = 100 and 50 μm (inset). (**d and e**) The total number and percentage of double nucleated oocytes (DNOs) classified as each developing follicle in neonate HIS/Hiph ovaries at PND0, PND4, and PND7. Values are the mean $\pm$ standard error. Pr, P, and S denote the significance compared with PrF, PF, and SF, respectively, at PND7 (Kruskal-Wallis test followed by the Scheffé method, $P < 0.05$). ND: not detected. N = 5 at each age.

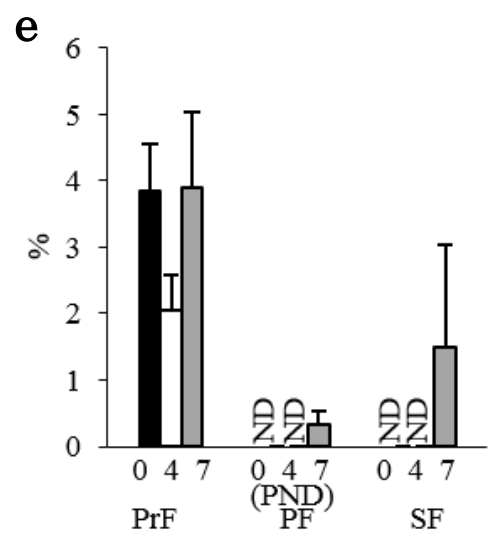
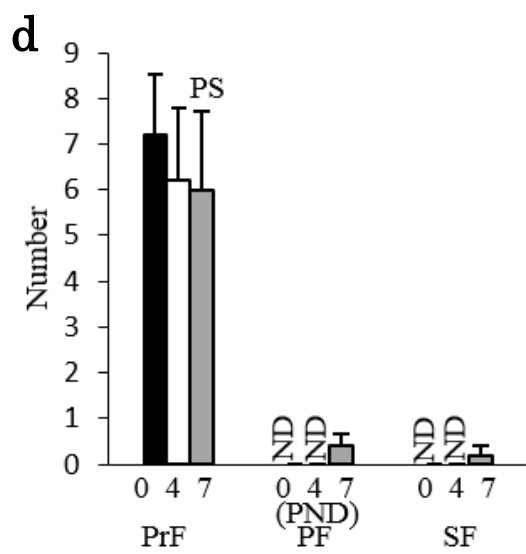
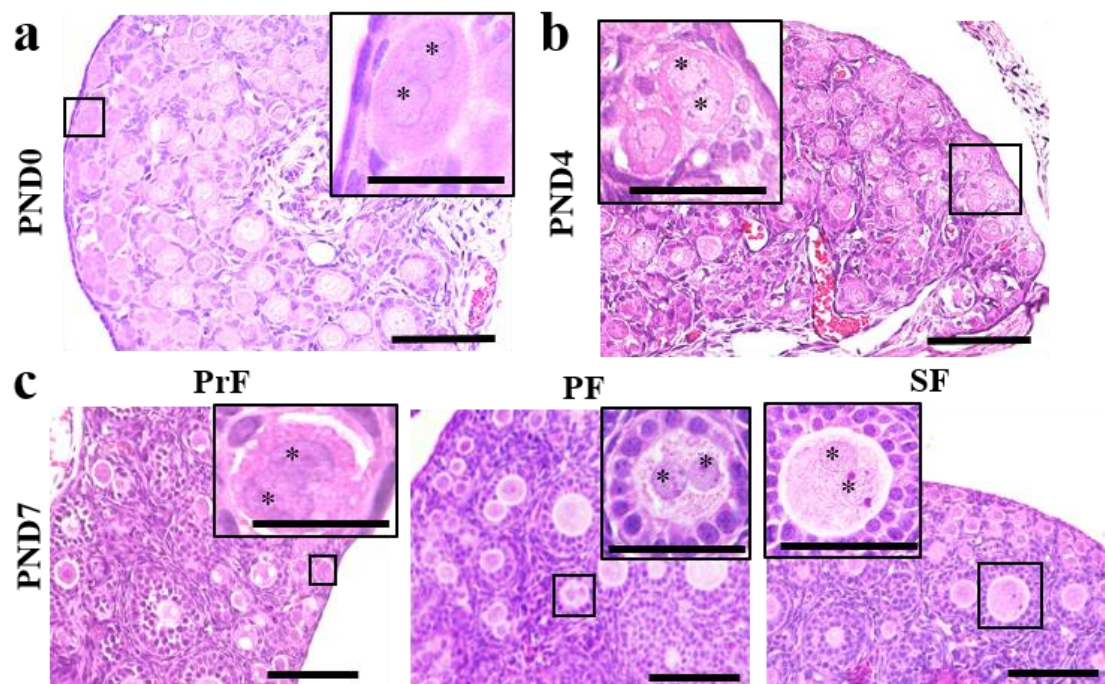


Figure 1-3. Development of multi-oocyte follicles in the neonatal ovary of HIS/Hiph

(a and b) Histological observation of hematoxylin and eosin-stained sections in the neonatal ovary of HIS/Hiph at postnatal days (PND)4 (a) and PND7 (b). Multi-oocyte follicles (MOFs) denoted by the arrows were observed in follicles classified as primordial follicle (PrF), primary follicle (PF), and secondary follicle (SF) (Panels a-b) at PND4 and PND7, respectively. Bars = 100 and 50 μm (inset). (c and d) The total number and percentage of MOFs classified as each developing follicle in the neonate HIS/Hiph ovary at PND4 and PND7. Values the mean $\pm$ standard error. * indicates significant differences between PND4 and PND7 within follicle categories (Mann-Whitney *U*-test, $*P < 0.05$ and $**P < 0.01$). Pr, P, and S denote the significance with PrF, PF, and SF, respectively, at PND4, and PND7 (Kruskal-Wallis test followed by the Scheffé method, $P < 0.05$). Apostrophes indicate a highly significant difference ($P < 0.01$). N = 5 at each age.

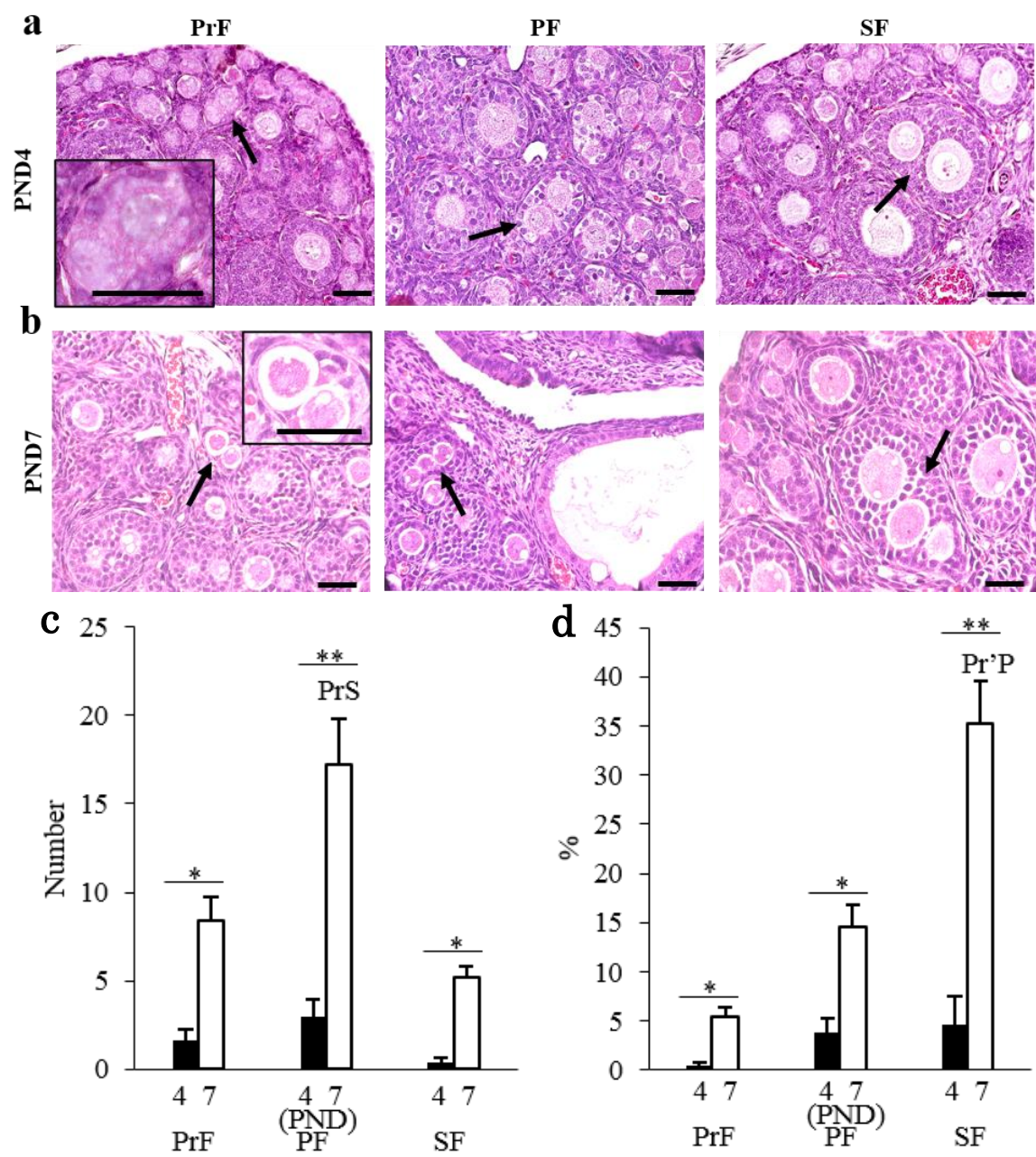


Figure 1-4. Characteristics of multi-oocyte follicles in the neonate ovary of HIS/Hiph at postnatal day 4 and 7

(a-f) Histological observation of hematoxylin and eosin-stained section in the neonate ovary of HIS/Hiph at postnatal day (PND)4 and 7. More than three oocytes are closely attached (arrow and arrowhead) with each other to form multi-oocyte follicles (MOFs) in the single follicles classified as primary follicle (PF) (panel a; PND4), two follicles (SF and PF) joined but they are separated by granulosa cells (arrows) (panel d; PND7). Granulosa cell invasiveness (GCI) denoted by the arrows of two or more follicles in a single follicle to appear as MOFs (panels b, c: PND4; e, f: PND7). Scale bar = 100 μ m.

(g-i) Hematoxylin and eosin-stained semi-serial sections of ovary at PND7. Two MOFs (red circle and arrow, panel g) and one single oocyte follicle (SOF; arrowhead in panel g). The SOF is connected with nearby MOFs (yellow circle) via fine connections of granulosa cells (yellow circle, panel h) but is separated from MOFs (red circle). The MOFs are then connected via shared GCs (asterisk) between adjacent MOFs (yellow circle) to form single MOFs (blue circle in panel i). **(j-l)** Periodic acid Schiff-hematoxylin-stained section of the ovary at PND7. The outline of MOF (red circle, panels j-l) and SOF (yellow circle, panel j) shows the bulging of follicle surface (arrows in panels j-l) within the basement membrane.

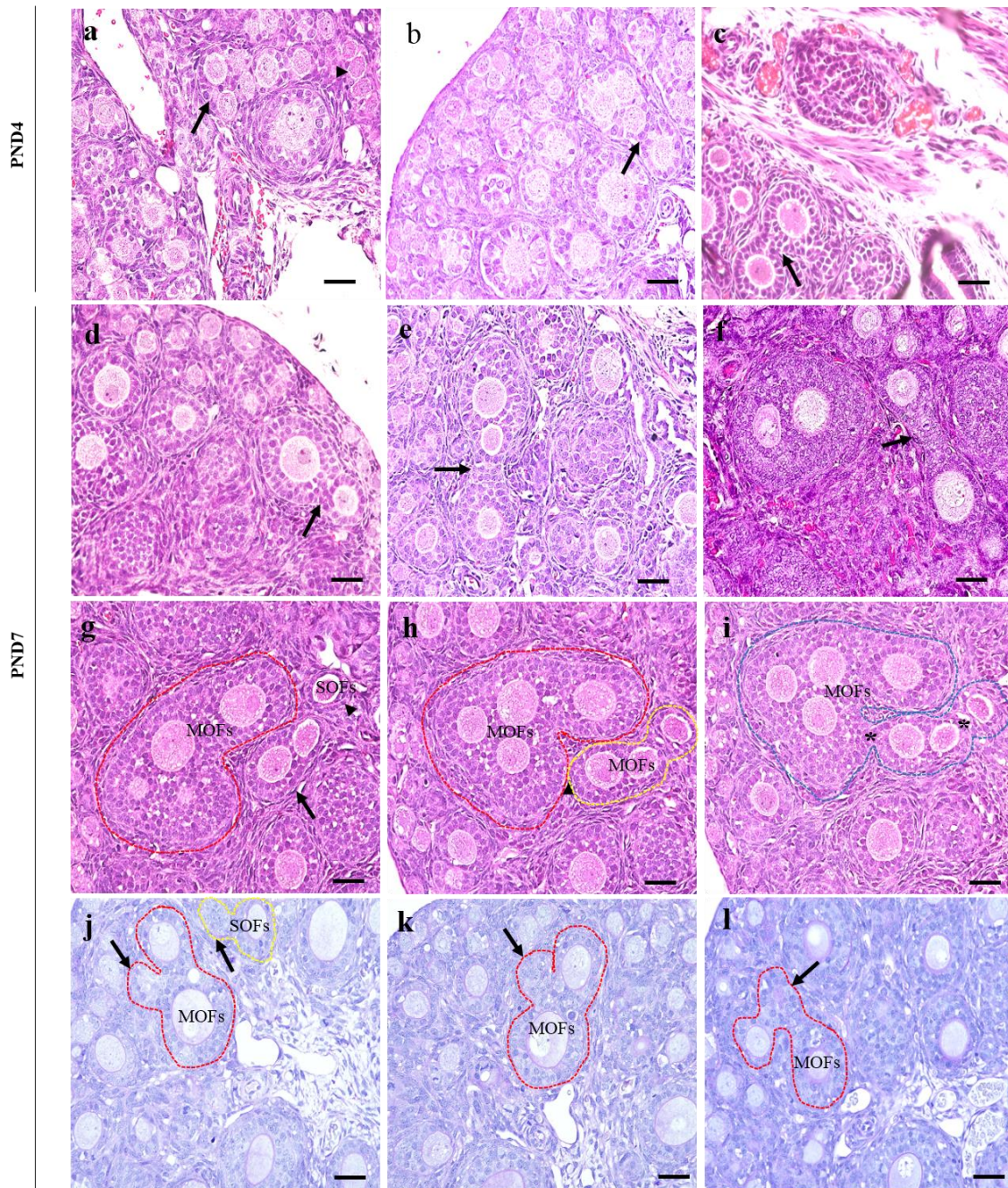


Figure 1-5. Follicular and oocyte development along with double nucleated oocytes and multi-oocyte follicles in neonate HIS/Hiph

(a-c) Double immunofluorescence staining by DEAD - box helicase 4 (DDX4) and Forkhead box L2 (FOXL2) in ovaries of neonate HIS/Hiph at postnatal day (PND)0 (a), PND4 (b), and PND7 (c). The positive signals for DDX4 (green) and FOXL2 (red) are detected in the cytoplasm of oocytes and the nuclei of follicular epithelial cells, respectively. Higher magnification (square area) of panels a, b, and c indicate the development of single nucleated follicles (red arrows) and MOFs (white arrows). Nuclei are stained by Hoechst 33342 (blue). Bars = 100 and 50 μ m (inset).

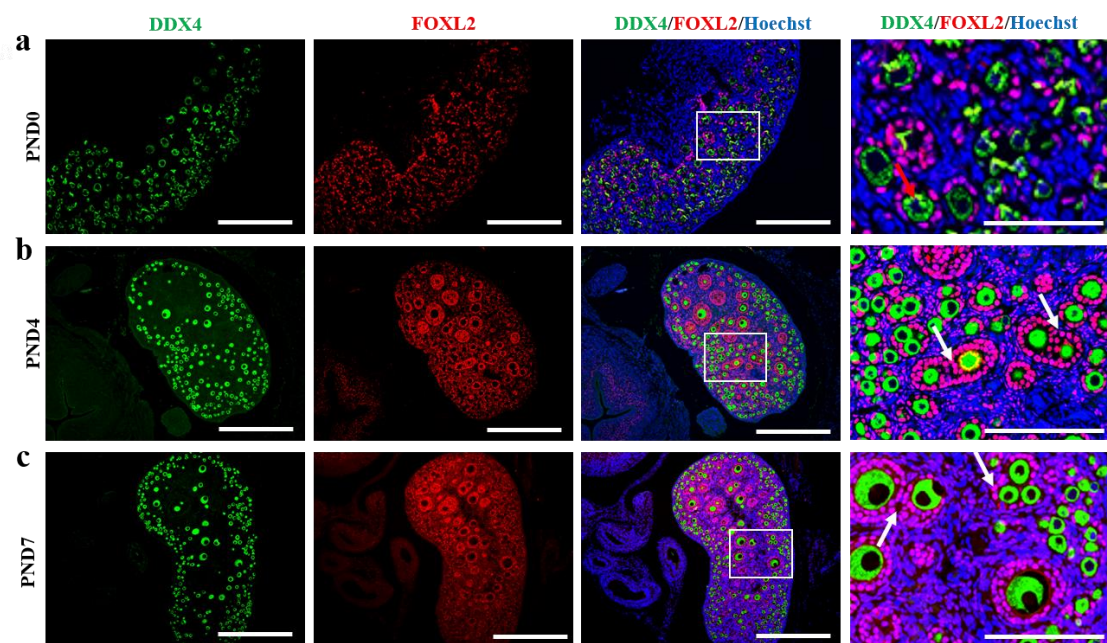


Figure 1-6. Proliferative activity of developing follicles in the neonatal ovary of HIS/Hiph

(a-c) Immunohistochemistry for the assessment of proliferative activity in the developing follicles in ovaries (serial sections) of neonate HIS/Hiph at postnatal day (PND)0 (a), PND4 (b), and PND7 (c) by Ki67 staining. Ki67-positive reactions (arrows) are observed only in granulosa cells, but in single oocyte follicles (SOFs), double nucleated oocytes (DNOs), and multi-oocyte follicles (MOFs) at PND0, PND4, and PND7 (panels a-c, respectively). Panels d-f are normal rabbit IgG controls for PND0, PND4, and PND7, respectively. Scale bar = 100 μ m.

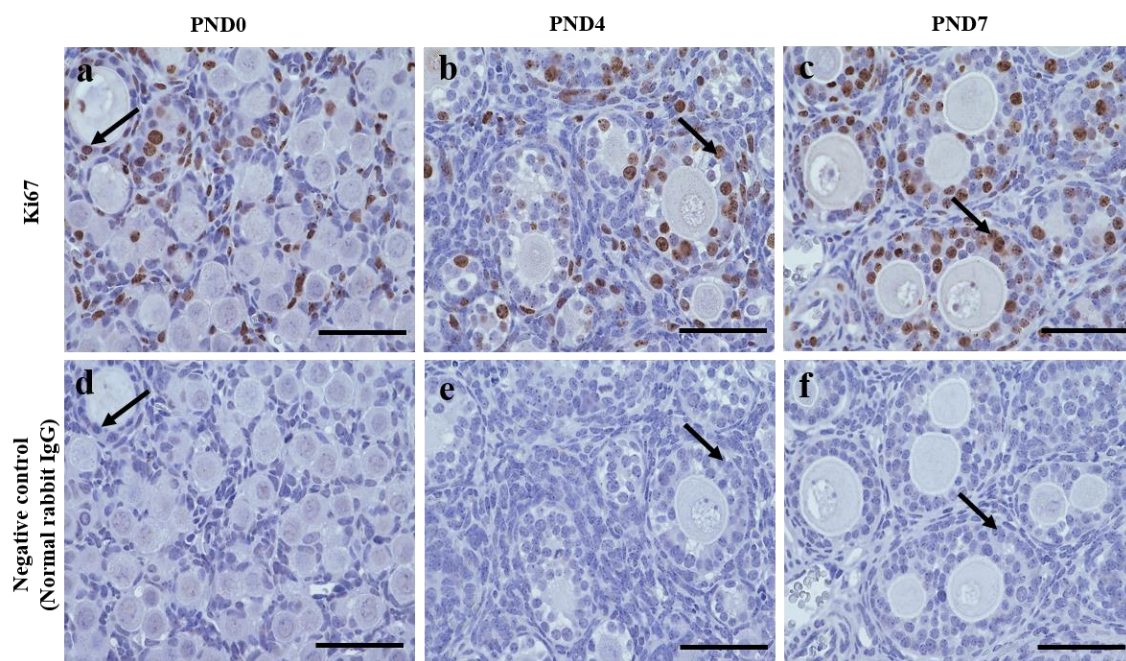


Figure 1-7. Oocyte apoptosis in neonate HIS/Hiph compared with other rodents (mice)

(a) Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive oocytes (arrows) at postnatal days (PND)0, 4, and 7 in the ovaries of neonate HIS/Hiph and ICR mice, respectively. **(b)** Number of TUNEL-positive apoptotic oocytes at PND0, PND4, and PND7 of HIS/Hiph and ICR mice, based on the analysis of five sections at each age of different ovaries. Values are the mean $\pm$ standard error. Letters (a, b, c, d, e, f) denote significance with PND0, PND4, and PND7 TUNEL-positive status in HIS/Hiph and ICR mice, respectively (Kruskal-Wallis test followed by the Scheffé method, $P < 0.05$). Apostrophe beside the letters indicate a highly significant difference in ICR mice and HIS/Hiph at different stages ($P < 0.01$). * indicates significant difference between PND0- HIS/Hiph vs ICR, PND4- HIS/Hiph vs ICR and PND7- HIS/Hiph vs ICR according to the Mann-Whitney *U*-test (* $P < 0.05$ and ** $P < 0.01$). N = five sections at each age.

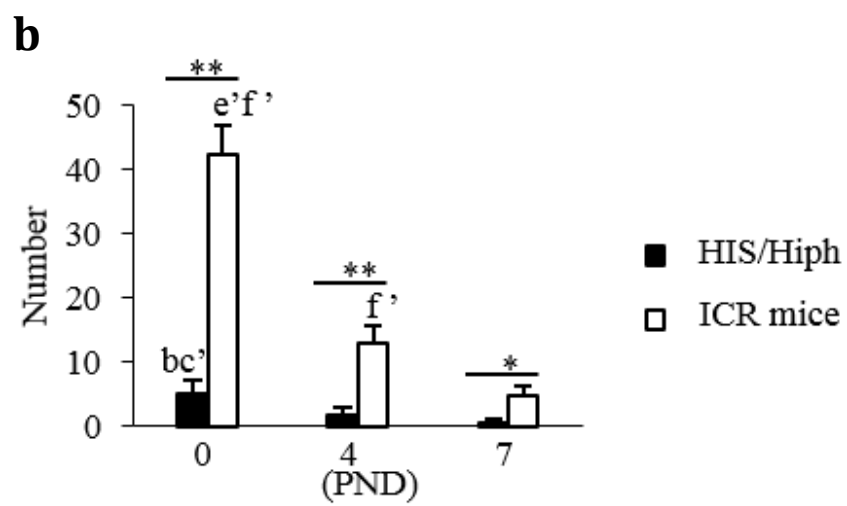
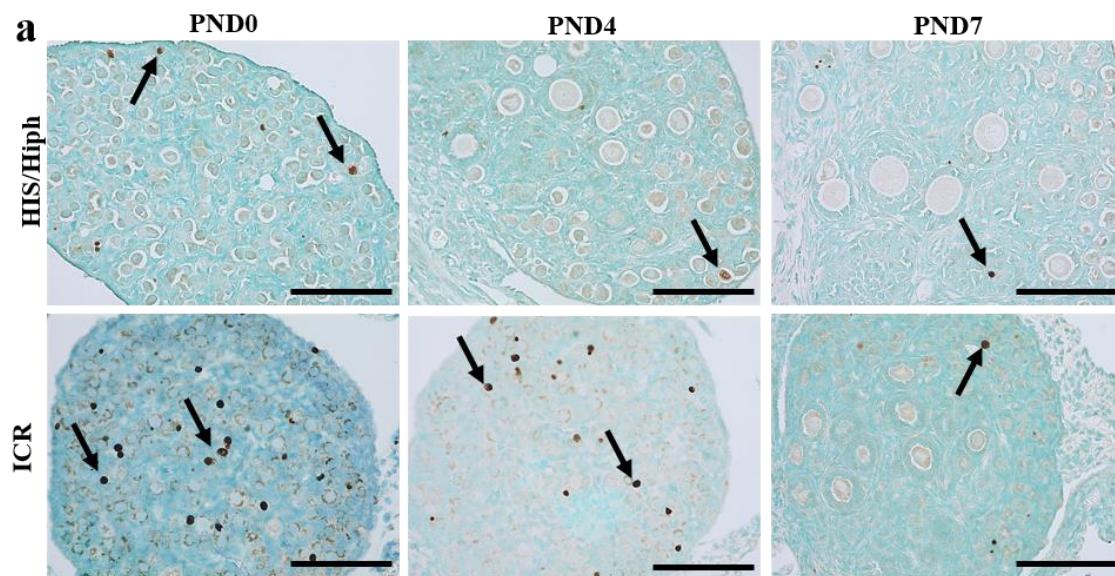


Figure 1-8. Ultrastructural features of the neonatal HIS/Hiph ovary

Transmission electron microscopy images of the neonate ovary of HIS/Hiph at postnatal days (PND)0, 4, and 7. Panels a, b, and c are the images of SNOs, where panel a indicates the representative primordial follicle (PrF) with a single oocyte with a clear round nucleus (N) and mitochondrial clouds (MCs, denoted by arrows) are dispersed throughout the cytoplasm of oocytes at PND0. Panel b depicts the PF SNOs and MC aggregated to one side of the cytoplasm of follicles at PND4 and panel c at PND7 PF SNOs with clearly visible zona pellucida (ZP) and GCs. Panels d, e, and f are the images of MOFs. Panel d depicts PrF MOFs surrounded by a common follicular epithelial cell layer and the cytoplasm (C1 and C2) are closely connected at PND4. Oocytes (O1 and O2) with a common ZP in the same cytoplasm of PF MOFs in panel e at PND7. With age (PND7), panel f shows the granulosa cell extensions, one of the developmental characteristic features of MOFs. Panels g and h are images of DNOs. Panel g shows DNOs (N1 and N2) that are clearly separated by rough endoplasmic reticulum (RER, denoted by arrows) and nuclei that do not have the same shape at PND0. The nuclei (N1 and N2) are in the same cytoplasm of PF DNOs separated by MC (inset) in panel h at PND7. Scale bar = 2 μm in panel g, 5 μm in panel b, and 10 μm in panels a, c, d, e, f (inset 1 μm), and h (inset 5 μm).

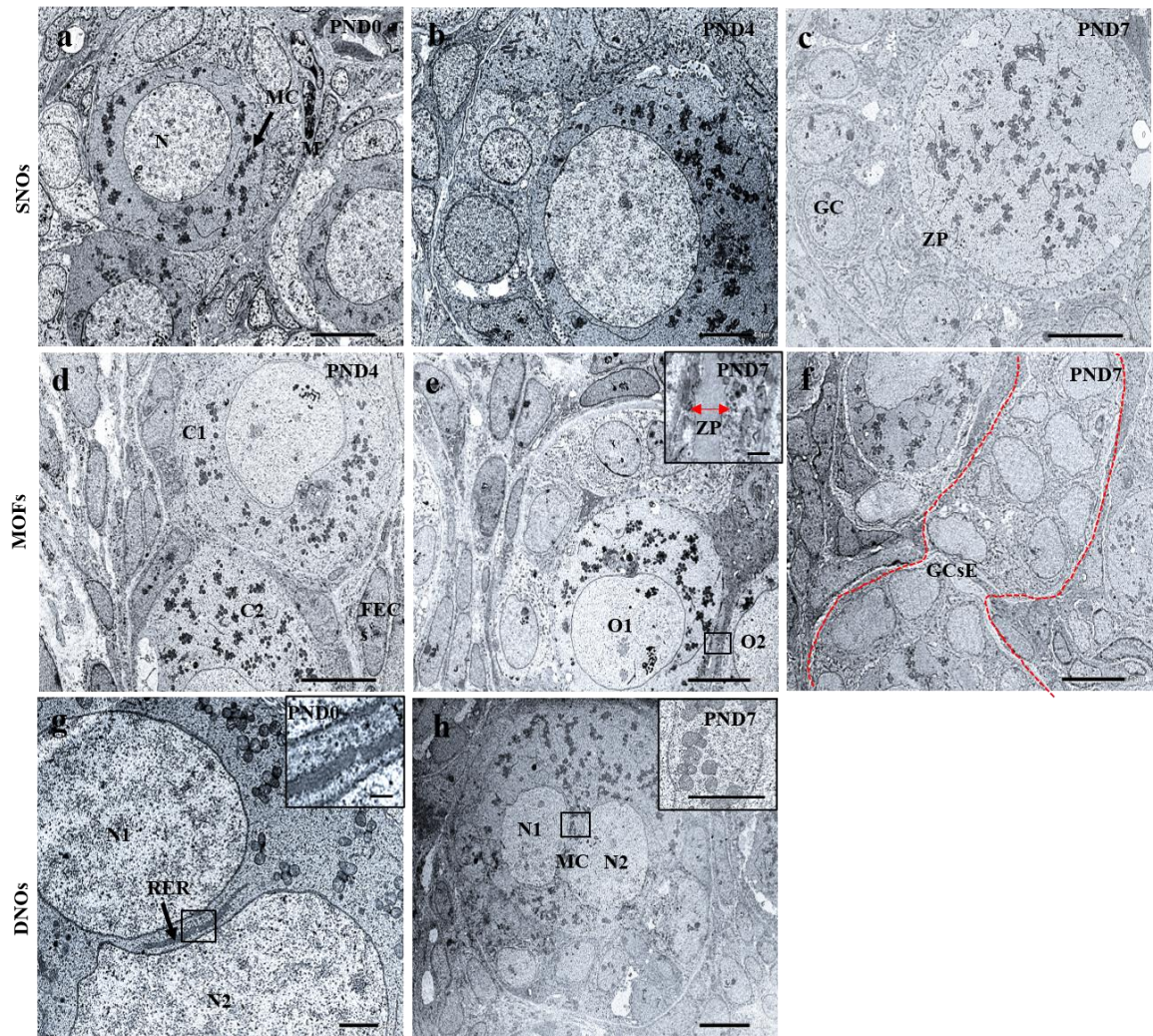
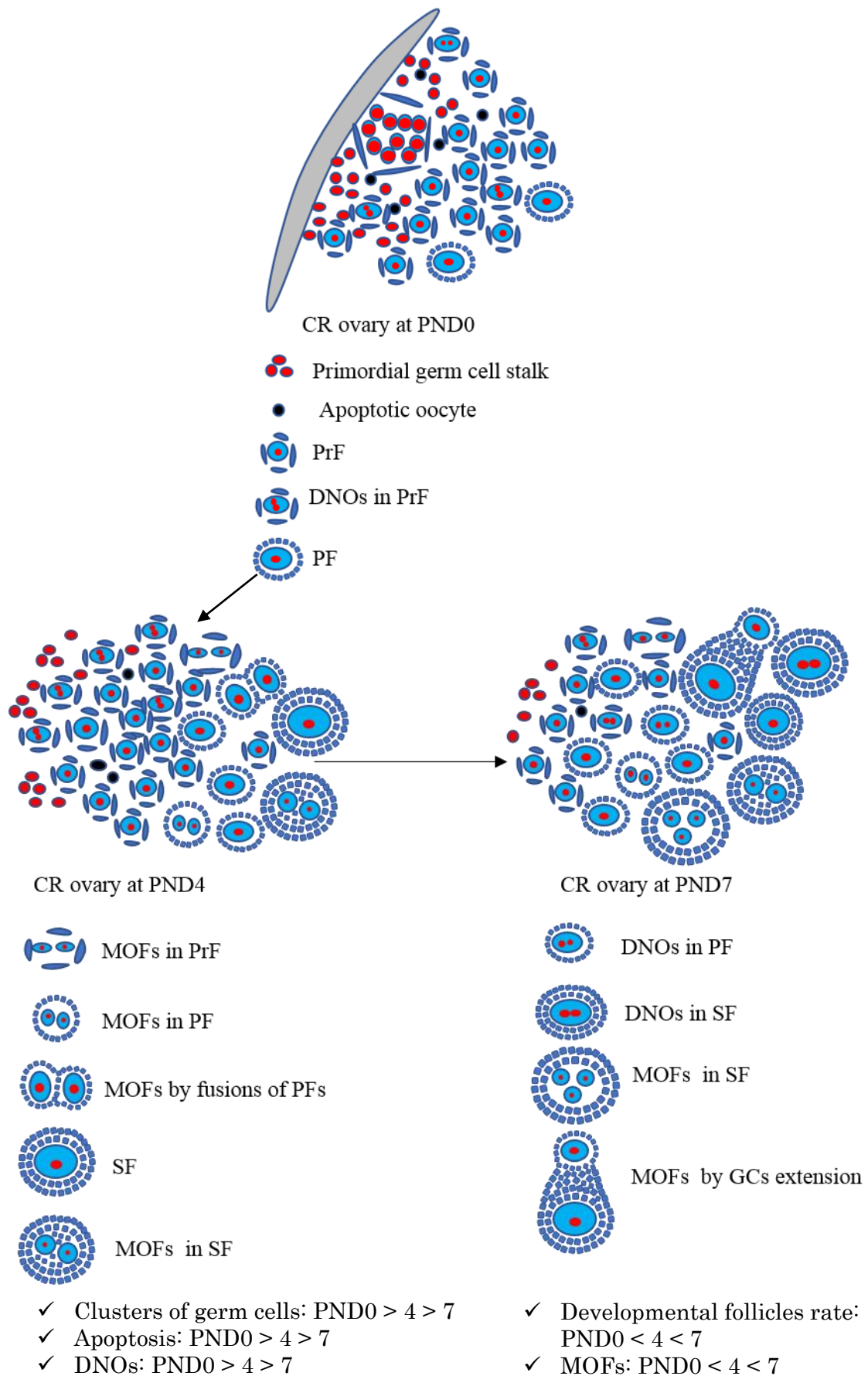


Figure 1-9. Schematic presentation of development of unique characteristics along with developing follicles in neonatal HIS/Hiph

At postnatal day (PND)0, follicles are dispersed throughout the ovary with few apoptotic oocytes. The follicles appear as clusters and develop into primordial follicles (PrFs). A few are primary follicles (PF) surrounded by single layer squamous and cuboidal follicular epithelial cells (FECs), respectively. With age, at PND4, follicles progress to develop into secondary follicles (SF). One of the unique characteristics of PrF is double nucleated oocytes (DNOs). Moreover, at PND4, besides DNOs in PrF, another important characteristic of multi-oocyte follicles (MOFs) is the formation in PrF, PF, and SF. At PND7, DNOs and MOFs are formed in all developing stages of follicles on SF.



Chapter 2

Comparison of ovarian morphology and follicular disturbances between two inbred strains of cotton rats

Introduction

In the mammalian ovary, the reserve of PrFs serves as the source of developing follicles throughout the reproductive lifespan. The growing follicles that develop from this reserve constitute early in life, and the reserve of the PrFs gradually decreases with both follicle growth activation and follicle degeneration during normal folliculogenesis. The process of folliculogenesis is similar in almost all mammals, but the timing of the formation of the reserve is species-specific manner (Monniaux et al., 2014). Although folliculogenesis is a key event in reproductive physiology, the mechanisms involved in processes such as the appearance of MOFs in cattle (Ireland et al., 2008; Silva-Santos et al., 2011), dogs (Payan-Carreiraa and Pires, 2008; Reynaud et al., 2009), pigs (Greenwald and Moor, 1989; Stankiewicz et al., 2009), sheep (Hadek, 1958; Oliveira et al., 2017a), and DNOs in neonate SH still remain unclear.

Rodents, especially laboratory mice belonging to the family Muridae, serve as an animal model for the study of folliculogenesis, but there have been no reports on the abundance of MOFs and DNOs. In CRs, the first estrus cycle starts between 20 and 30 days of age (puberty onset is at 30-40 days of age) and the estrus cycle averages 9 days (4-20 days; not stable), gestation length is 27 days, and hair growth is present at birth (Faith et al., 1997). Laboratory-reared SH females also undergo first estrus between 20 and 30 days of age and generally attain puberty between 30 and 40 days of age. SH is prolific in nature and produces nine litters per year, averaging five to six pups per litter (Faith et al., 1997).

In Chapter 1, the author found several follicular disturbances, including MOFs and DNOs in neonatal inbred strain of SH, HIS/Hiph, maintained at the Hokkaido Institute of

Public Health. However, it is still unknown whether these phenotypes are preserved in other SH strains or other CRs, and a comparative study would be helpful to elucidate HIS/Hiph as an appropriate animal model to study folliculogenesis and the molecular mechanisms of these disturbances. Therefore, in Chapter 2, the author focused on the 4-week-old age as the time when the first estrus and puberty onset of CRs are observed, and to clarify the strain differences in the SH, the author used HIS/Hiph and HIS/Mz and compared their pup numbers and ovarian morphology.

Materials and Methods

Ethical statements

All animal and experimental procedures were followed the ethical principles and guideline outlined by the Hokkaido Institute of Public Health (Sapporo, Japan; approval no. K27-03) and the University of Miyazaki (Miyazaki, Japan; approval no. 2020-503). The details described in Chapter 1.

Animals and ovary processing

Inbred strains of SH, HIS/Hiph and HIS/Mz, maintained at the Hokkaido Institute of Public Health (HIS/Hiph) and the University of Miyazaki (HIS/Mz), respectively, were used in the present study. All SH were maintained in conventional room, and food and water were provided *ad libitum*. The author did not examine estrus cycles at the time of sampling, because the effect of the estrous cycles is minimal since HIS/Hiph has MOFs and DNOs at neonatal age. They were euthanized by cutting the abdominal aorta while deeply anesthetized with isoflurane. The ovaries from 12 HIS/Hiph and 15 HIS/Mz were harvested at four weeks of age. Sample collection and processing methods were performed as described in Chapter 1. The author collected 287 HIS/Hiph delivery records from October 2015 to January 2021 and 51 HIS/Mz delivery records from December 2019 to December 2020. From these, the author analyzed the number of pups, their sex ratio, approximate age of mother, and the correlation between the number of pups and mother ages. In chapter 1, the author used neonatal HIS/Hiph up to 7 days and the appearance of follicular disturbances (DNOs or MOFs) varies with the development of follicular stages, and neonatal ages. Furthermore, MOFs occur more frequently in the

ovaries of fetal and juvenile females than in adults (Stankiewicz et al., 2009). In this study, the author compared their litter numbers and ovarian histology after reaching puberty between the HIS/Hiph and HIS/Mz strains at four weeks of age.

Histomorphology and histoplanimetry

These methods were performed as described in Chapter 1.

Immunohistochemistry

Immunohistochemistry was performed to evaluate cell proliferation by targeting the marker of proliferation Ki67 (described in Chapter 1) and proliferating cell nuclear antigen (PCNA). Positive oocytes for tumor protein p63, associated with female fertility (Lena et al., 2021), were also examined. The staining conditions are listed in Table 2-1. Deparaffinized 3 μ m thick sections of the ovaries of HIS/Hiph and HIS/Mz were incubated in 10 mM citrate buffer (pH 6.0) for 15 min at 110°C. The sections were treated with 0.3% H₂O₂/methanol solution for 20 min to quench the endogenous peroxidase activity. Sections were blocked using 10% NGS (SABPO kit; Nichirei Bioscience, Tokyo, Japan) for p63 or 5% NDS (Sigma Aldrich, St. Louis, USA) for PCNA. Sections were incubated overnight with goat anti-PCNA (1:3000; sc9857; Santa Cruz Biotechnology, California, USA), and mouse anti-p63 (1:50; sc8431; Santa Cruz Biotechnology) at 4°C. The sections were then treated with donkey anti-goat IgG (1:400; Santa Cruz) (for PCNA), or biotinylated goat anti-mouse IgG (1:200; Southern Biotech) (for p63) for 30 min at room temperature. This was followed by incubation with streptavidin-horseradish peroxidase using the SABPO kit (Nichirei Bioscience) for 30 min, followed by incubation with DAB. Finally, the sections were counterstained with

hematoxylin and dehydrated using ascending grades of alcohol. The stained sections were examined using a BZ-X710 microscope (Keyence).

TUNEL assay

This method was described in Chapter 1.

Electron microscopy

This method was described in Chapter 1.

Statistical analysis

The results were expressed as the mean $\pm$ standard error. Statistical analysis was performed as described in Chapter 1. The correlation between two groups was analyzed by Spearman's rank correlation coefficient test.

Results

Comparative results of recorded data of HIS/Hiph and HIS/Mz

Table 2-2 shows, the numbers of HIS/Hiph and HIS/Mz pups produced, averaging five to six pups per litter, which was within normal limits. Briefly, 287 HIS/Hiph deliveries were recorded between October 2015 and January 2021, when the highest number of pups per individual was 13 the lowest was 1 and 51 HIS/Mz deliveries were recorded between December 2019 and December 2020, when the highest number of pups per individual was 10, the lowest was 1. The mother age (days) and sex ratio and were almost the same.

Figure 2-1 shows, the parent's age in days of delivery, and their relationships with the number of pups based on recorded history. There was no correlation between both parameters in both strains. Although the purpose of breeding and recording was different, they did not measure the reproductive ability. In CRs, the average number of pups was 5.0 at the first birth, 5.9 at the 2nd birth, 6.6 at the 3rd and 4th births, and 6.3 at the 5th birth (Tanaka and Arai, 1967). The results approximately match the number of weeks to the first birth and the description in the review.

Comparative ovarian histology of HIS/Hiph and HIS/Mz

Figure 2-2 shows the ovarian histology of HIS/Hiph and HIS/Mz. Early developing follicles, such as PrF and PF, were found in the cortex of both strains. Furthermore, the follicles that progressed to later developing stages, such as SF and TF, were localized in the superficial or deep regions of the cortex. In the medulla, the ductal structures resembling remnants of rete ovarii (Kon et al., 2007) were frequently found in HIS/Hiph

(Fig. 2-2a), whereas more numerous interstitial cells (ICs) were found in the medullary stroma of HIS/Mz (Fig. 2-2b) than in HIS/Hiph.

For histoplanimetry, the author quantified the number and percentage of each developing follicle in HIS/Hiph and HIS/Mz. In both strains, the total number and percentage of developing follicles significantly decreased with the progression of folliculogenesis (Fig. 2-2c and 1d). HIS/Hiph revealed nearly two-fold higher numbers of PrF, PF, and SF than those of HIS/Mz, with significant differences, while TF was comparable in both strains (Fig. 2-2c). Conversely, for the number of follicles in each follicle stage to total number of follicles (indicated as a percentage), a significant difference was observed only in PF between HIS/Hiph and HIS/Mz (Fig. 2-2d).

MOFs found in the ovaries of HIS/Hiph and HIS/Mz

Figure 2-3 shows the appearance of MOFs. The ovaries of the HIS/Hiph strain possessed MOFs in PrF, PF, SF, and TF stages (Fig. 2-3a-d). The oocytes contained in the same MOFs were closely arranged, but each oocyte was surrounded by ZP (Fig. 2-3a-c) or clearly separated by GCs (Fig. 2-3d). In the ovaries of HIS/Mz, MOFs were found up to SF but not in TF (Fig. 2-3e-h). Most of the oocytes were closely arranged in the same MOFs (Fig. 2-3e-g) but rarely separated by GCs. The oocytes of MOFs separated by either ZP or GCs were more frequently observed in HIS/Hiph than in HIS/Mz.

Histoplanimetry analysis revealed that HIS/Hiph had 2–6 folds higher number of MOFs showing the features of PrF, PF, and SF than HIS/Mz ($P < 0.01$, Fig. 2-3i). Within the HIS/Hiph strain, the PF-type MOFs were significantly higher in number than other types of MOFs ($P < 0.01$), whereas PrF- or PF-type MOFs were significantly higher in number than the SF-type MOFs in the HIS/Mz strain (Fig. 2-3i). The ratio of number of

PF- or SF-type MOFs to total number of follicles was significantly higher in HIS/Hiph than in HIS/Mz ($P < 0.01$), although PrF-type MOFs showed no strain difference (Fig. 2-3j). Moreover, in HIS/Hiph, the number of SF-type MOFs was significantly higher than the number of PrF- or TF-type MOFs ($P < 0.01$), and the number of PF-type MOFs was also significantly higher than that of PrF-type MOFs ($P < 0.05$). No significant differences were observed among the follicle stages of MOFs in HIS/Mz (Fig. 2-3j).

Table 2-3 summarizes the number of oocytes observed in the single MOF of each follicular development stage in the ovaries of HIS/Hiph and HIS/Mz. In both strains, most MOFs had two oocytes in a single follicle, and the appearance frequency of MOFs containing two oocytes increased with the progression of folliculogenesis, but TF in HIS/Mz did not contain multiple oocytes. Interestingly, HIS/Hiph tended to have more oocytes in a single MOF than HIS/Mz. Although four or more oocytes were only found in PrF-type MOFs in HIS/Mz, they were observed up to the TF stage in HIS/Hiph.

Unique morphologies found in the MOF of HIS/Hiph and HIS/Mz

Although MOFs were mostly round to oval in shape (Fig. 2-3a-h), two types of characteristic structures were also found in them (Fig. 2-4). First, two individual follicles were partially fused by sharing GCs, and the two follicles were surrounded by the same BL (Fig. 2-4a-c). The connecting region of each follicle was mildly constricted, and they were found in later stages of developing follicles, including SF and TF. This type of MOF was seen in HIS/Hiph (Fig. 2-4a-c), whereas it was partial in HIS/Mz MOFs (Fig. 2-4d-f), observed from serially sectioned ovaries.

The other structure was that two individual follicles were connected by a narrow bridge of GCs (Fig. 2-4g). These MOFs showed more irregular shapes, and the

connecting region of each follicle was severely constricted compared to BL constricted-type MOFs, as described above. Interestingly, these GC bridge-type MOFs were frequently observed in SF and TF stages of MOFs. These GC bridge-type MOFs were frequently observed only in HIS/Hiph and not in HIS/Mz.

DNOs found in the ovaries of HIS/Hiph and HIS/Mz

DNOs, which are defined as oocytes of SOF containing two nuclei, were found in HIS/Hiph but not in HIS/Mz (Fig. 2-5a-d). In Chapter 1 study showed that DNOs were found up to SF in neonate HIS/Hiph (0–7 days after birth). In four weeks old HIS/Hiph, DNOs were found in all types of developing follicles (Fig. 2-5a-d). The nuclei of DNOs were closely localized to each other in the oocytes within the early developing follicles, including PrF and PF (Fig. 2-5a and b), but were separated in SF and TF (Fig. 2-5c and d). Most DNOs in the PrF and PF were localized in the peripheral region of the cortex, and those of later stages were found in the superficial and deep cortex areas, with single nucleated oocytes.

Next, the author enumerated the number and percentage of DNOs. In HIS/Hiph, PrF showed the highest number of DNOs, whereas the number of DNOs significantly decreased in the consecutive follicles ($P < 0.01$; Fig. 2-5e). Furthermore, the percentage of DNOs in each follicular stage appeared to be higher in TF than in other types of follicles without significant difference ($P = 0.167$). DNOs were not detected in HIS/Mz.

Follicular cell cycle arrest and oocyte death in the ovaries of HIS/Hiph and HIS/Mz

The author then evaluated oocyte death as a factor affecting the severe difference in the total number of each follicle type and the appearance of MOFs and DNOs in both

strains. First, the author examined the expression of p63 in oocytes, which promotes cell cycle arrest and inhibits apoptosis of oocytes (Nakamuta and Kobayashi, 2004), in the ovaries of HIS/Hiph (Fig. 2-6a and b) and HIS/Mz (Fig. 2-6c). Oocytes of the early developing follicles, including PrF, PF, and SF, with two layers of GCs expressed p63 in both strains (Fig. 2-6a and c). Oocytes in MOFs showed the same positivity for p63 as SOFs in both strains (Fig. 2-6a and c). In the HIS/Hiph strain, the nuclei of DNOs in the SOF of PrF, PF, and two layers of GCs also showed positivity for p63 (Fig. 2-6b). Consistent with this, the author did not observe any apoptotic oocytes in SOF, MOF, or DNOs, but luteinized GCs showed positive reactions in both strains by TUNEL assay (Fig. 2-6d).

Proliferative activity of the oocytes in the ovaries of HIS/Hiph and HIS/Mz

The author also evaluated the proliferative activities in the oocytes of SOF, MOF, and DNOs (Figure 2-7). In mammalian ovaries, although immunoreactivity for PCNA, a cell proliferation marker, was detected in the oocytes of ruminants such as Sheep (Oliveira et al., 2017a) and cattle (Oliveira et al., 2017b), it was observed in the GCs and theca cells of mice (Xu et al., 2011). In HIS/Hiph, oocytes in SOFs and MOFs or DNOs also showed positivity for PCNA regardless of follicular developmental stage (Fig. 2-7a-c). The positivity for PCNA was clearer in the nuclei of oocytes of SOFs or MOFs and of DNOs in HIS/Hiph (Fig. 2-7a-c) than in those of SOFs and MOFs in HIS/Mz (Fig. 2-7d and e).

Conversely, Ki67, another cell proliferation marker, was only observed in the nuclei of GCs, but not in those of oocytes in the HIS/Hiph strain, as shown in TF-type SOFs and SF-type MOFs (Fig. 2-7f and g). No positive reactions were observed in the DNOs of

HIS/Hiph (Fig. 2-7h). In HIS/Mz, Ki67 positive reactions were not observed in the nuclei of oocytes, and those of GCs were also faint in SOFs and MOFs (Fig. 2-7i and j).

Ultrastructures of SOFs, MOFs, and DNOs in the ovaries of HIS/Hiph and HIS/Mz

Figure 2-8 shows the results of TEM analysis for SOFs, MOFs, and DNOs in the ovaries of HIS/Hiph and HIS/Mz at four weeks of age. In HIS/Hiph, SOF containing SNOs, determined as PrF, contained abundant MCs dispersed throughout the cytoplasm, and MCs showed grape-like structures (Fig. 2-8a). In MOF, MCs were also abundant in each oocyte, and these oocytes were clearly distinguished by ZP (Fig. 2-8c). In DNOs, MCs were observed between double nuclei (Fig. 2-8e). In SOFs of HIS/Mz classified as PF, SNOs were surrounded by cuboidal GCs, and they had a few MCs with lower electron density than those in HIS/Hiph (Fig. 2-8b). MOF of HIS/Mz also showed fewer numbers and lower electron density of MCs than HIS/Hiph (Fig. 2-8d). No DNOs were observed in HIS/Mz, similar to the histology results.

Discussion

This study demonstrated the strain-specific histological differences in the ovaries of two inbred strains of SH. HIS/Hiph had MOFs and DNOs in all stages of developing follicles, but HIS/Mz had fewer MOFs and no DNOs (Fig. 2-3 and 5). Moreover, the estimated follicle population of the HIS/Mz strain was almost half that of the HIS/Hiph strain (Fig. 2-2). Higher number of MCs were also observed in the HIS/Hiph strain than in HIS/Mz (Fig. 2-8). These results indicate that ovarian morphology differed between SH strains, and that HIS/Hiph clearly showed unique ovarian phenotypes, such as numerous MOFs and the presence of DNOs.

During folliculogenesis, follicular morphology changes as the oocyte grows and the surrounding cells proliferate. The major morphological distinction between domestic animals and rodents is the sheer abundance of PrFs in the latter, located throughout the ovarian cortex. The ovaries of laboratory mouse strains contain follicles of varying stages of development in the order of 3,000 to 5,000 (Tilly, 2003). This number declines over time as most follicles die *via* atresia, with a small number surviving to ovulation in each estrus cycle (Uslu et al., 2017). In fact, ICs, derived from the internal theca of atretic follicles in rodents (Aoyama et al., 2019; Tian et al., 2015), were more abundant in HIS/Mz than in HIS/Hiph. The author considered that this result might reflect the difference in folliculogenesis between SH strains, including follicle atresia and oocyte death. However, there were no clear strain-specific differences in the positivity of oocytes for p63 (Fig. 2-6a-c), a regulator of the cell cycle in mouse germ cells (Nakamuta and Kobayashi, 2004), as well as oocyte death examined by TUNEL assay (Fig. 2-6d) at four-weeks of age. Therefore, the author considered that the differences in the number of

developing follicles would be formed during the embryonic or neonatal period, but not at four weeks of age.

Furthermore, litters averaged five to six pups in CRs (Faith et al., 1997), and the recorded results indicated that the number of pups was within the normal range. In this study, there was no age-related decline of pup numbers in both strains. Although the history of breeding for HIS/Hiph 5 years and HIS/Mz 2 years was recorded for the maintenance of each strain, it did not include an evaluation of reproductive ability. To examine reproductive ability, the author has to compare the number of pups in the 1st delivery from the mating of sexually mature males and immature females. Table 2-2 did not take this point into account; therefore, the number of deliveries might have affected this result. Therefore, these points are considered to be limitations of this study. This information would be helpful for future studies to determine the fertility status and its age-related alternations in HIS/Hiph and and HIS/Mz.

The frequency of MOFs is important for further studies to evaluate the mechanisms and fertility status of such disturbances in mammals. However, the frequency of MOFs in the ovaries is species- and individual-dependent. In opossum (< 3 months), dogs (< 7 months), and pigs (< 5 months), MOFs are more common in the ovaries of younger than older individuals (Hartman, 1926; Payan-Carreiraa and Pires, 2008; Stankiewicz et al., 2009). Moreover, some researchers found MOFs in rodents upon treatment with synthetic estrogenic hormones (Jefferson et al., 2002) or the estrogen receptor β agonist 2,3-bis(4-hydroxyphenyl)-propionitrile (Sato et al., 2018), as well as in ovaries of aquaporin-8 (AQP8) knockout (Su et al., 2013) or protein 27 Kip subfamily of cyclin-dependent kinase inhibitors (p27Kip1) mutant mice (Pérez-Sanz et al., 2013). Furthermore, inbred SH develops unique phenotypes such as pharyngeal pouch remnants

and metabolic disorders, carcinomas, as well as anemia with renal inflammation and chronic kidney diseases (Waldum et al., 1999; Ichii et al., 2016; Nakamura et al., 2018; Nakamura et al., 2019). For dogs, there is disagreement on this topic. Some authors have shown that MOFs are less common in older dogs than at the beginning of their reproductive lives (Payan-Carreiraa and Pires, 2008; Telfer and Gosden, 1987), but other authors have reported no difference in the numbers of MOFs between young and adult females (McDougall et al., 1997). The formation of these disturbances in mammals occurs owing to the failure of germ cell division during the early stages of folliculogenesis, such as in human (Zeilmaker et al., 1983) or bovine (Tingen et al., 2009). Recently, a new theory for MOF formation suggested that follicles are fused by the invasion of GCs toward other follicles (Gaytán et al., 2014). In SH, the strain difference in the frequency of MOFs was first demonstrated in this study. Regarding breed, these structures were observed more frequently in mongrels (52.3%) than in purebred animals (25.5%) (Payan-Carreiraa and Pires, 2008). Notably, the number and percentage of PF-type MOFs were higher than that of PrF only in HIS/Hiph (Fig. 2-3i-j), indicating that MOFs can develop after the formation of follicles. Furthermore, BL constricted-type and GC bridge-type MOFs were also found in SH; in particular, these were clearer in HIS/Hiph than in HIS/Mz. The author considered that BL constricted-follicle structures indicated “BL breaching (BLB),” which is one of the most important characteristics for the formation of MOFs. The BLB might be a result of (i) decreased synthesis of extracellular matrix proteins that comprise the BL, and (ii) increased degradation of BL more than that required for normal remodeling during follicular growth (Williams et al., 2008). Further, GC bridge-structure resembling “GCI” has been reported in inbred rat (Gaytán et al., 2014), and the aberrant initiation of cellular invasion by the GCs result in

the eventual breaching of the follicle wall allowing GCs to invade the perifollicular stroma or joining of adjacent follicles to form MOFs. In fact, the expression of proliferative cell markers, PCNA and Ki67, was detected in GCs of both strains, and Ki67 positivity was stronger in HIS/Hiph than in HIS/Mz (Fig. 2-7). These results indicate that fusion of two or more SOFs, or BLB or GCI, occurs in SH, especially in HIS/Hiph, showing higher proliferating activities of GCs, to form MOFs. Furthermore, in the giant panda the existence of twin oocytes in the follicle increases the rate of twinning (Zhang et al., 2001), and recently, in humans, successful live births from conjoined oocytes have been reported (Yano et al., 2017). Therefore, the difference between strains in abundance of MOFs and DNOs may play a role in twin research.

During comparative histological analysis of SH strains, the author found another important strain difference that DNOs, found in all types of follicles in HIS/Hiph ovaries, was lacking in HIS/Mz. Double nucleated cells can also be observed in other cell types, such as hepatocytes or trophoblasts, and these double nuclei indicate intensive transcriptional activity (Donne et al., 2020). There are no reports of DNOs in other mammals, except that binuclear oocyte are usually found in opossums (Hartman, 1926). Multi-nucleated oocytes have also been described during the initial stages of oogenesis in some amphibian species, such as *Ascaphus truei*, in which oogenesis involves eight nuclei (Macgregor and Kezer, 1970) whereas in *Leiopelma hochstetteri*, oocytes with two nuclei have been described (Robinson et al., 1973). Moreover, the number and percentage of DNOs at four weeks of age were lower than those of neonates. Therefore, the DNOs might decrease with the development of ovaries. Unlike MOFs, the number of DNOs was the highest in PrF and declined with follicle development.

In addition, oocytes were Ki67-negative/PCNA-positive in the examined SH, and PCNA positivity was clearer in HIS/Hiph than in HIS/Mz. These results indicated that PCNA-positive oocytes did not reflect proliferative cells, but the other cell types. Although PCNA is a general proliferation marker, PCNA expression in oocytes is associated with apoptosis rather than proliferation (Muskhelishvili et al., 2005; Xu et al., 2011). Thus, strong expression of PCNA in HIS/Hiph ovaries (Fig. 2-7a) alleviates apoptosis of oocytes and increases PrF assembly and formation of follicles with DNOs, as compared to HIS/Mz ovaries showing weak expression.

The presence of MCs in the SOFs, MOFs, and DNOs was clarified by TEM analysis of ovaries of HIS/Hiph and HIS/Mz strains (Fig. 2-8). Higher number of MCs were observed in the HIS/Hiph strain oriented throughout the cytoplasm as a grape-like structure than in the HIS/Mz strain. It is still not known how the structural (and molecular) components of MCs are assembled into such morphologically intricate complexes. The most important function of mitochondria is to supply cells with metabolic energy generated by oxidative phosphorylation (Sekula et al., 2020). Therefore, the author hypothesized that the presence of more MCs might contribute to more oocytes in the HIS/Hiph strain than in the HIS/Mz strain by maintaining oocyte reserves.

Follicular development of CR ovary in the strains of HIS/Hiph and HIS/Mz is hypothesized in Figure 2-9. PGC migrate to, and trespass the ovary to form germ cell nest. They proliferate at high rate, then enter meiosis and form PrF by germ cell nests break down. During this time, MOFs and DNOs might be formed in SH ovary. The MOFs survive through all types of developing follicles at HIS/Hiph strain ovaries whereas HIS/Mz MOFs upto secondary stages. In addition, MOFs might also be formed

by the fusion of two or more follicles, BLB and GCI after nest breakdown. The formation characteristics might be the same in both strains except GCI characteristic is observed only in HIS/Hiph MOFs. On the other hand, DNOs are formed only in HIS/Hiph strain might be the fusion of two oogonia or nuclear division without cytoplasmic cleavage of follicles during folliculogenesis, and frequently observed in primordial ones.

In conclusion, these findings clarify the comparative histology of SH strains, especially focusing on the quantity and quality of normal oocytes with MOFs and DNOs. Therefore, these results would be helpful in understanding folliculogenesis, molecular mechanisms, and fertility status, as well as their disturbances, including the appearance of MOFs and DNOs in several mammalian species, including humans.

Summary

In Chapter 2, the author focused on the 4-week-old age as the time when the first estrus and spring onset of CRs are observed. Hence, the author clarified the strain differences in CRs, used HIS/Hiph and HIS/Mz and compared their pup numbers and ovarian morphology at 4 weeks of age when the first estrus and spring onset of CR are observed.

At four weeks of age, there was no difference in the number of pups or the sex ratio of pups between the two strains. The complete folliculogenesis was observed and found all stages of developmental follicles in both strains while neonate ages developed up to secondary stages of the follicle. The follicle population was also greatly reduced compared with neonate ages and this was a common phenomenon in all mammals because of cell death. Interestingly, the follicle population in HIS/Mz strain was almost half of the HIS/Hiph strain. Even though, the proliferative activities and apoptosis were normal in both strains, MOFs were also observed. In neonate, HIS/Hiph MOFs started to develop at PND4 and the frequency was increased with ages. Compare with neonates, MOFs frequently appeared at later stages of developing follicles in both strains and the frequency of HIS/Hiph MOFs was 2-6 folds compared with HIS/Mz. Moreover, DNOs were observed in all stages of developing follicles in HIS/Hiph and lacked DNOs in HIS/Mz strain while in neonate HIS/Hiph DNOs found up to secondary stages. Also, like neonate HIS/Hiph, plenty of MCs were observed in HIS/Hiph SOFs, MOFs, and DNOs compared with HIS/Mz.

Conclusively, the author found that the neonate phenotype of HIS/Hiph ovaries was also observed at 4 weeks of age and was partially common among other strains, while

the number of follicles and the appearance of MOFs and DNOs depended on the genetic background of CR. Therefore, this study results would be helpful to analyze species and individual dependent appearance of follicular disturbances in other mammals as well as help to analyze breeding performance of CRs.

Tables and Figure

Table 2-1: Summary of immunostaining (immunohistochemistry/ immunofluorescence) conditions used for the analysis of ovarian follicles.

Target	Marker	Antigen retrieval	Blocking	Primary antibody	Secondary antibody
Oocyte	p63	CB 115°C, 15 min	10% NGS	Rabbit anti-p63antibodies (Santa Cruz Biotechnology, California, USA) 1: 50	Goat anti-mouse (SABPO kit, Nichirei) 1:100 (Biotinylated) for IHC
Proliferative cells	PCNA	CB 115°C, 15 min	5% NDS	Goat anti-PCNA antibodies (Santa Cruz Biotechnology, California, USA) 1: 3000	Donkey anti-goat IgG antibody (1:400; Santa Cruz, Dallas, TX, USA) (Biotinylated) for IHC
p63: Protein (Tumor) 63, PCNA: Proliferating cell nuclear antigen, CB: Citrate buffer (pH 6.0), NGS: Normal goat serum, NDS: Normal donkey serum, and IHC: Immunohistochemistry					

Table 2-2: The number of pups recorded period in the inbred SH strains, HIS/Hiph and HIS/Mz.

SH strain	Recorded period	Mother age (days)	Number of pups		
			Total	Male	Female
HIS/Hiph	2015-2021	97.8 ± 1.6	5.2 ± 0.1 (1-13)	2.8 ± 0.1 (0-8)	2.4 ± 0.2 (0-8)
HIS/Mz	2019-2020	96.6 ± 2.7	5.7 ± 0.3 (1-10)	3.0 ± 0.2 (1-7)	2.7 ± 0.2 (0-6)
Values are mean ± SE. n = 287 deliveries in HIS/Hiph and 51 deliveries in HIS/Mz. Parentheses show minimum to maximum values.					

Table 2-3: The number of oocytes included in the multi-oocyte follicles of each follicular development stage in the ovary of HIS/Hiph and HIS/Mz inbred strain.

Follicular type	Multi-oocyte follicles					
	2 Oocytes (%)		3 Oocytes (%)		> 4 Oocytes (%)	
	HIS/Hiph	HIS/Mz	HIS/Hiph	HIS/Mz	HIS/Hiph	HIS/Mz
Primordial	52.7	82.0	43.3	16.0	4.0	2.0
Primary	62.5	94.5	35.0	5.5	2.5	0.0
Secondary	68.0	100.0	27.5	0.0	4.5	0.0
Tertiary	82.5	0.0	15.8	0.0	1.7	0.0
The results are based on the percentages of multi-oocyte of follicles of each follicular type in the ovary of HIS/Hiph and HIS/Mz inbred strain						

Figure 2-1. Numbers of pups and parent's age in days of delivery.

The total number of pups and parent's age in days of delivery in the HIS/Hiph and HIS/Mz strains. Values are the mean $\pm$ standard error. Correlation between the number of pups and parent's age in days of delivery in the HIS/Hiph and HIS/Mz strains. Spearman's rank correlation coefficient ($n = 234$ deliveries in HIS/Hiph and 49 deliveries in HIS/Mz).

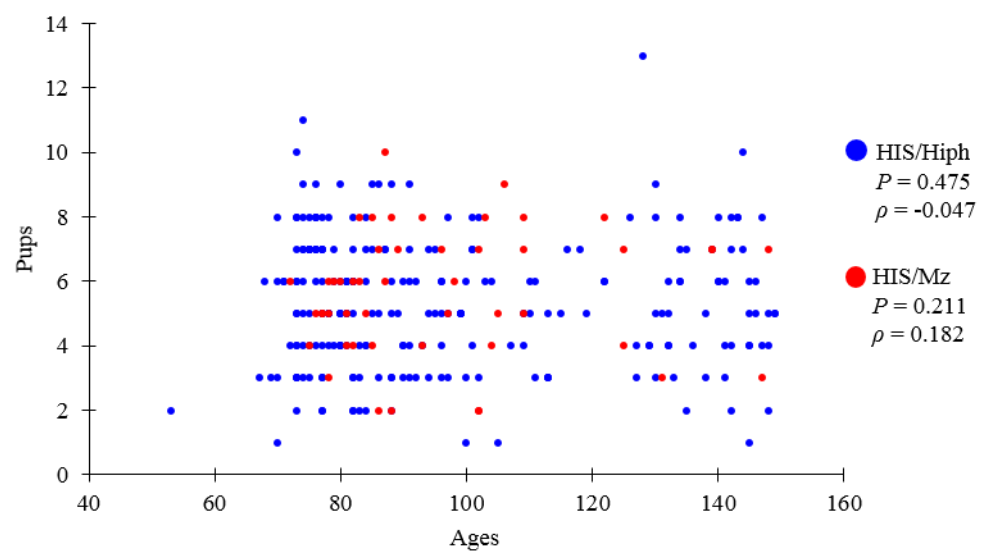
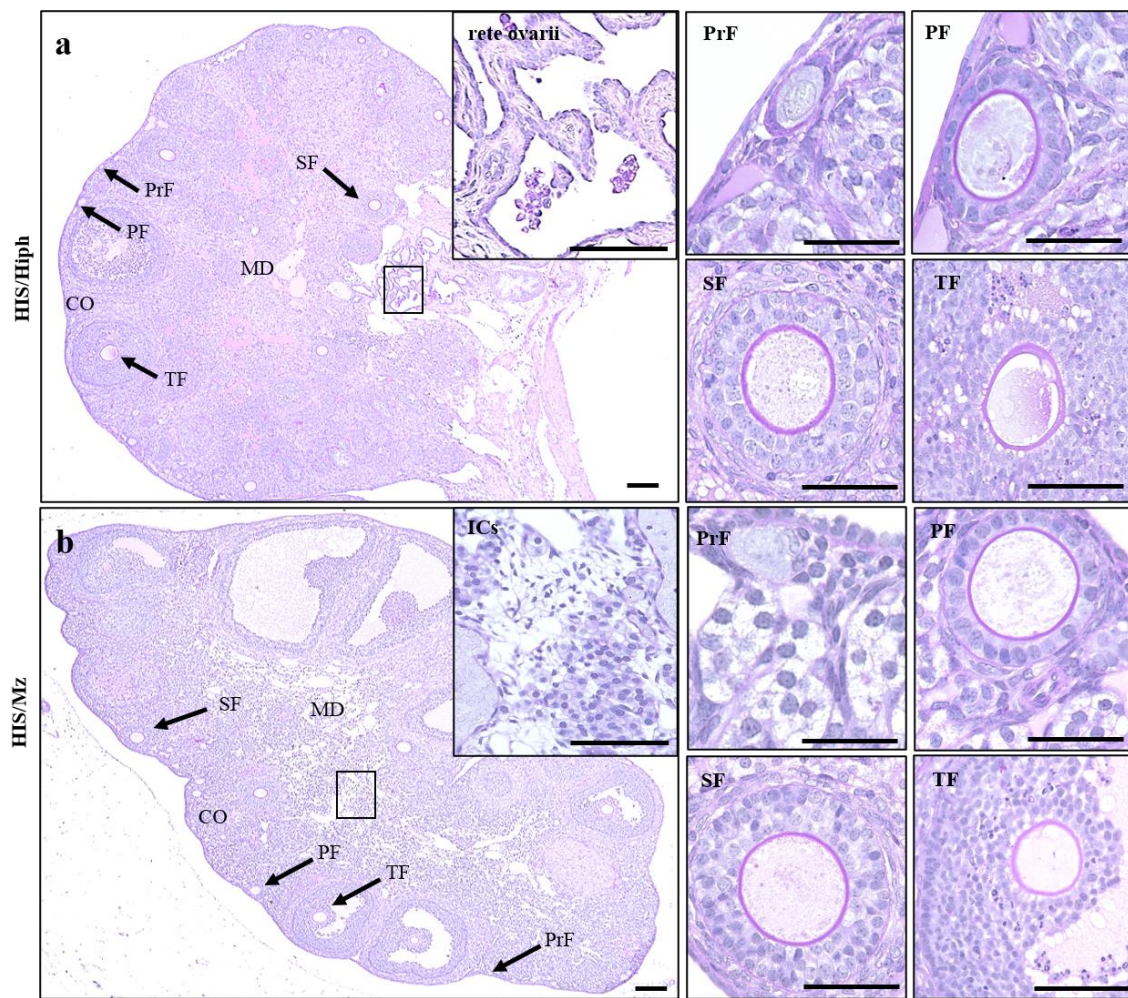
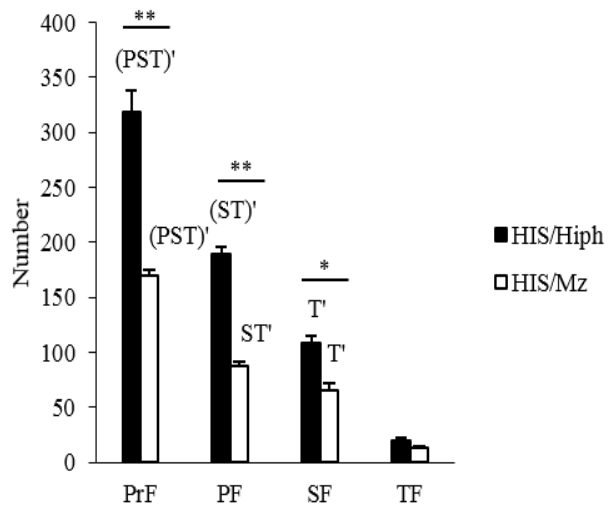


Figure 2-2. Histology of the ovaries of HIS/Hiph and HIS/Mz.

(a-b) Histological observation of periodic acid Schiff-hematoxylin-stained ovary section from four-weeks old HIS/Hiph and HIS/Mz rats. The classified follicles shown in panels a and b (arrows) are further magnified to highlight primordial (PrF), primary (PF), secondary (SF), and tertiary (TF) follicles in both strains. Panels a and b show the regions of the ovary divided as cortex (CO) and medulla (MD). Panel a also shows the remnants of the rete ovarii (inset). Panel b shows the interstitial cells (ICs) (inset). Square area indicates the same area of insets. Scale bars = 200 μm or 50 μm (magnified and inset). **(c and d)** The total number and percentage of each follicle type classified as PrF, PF, SF, and TF in the ovary of HIS/Hiph and HIS/Mz. The percentage was calculated from the number of each follicle type/total number of follicles. Values are the mean $\pm$ standard error. * indicates significant differences between HIS/Hiph and HIS/Mz within a follicle type (Mann-Whitney *U*-test, $*P < 0.05$ and $**P < 0.01$), and alphabets (Pr, P, S, and T) denote the significant difference of PrF, PF, SF, and TF in same strain (Kruskal-Wallis test followed by Scheffe's method, $P < 0.05$). Apostrophe beside letters indicates a highly significant difference ($P < 0.01$). N = 5 in each strain.



c



d

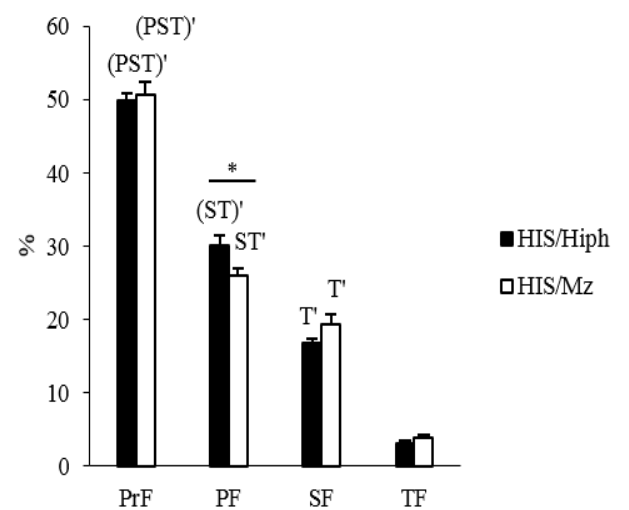


Figure 2-3. Multi-oocyte follicles (MOFs) in the ovaries of HIS/Hiph and HIS/Mz.

(a-h) Histological observation of MOFs from hematoxylin-eosin-stained sections of the ovaries of four-weeks old HIS/Hiph (a-d) and HIS/Mz (e-g). MOFs denoted by the arrows are observed in follicle types classified as primordial follicle (PrF), primary follicle (PF), secondary follicle (SF), and tertiary (TF) follicle in HIS/Hiph (a-d) and PrF, PF, and SF in HIS/Mz (e-g). Panel h shows the TF-type single oocyte follicle (SOF). Scale bars = 100 μ m. **(i and j)** The total number and percentage of MOFs (follicular type MOFs/number of follicles in each type) classified as each follicle type in the ovary of HIS/Hiph and HIS/Mz. Values are the mean $\pm$ standard error. * indicates significant differences within follicle types between HIS/Hiph and HIS/Mz strains (Mann-Whitney *U*-test, $*P < 0.05$ and $**P < 0.01$). Pr, P, S, and T denote the significant difference of PrF, PF, SF, and TF, respectively, in HIS/Hiph and HIS/Mz (Kruskal-Wallis test followed by the Scheffé method, $P < 0.05$). Apostrophe beside the letter indicates a highly significant difference ($P < 0.01$). N = 5 in each strain. ND: Not detected.

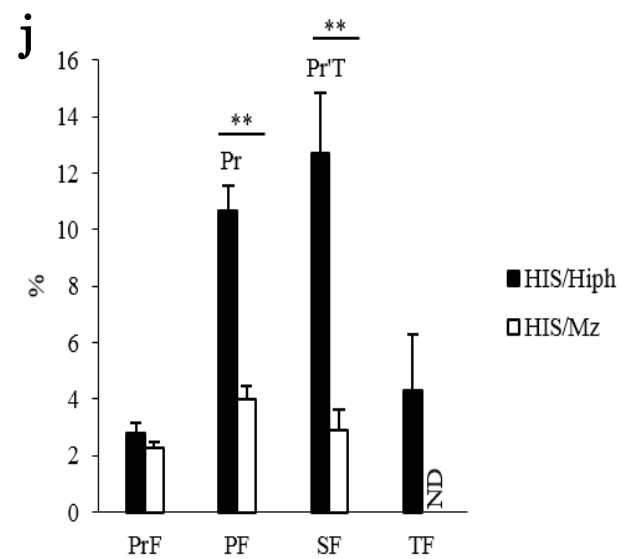
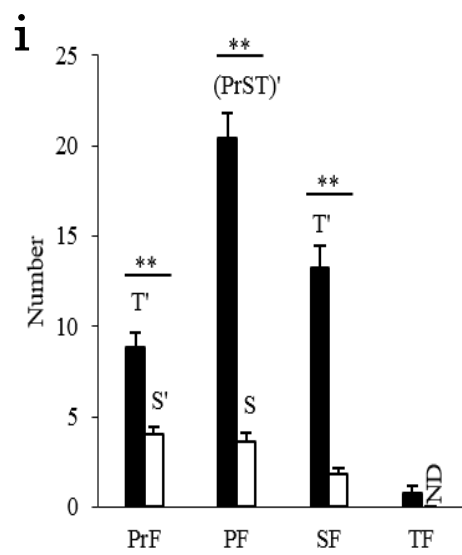
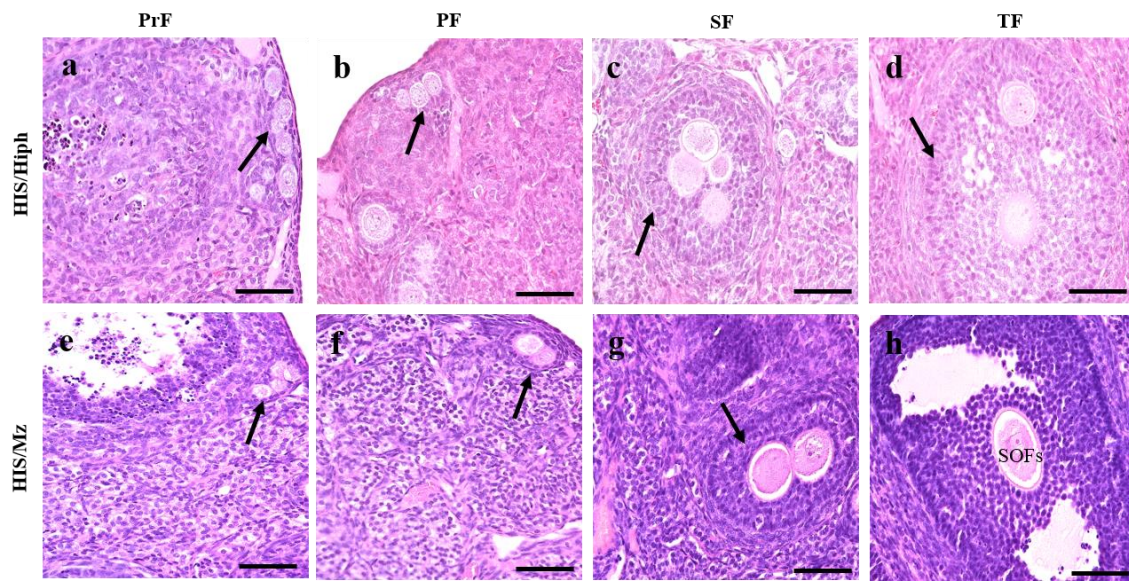


Figure 2-4. Unique morphological characteristics of the multi-oocyte follicles (MOFs) in the ovaries of HIS/Hiph and HIS/Mz.

(a-f) Histological observation of basal lamina (BL) constricted-type MOFs in periodic acid Schiff (PAS)-hematoxylin -stained serial sections from the ovaries of four-weeks old HIS/Hiph (a-c) and HIS/Mz (d-f). BL constricted-type MOFs are clearly observed in HIS/Hiph (a-c), but they are not clear in HIS/Mz (d-f). **(g)** Granulosa cell (GC) bridge-type MOFs in PAS-stained serial sections in the ovary of HIS/Hiph. Arrows indicate the order to next serial sections. Arrowhead shows GC bridge regions. Scale bars = 100 μm or 50 μm (inset).

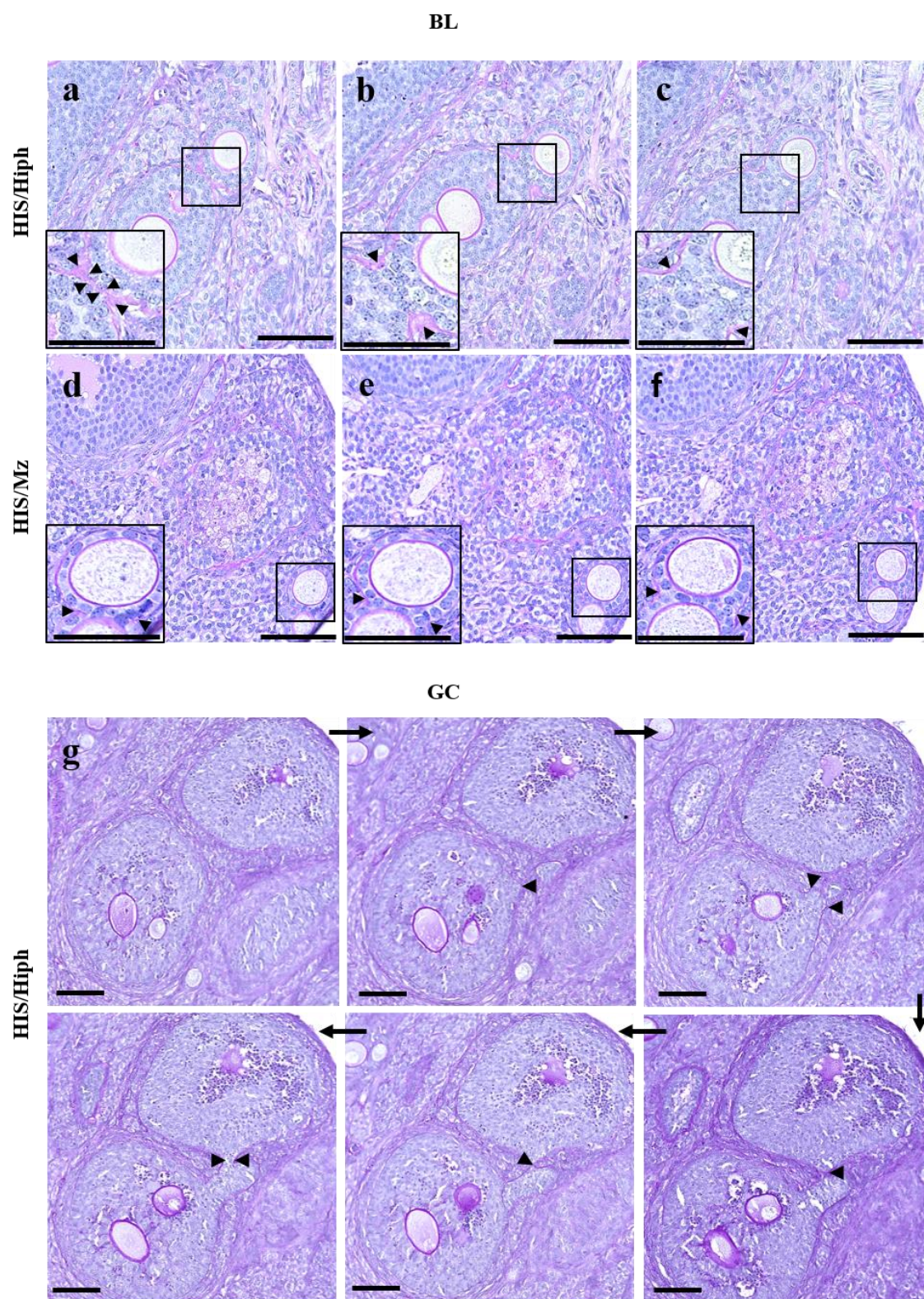


Figure 2-5. Double nucleated oocytes (DNOs) in the ovaries of HIS/Hiph and HIS/Mz.

(a-d and a'-d') Histological observation of DNOs from hematoxylin-eosin-stained sections of the ovaries of four-weeks old HIS/Hiph and HIS/Mz. Two nuclei are observed within a single oocyte classified as primordial follicle (PrF), primary follicle (PF), secondary follicle (SF), and tertiary follicle (TF) in panels a-d (arrows), and a'-d' (magnified), respectively, but DNOs are absent in HIS/Mz. Each nucleus (asterisk) shares the same cytoplasm. Scale bars = 100 μ m or 50 μ m (magnified). **(e and f)** The total number and percentage of DNOs classified as developing follicles in the ovary of HIS/Hiph. Values are presented as the mean $\pm$ standard error. Pr, P, S, and T denote the significant difference of PrF, PF, SF, and TF, respectively, in HIS/Hiph (Kruskal-Wallis test followed by the Scheffé method, $P < 0.05$). Apostrophe beside the letters indicates a highly significant difference ($P < 0.01$). N = 5 in each age. ND: Not detected.

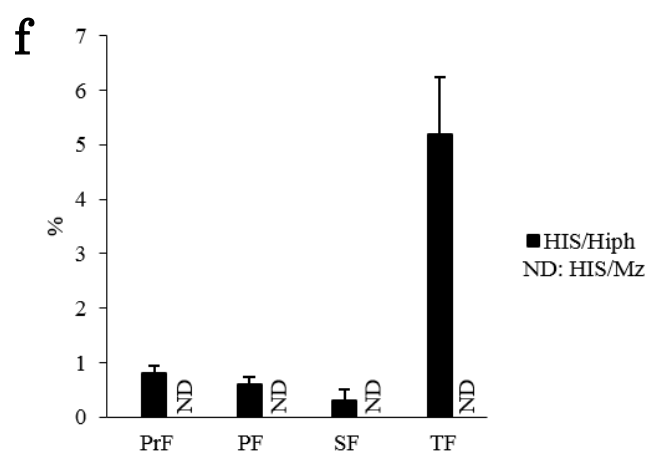
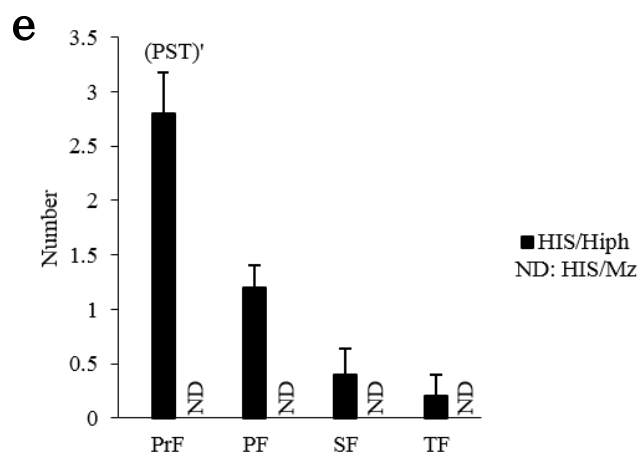
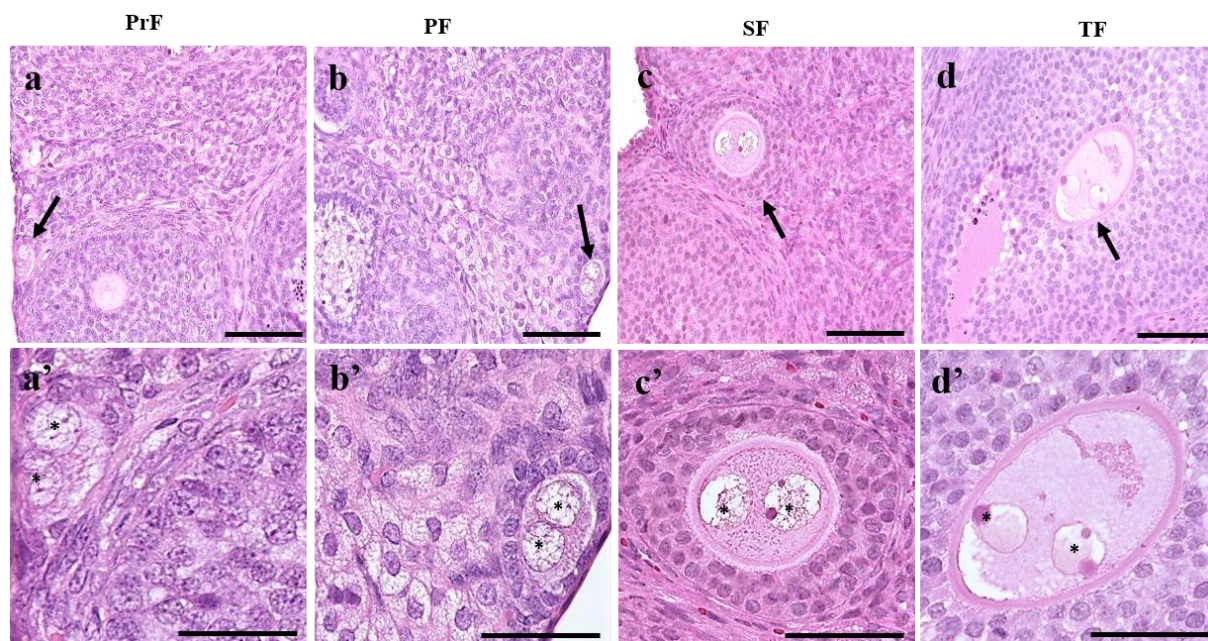


Figure 2-6. Evaluation of oocyte death in the ovaries of HIS/Hiph and HIS/Mz.

(a-c) Immunohistochemistry for p63. Single oocyte follicles (SOF) classified as primordial (PrF), primary (PF), and secondary (SF) follicles (arrowheads), and multi-oocyte follicles (MOFs) (arrows), and double nucleated oocytes (DNOs) (white arrows, found only in HIS/Hiph) contain p63-positive oocytes in both four-weeks old HIS/Hiph (a and b) and HIS/Mz (c). **(d)** TUNEL assay. Panel shows lack of apoptotic oocytes, but granulosa cells (arrows) in the luteinized follicles show positive reactions in both strains. Scale bars = 100 μm (a-c) or 200 μm (d).

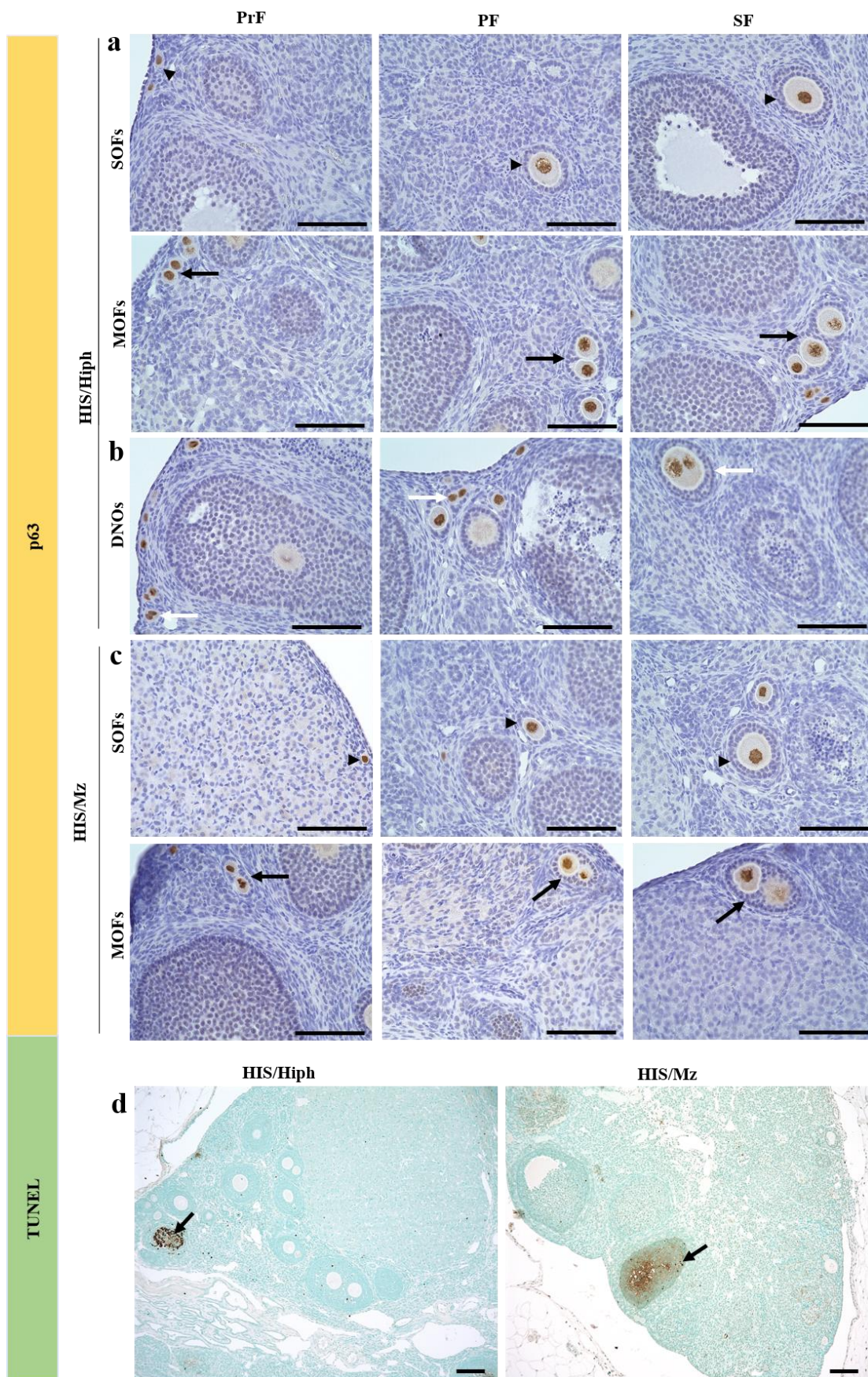


Figure 2-7. Evaluation of proliferative activity of oocytes in HIS/Hiph and HIS/Mz.

(a-e) Immunohistochemistry for PCNA. PCNA-positive oocytes in single oocyte follicles (SOF, a), multi-oocyte follicles (MOFs, b) and double nucleated oocytes (DNOs, c) are observed in the primordial follicles (PrFs) in HIS/Hiph (a-c). PCNA-positive SOFs and MOFs (weak expression) are observed in PrFs in HIS/Mz (d and e). **(f-j)** Immunohistochemistry for Ki67. Ki67-positivities are clearly observed in the granulosa cells (GCs) in tertiary follicle-type SOF (f) and secondary follicle type-MOFs (g) in HIS/Hiph. No positive reactions are observed in oocytes (f and g), GCs in primordial follicles, and DNOs (h) in HIS/Hiph. A few Ki-67-positive GCs are observed in the SOFs and MOFs (arrows) of HIS/Mz. Scale bars 100 μm or 50 μm (inset). ND: Not detected.

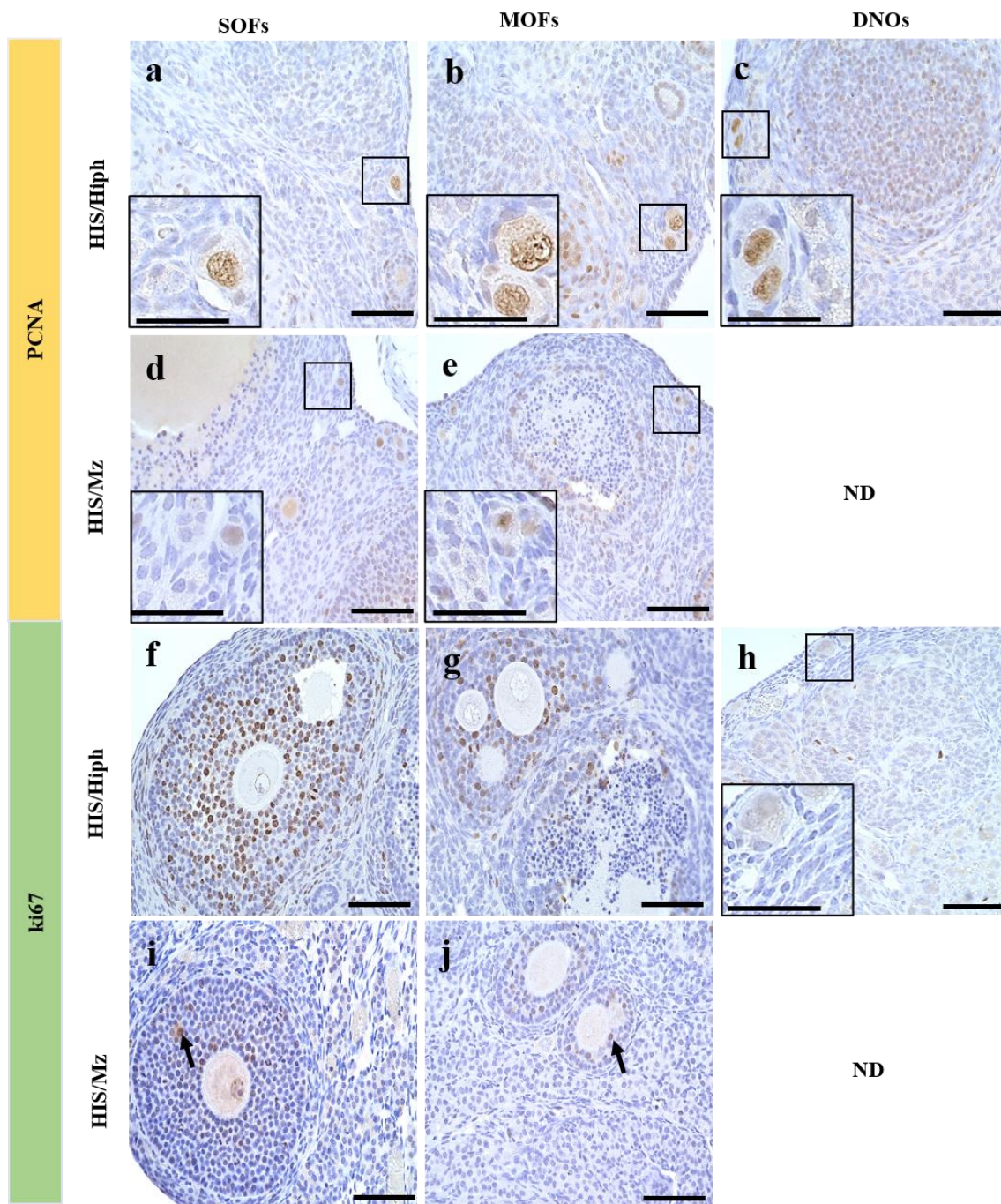


Figure 2-8. Ultrastructures of ovarian follicles and oocytes in HIS/Hiph and HIS/Mz.

Panels are transmission electron microscopy images of the ovary sections of four-weeks old HIS/Hiph and HIS/Mz. Panels a, c, e and b, d show representative images of HIS/Hiph and HIS/Mz ovaries, respectively. Panel a indicates a single oocyte follicle (SOF) with a clear round-shaped nucleus (N) and mitochondrial clouds (arrows) distributed throughout the cytoplasm and oriented as a grape-like structure. Panel c shows a multi-oocyte follicle (MOF) with differentiated oocytes (O1 and O2) separated by the clear zona pellucida (ZP) (arrowhead). Panel e shows double-nucleated oocytes (DNOs), and the nuclei (N1 and N2) that are separated by mitochondrial clouds (MCs) (arrows). Panels b and d show the SOF and MOF in the HIS/Mz ovary, and a few MCs (arrows) are observed in SOF and MOF, and each MC shows a low electron density as compared to that of HIS/Hiph MCs. Scale bars = 5 μm in panel a; 2 μm in panels b, c, and e; and 10 μm in panel d. ND: Not detected.

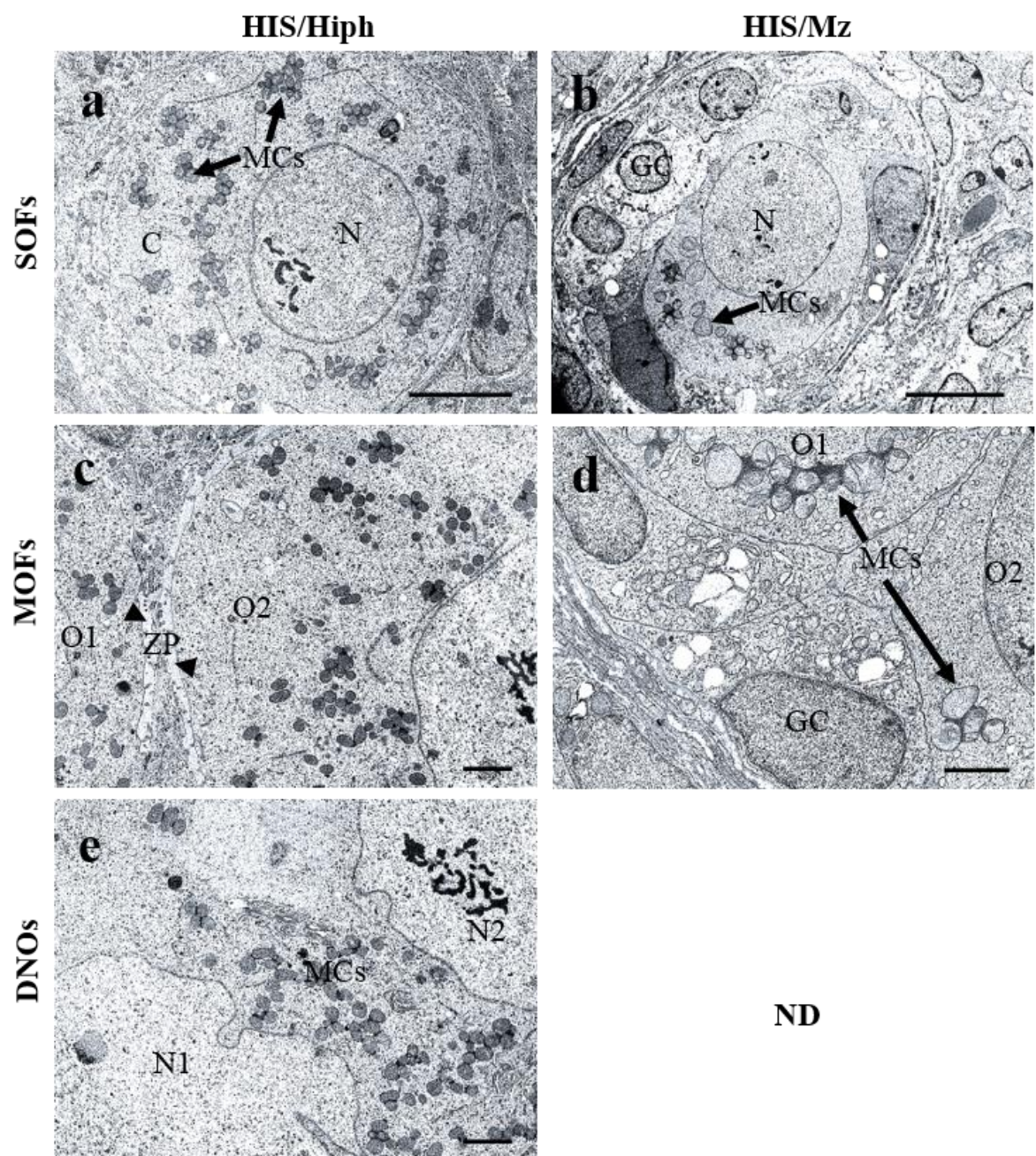
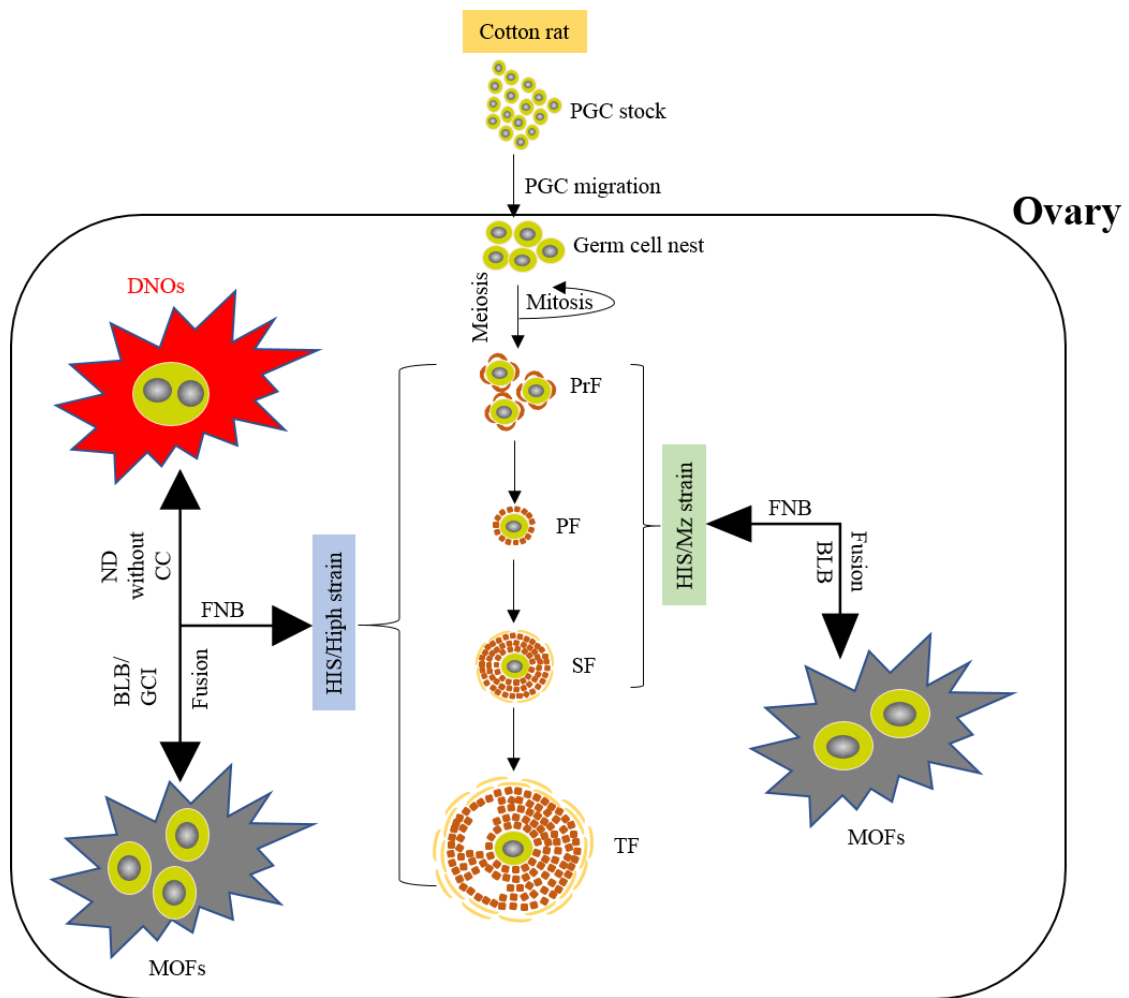


Figure 2-9. Schematic presentation of cotton rats ovarian morphology and it's disturbances in between HIS/Hiph and HIS/Mz inbred strains.

The cotton rat ovary follicular growth and development of follicular disturbances in between HIS/Hiph and HIS/Mz inbred strains at four weeks of age. In general, proliferating germ cells (PGCs) migrate to the ovary to form germ cell nest. Nests breaking germ cells enter meiosis and form primordial follicles (PrF). After that, formation of multi-oocyte follicles (MOFs) might be the fusion of two individual oogonia or failure of nest breaking (FNB) or fusion of follicles or basal lamina breaching (BLB) or granulosa cell invasiveness (GCI), and double nucleated oocytes (DNOs) by fusion of two individual oogonia or nuclear division (ND) of oocytes without cytoplasmic cleavage (CC) in all types of follicles including PrF, primary (PF), secondary (SF), and tertiary (TF) at HIS/Hiph strain during folliculogenesis . In contrast, the formation of MOFs upto SF at HIS/Mz strain might be caused by same reasons of HIS/Hiph strain except GCI, but there is no development of DNOs.



Chapter 3

Unique morphological characteristics in the ovary of cotton rat

Introduction

Follicular development from primordial to antral begins at the early postnatal development stage and continues until the late term of the life span (Oktem and Urman, 2010). Significant efforts have been made in recent years to elucidate the factors and mechanisms regulating folliculogenesis, since the size of the PrF population is determined, at least partly, by the reproductive life span of female mammals.

The appearance of follicular disturbances (DNOs or MOFs) varies with the development of follicular stages, age, puberty, estrous cycles, and other unknown factors. In Chapter 1, the author clarified neonate HIS/Hiph up to 7 days of age when the follicles develop up to secondary ones, MOFs and DNOs observed in all stages, and with ages MOFs increases but DNOs decreased. On the other hand, in Chapter 2, the author compared the strain differences between HIS/Hiph and HIS/Mz at four weeks of age where the difference was observed in follicle population, MOFs, DNOs and MCs.

As the appearance of MOFs and DNOs varied in neonates and puberty ages of two strains, and also the frequency of MOFs in the ovaries is species- and individual dependent. For the opossum, mouse, pig and goat, MOFs are common in the ovaries of younger rather than older females (Hartman 1926; Davis and Hall 1950; Lucci et al., 1999; Stankiewicz et al., 2009). In CRs, the females usually undergo their first estrus between 20 and 30 days of age (spring onset is at 30-40 days of age). The estrus cycle averages 9 days with a range of 4 to 20 days (not stable), gestation length is 27 days, and hair growth is present at birth (Faith et al., 1997). Laboratory-reared SH females also undergo first estrus between 20 and 30 days of age and generally attain puberty between 30 and 40 days of age. CR is prolific in nature and produces nine litters per

year, averaging five to six pups per litter (Faith et al., 1997). Further, in chapter 2 showed, there was no difference in the number of pups or their sex ratio between the two strains, but the number of HIS/Mz follicles was significantly lower than that of HIS/Hiph, almost half.

Therefore, in this Chapter, the author examined the ovaries of HIS/Hiph at 6-8 weeks of age that had reached the fertile stage were scrutinized.

Materials and Methods

Ethical statements

The author was adopted to ethical guideline during experimental procedures of all animals as described in Chapter 1.

Animals and tissue processing

In this Chapter, the author used HIS/Hiph at 6–8 weeks old of age, previously classified as the young period (Ichii et al., 2018), after obtaining puberty and experiencing several estrus cycles. Animals rearing, sample collection and processing methods were performed as described in Chapter 1.

Light microscopy and histomorphometry

The paraffin blocks of 5 ovarian specimens were cut at a thickness of 5 μm to obtain whole ovarian semi-serial sections and stained with HE or PAS-H to examine the morphological characteristics of the HIS/Hiph ovarian follicles.

For histoplanimetry using HE stained sections, ovarian follicles were classified as described in Chapter 1 but their numbers were counted at 7-section intervals (whole ovaries were sectioned, resulting in at least 50 sections per ovary), and the number of various developmental follicles was multiplied by 7 to get an assessment of the total number of follicles per ovary (Gaytán et al., 2014; Su et al., 2013). The number and percentage of MOFs or DNOs were also counted as Chapter 1.

For the determination of oocyte polarity, 10 PFs and SFs containing single and multiple oocytes were randomly selected from each ovarian specimen. Selected oocytes

were split by a line passing by the center of the nucleus with the NDP.view2 software, and such a line was divided into two sections, indicating the long distance (LD) and short distance (SD) between the nuclear membrane and the plasma membrane of oocytes. Measurements were taken for both LD and SD.

Immunohistochemistry

This method was performed as described in Chapter 1.

Electron microscopy

This method was performed as described in Chapter 1.

Statistical analysis

The results were expressed as the mean $\pm$ standard error. Statistical analysis was performed as described in Chapter 1.

Results

General histology of the HIS/Hiph ovary

Figure 3-1 shows the histological characteristics of the HIS/Hiph ovary. The ovarian cortex and medulla developing follicles, and the corpus luteum were observed in the cortex, similar to other rodent species (Fig. 3-1a). Furthermore, the follicles which progressed to later developmental stages, such as SF, localized to the superficial or deep region of the cortex, as previously reported in rodents (Francis and Brambell, 1997) or rabbits (Al-Mufti et al., 1988). Moreover, the interstitial gland, a characteristic of the rodent ovary (Miyabayashi et al., 2015), was observed throughout the cortical stroma (Fig. 3-1b) and clusters of epithelioid cells resembled luteal cells in the magnified area of panel b. According to the criteria of Chapter 1 and 2, the developing follicles were classified as PrF, PF, SF, and TF (Fig. 3-1c-f).

For histoplanimetry (Fig. 3-1g and h), the follicle number at each developmental stage decreased as follicular development progressed. PrF showed the highest number and percentage of follicles, and a significant difference was detected compared with those of TF ($P < 0.01$). PrF also showed the higher percentage of follicles compared to SF ($P < 0.01$). PF showed a significantly higher number ($P < 0.05$) and percentage ($P < 0.01$) of follicles compared to TF. Further, PF showed a significantly higher percentage of follicles compared to SF ($P < 0.01$).

MOFs in the HIS/Hiph ovary

A MOF is defined as a SOF containing two or more oocytes (Fig. 3-2). The HIS/Hiph ovary showed MOFs in all the developmental stages of follicles, including

PrF, PF, SF, and TF (Fig. 3-2a-d). MOFs classified as PrF (Fig. 3-2a) were similar to oocyte nests as found in prenatal or neonatal ovaries (Tingen et al., 2009). In particular, the ZP was clearly observed in MOFs classified as SF or TF, similar to SOFs (Fig. 3-2c and d). The MOFs frequently observed in histology images seemed to be unique histological features of the HIS/Hiph ovary.

In Figure 3-2e and f, the highest number of MOFs was observed in those classified as PF, compared to that observed in other developmental stages of the ovarian follicle in the histoplanimetric analysis. Significant differences were observed in PF and SF compared with PrF and TF MOFs ($P < 0.01$). Furthermore, MOFs classified as SF also showed significantly higher values compared with those of TF ($P < 0.01$), while the percentage of MOFs classified as SF was higher compared with the percentage of those classified as PrF, PF and TF.

Most MOFs had 2 oocytes in a single follicle, but some of them had 3 or more (Fig. 3-3a-c). The sizes of MOFs were 2 to 3 times larger compared to the follicles containing single oocytes because of the increased number of oocytes and GCs. The shape of the MOFs in CRs also showed morphological variation, especially those in PrF, which were elongated, triangular, or rounded (Fig. 3-3a). The oocytes of MOFs seemed to be separated by FECs or GCs (Fig. 3-3c). An observation of semi-serial sections showed that the apparently single follicles (Fig. 3-3d and e) in one section were connected to MOFs in the next section (Fig. 3-3f), indicating that some MOFs have fine interconnections via GCs.

Characterization of SOFs and MOFs by immunostaining

The author immunohistochemically examined the expression of the pan-oocyte

marker DDX4 and the FEC marker FOXL2 in the HIS/Hiph ovary. As shown in Figure 3-3g-i, in addition to the single follicles (Fig. 3-3g and i), the cytoplasm of oocytes in the MOFs classified as PrF (Fig. 3-3g), PF (Fig. 3-3h), SF and TF (Fig. 3-3i) showed positive expression for DDX4. Furthermore, the oocyte nucleus in MOFs showed polarity of localization in the cytoplasm (Fig. 3-3g-i). As shown in Table 3-1, the measured LD between the nuclear membrane and the plasma membrane of oocytes in PFs and SFs containing single and multiple oocytes was significantly greater than the respective SD ($P < 0.01$). Moreover, FOXL2 expression was observed in the nuclei of FECs in SOFs (Fig. 3-3j) and MOFs (Fig. 3-3k-l). However, Ki67 staining demonstrated the proliferative activity of GCs, but not of oocytes, in the follicles, described in previous Chapters. The majority of the MOFs had been developed before the formation of the theca cell layer. However, the role of theca cells in follicular function has received less attention compared with intensive investigations into the role of GCs (Tajima et al., 2007). There were no visible differences in theca cell layer thickness between SOFs and MOFs, with SF and TF having SOFs (Fig. 3-3m), MOFs in SF (Fig. 3-3n) and TF (Fig. 3-3o).

DNOs in the CR ovary

Figure 3-4 shows the appearance of DNOs, containing two nuclei, in developing follicles including PrF (Fig. 3-4a), PF (Fig. 3-4b), and SF (Fig. 3-4c). The nuclei are closely attached (Fig. 3-4a-b) or separated (Fig. 3-4c) within the same cytoplasm of follicles. Normally, SOF contained single nucleus and size of the nucleus seemed to be largest in initial stages as PrF (Fig. 3-4d). The majority of them were found in PrF which was beneath the peripheral area and the remaining DNOs lied either superficially

or deep in the cortex of HIS/Hiph ovaries. Notably, mature follicles such as TF did not contain any DNOs (Fig. 3-4d and e). DNOs also showed DDX4-positivity in the cytoplasm of PrF, PF and SF (Fig. 3-4d-f), respectively.

The histoplanimetric data for DNOs are shown in Figure 3-4g and h. Briefly, from all the examined sections, the PrF showed the highest number of DNOs, while the number of DNOs was sharply decreasing in the consecutive follicles. However, no significant differences were detected. Furthermore, while no significant differences were observed, the percentage of DNOs in each follicular stage appeared to be higher in PrF compared to that in other types of follicles.

Ultrastructure analysis of HIS/Hiph ovary

Figure 3-5 shows the ultrastructural analysis of follicles by TEM. Figure 3-5a shows a representative image of a PrF having a single oocyte. It has a clear round-shaped nucleus, and the MCs of the HIS/Hiph oocyte have a unique structure. Indeed, the MCs are distributed throughout the cytoplasm of the oocyte. On the other hand, the oocytes in MOFs classified as PrF were separated by FECs (Fig. 3-5b). Moreover, MCs aggregated to one side of the cytoplasm of MOF oocytes (Fig. 3-5b and c), in contrast with those observed in the follicles having a single oocyte (Fig. 3-5a). Figures 3-5d and e show a more developed MOF, where oocytes are separated by FECs or GCs. However, the border structure, such as the BL separating each oocyte is unclear, indicating that oocytes in MOF shared the GCs in the follicles. Furthermore, almost all oocytes of MOFs possessed a clear ZP, but their thickness differed among oocytes (Fig. 3-5d and e). Unfortunately, the author did not find DNOs during observation of ultrathin HIS/Hiph ovary sections by TEM.

Discussion

In this Chapter, the author clarified the abundance of MOFs and DNOs in HIS/Hiph at 6-8 weeks of age in relation with the findings of Chapter 1 and 2. Furthermore, MOFs appeared in all stages of follicles but DNOs upto SF stages.

The presence of MOFs in adult ovaries has been reported in mammals (Payan-Carreiraa and Pires, 2008; Ireland et al., 2008; Lucci et al., 1999). Moreover, some researchers found MOFs in rodents upon treatment with synthetic estrogenic hormones (Jefferson et al., 2002) or the estrogen receptor β agonist 2,3-bis(4-hydroxyphenyl)-propionitrile (Sato et al., 2018), as well as in ovaries of AQP8 knockout (Su et al., 2013) or p27Kip1 mutant mice (Pérez-Sanz et al., 2013). Furthermore, the frequency of MOFs differs among species or individuals (Table 3-2), with opossums showing high incidence of MOFs (e.g. approximately 100 oocytes per follicle), and almost 70% of female dogs having more than one MOF in their ovary (Reynaud et al., 2009). Since the appearance of MOFs in HIS/Hiph decreased in TF compared with PF and SF, almost all of them would disappear until the formation of the antral follicle. However, a few MOFs might be ovulated. It has been suggested that the smallest oocytes in a MOF might be arrested in development and probably degenerate, and all disappear except one, or that all might undergo atresia and never reach the stage of extrusion of the first polar body (Kennedy, 1924). Whenever the number of follicles in MOFs was higher than three, they would be present at different developmental stages. Some of these MOFs, where the differences among cells are more pronounced, would possibly undergo degeneration (Payan-Carreiraa and Pires, 2008). However, it has also been suggested that MOFs might be ovulated in female dogs (Reynaud et al., 2009).

Further studies focusing on the ovulation and fertility features of oocytes derived from MOFs are required to clarify the characteristics of the reproductive function of MOFs.

The characteristics and the hypotheses of mechanisms of MOFs and DNOs were similar as discussed in Chapter 1 and 2. Furthermore, the existence of twin oocytes in the follicle increasing the rate of twinning in the giant panda (Zhang et al., 2001), and recently in human reported successfully live birth from conjoined oocytes (Yano et al., 2017). Therefore, the abundance of MOFs and DNOs may play a role in twin research.

The ultrastructural analysis of HIS/Hiph ovaries shows that almost all oocytes of MOFs have a clear ZP. The thickness of the ZP in mammals is usually similar among oocytes (Payan-Carreiraa and Pires, 2008), but that of the MOF differs. These data indicate a partial dysfunction of oocytes in MOFs, even though these can still express the representative oocyte marker DDX4. However, the difference in polarity between MOFs and SOFs is clarified ultrastructurally by the presence of MCs in HIS/Hiph. The author hypothesized that such MCs aggregated toward the LD between the nucleus and the plasma membrane (Table 3-1). Oocyte polarity and embryonic patterning are well-established features of development in lower species (Edwards and Beard, 1997). In particular, the distribution of organelles in oocytes shows dramatic asymmetry resulting in a structural and molecular polarity (Wodarz, 2002). However, there is a profound conceptual difference between cell asymmetry and polarity: indeed, the asymmetrical distribution of molecules and/or organelles within a cell does not impose or necessarily indicate the existence of cell polarity (Wodarz, 2002).

Furthermore, MCs tended to aggregate to one side of the cytoplasm of MOF oocytes that was the completely different orientations from neonate and puberty ages, as MCs were distributed throughout the cytoplasm of oocytes. In mouse, fully developed and

easily recognizable MCs are present in the oocytes of 17.5 days embryos, persists up to PND3 and disperse in the majority of PND4 oocytes (Kloc et al., 2004). Concomitantly, in young High/Hiph MCs re-orientation might be affected by ovulatory cycle and hormonal activities and other unknown factors. Although the functional significance of MCs in HIS/Hiph is unclear, polarity located MCs in *Xenopus* oocytes is known to be associated with the germinal granules responsible for the determination of the germ cell fate (Malgozata et al., 2008).

In conclusion, the appearance of MOFs and DNOs, are maintained from the neonatal period to the fertile period, and the abundance of HIS/Hiph MOFs and DNOs may play a role in ovulation and fertility related research.

Summary

In Chapter 3, the author examined the ovaries of HIS/Hiph at 6-8 weeks of age that had reached the fertile stage were scrutinized, by using Chapter 1 and 2 methodology.

Similar with puberty age, MOFs found in all developing stages of follicles whereas DNOs up to secondary stages as found in neonate age. Moreover, MOFs tended to be frequently observed in PrF and PF while DNOs in PrF one. Similar characteristics of MOFs were also observed as described in neonate and puberty ages, but the GCI characteristics was more frequently observed in the later developing follicles including SF and TF. The proliferative activities and hypothesis of mechanisms of MOFs and DNOs seemed to be similar with Chapter 1 and 2. Moreover, with ages the MCs distributed to one side of the cytoplasm of oocytes in SOFs, MOFs and DNOs rather than throughout the cytoplasm of oocytes by TEM.

These results clarified the unique phenotypes found in the CR ovary are maintained from the neonatal period to the fertile period.

Tables and Figure

Table 3-1. The polarity of SOFs and MOFs as determined by the measurement of distances (long distance [LD] and short distance [SD]) between the nuclear membrane and the plasma membrane of oocytes in the HIS/Hiph ovary.

Follicle type	PF		SF	
	LD (µm)	SD (µm)	LD (µm)	SD (µm)
SOFs	10.8 ± 0.8 *	7.2 ± 1.0	18.4 ± 1.4 *	12.5 ± 0.8
MOFs	13.9 ± 1.0 #	4.2 ± 0.6	23.6 ± 3.0 #	9.0 ± 1.4

Values are reported as mean ± SE. * and # indicate significant differences between LD and SD, as found by Mann-Whitney *U*-test (*: SOF-LD vs SD and #: MOF-LD vs SD, $p < 0.01$). n = 50 oocytes (10 randomly selected oocytes/ovary).

Table 3-2. Frequency of MOFs in different mammalian species.

Animals	Category	Frequency (%) (observed/total follicles)	Reference
Goat	Prepubertal	100.0 (6/6)	Lucci et al., 1999
	Non pregnant	50.0 (3/6)	
	Pregnant	17.0 (1/6)	
Dog	Prepubertal	68.4 (13/19)	Payan-Carreira and Pires, 2008
	Mature	36.6 (48/131)	
Pig	Gilts	6.4 (49/819)	Stankiewicz et al., 2009
	Sows	1.4 (9/659)	
Cattle	Fetus	40.0 (8/20)	Silva-Santos et al., 2011
	Heifer	37.5 (9/24)	
	Cow	45.0 (9/20)	
The table shows previously published data on the frequency of multi-oocyte follicles (MOFs) in 4 mammalian species (goat, dog, pig, cattle).			

Figure 3-1. General histology of the ovary of HIS/Hiph.

(a-g) Histological observation of hematoxylin eosin-stained ovary section from HIS/Hiph at 6-8 weeks of age. The ovarian cortex (CO) and medulla (MD) are clearly observed in the HIS/Hiph ovary, and the vascular system entering the ovarian hilus (panel a). Panel b shows the interstitial glands. Panels c, d, e, and f show primordial (PrF), primary (PF), secondary (SF), and tertiary follicles (TF), respectively. Arrows in panels c-f indicate the PrF, PF, SF, and TF, respectively. Scale bar = 100 μ m. (g and h) The total number and percentage of each developing follicle in the HIS/Hiph ovary at 6-8 weeks. Values are mean $\pm$ SE. The letters (a, b, c, d) denote statistical significance with primordial (PrF), primary (PF), secondary (SF), and tertiary follicles (TF), respectively (Kruskal-Wallis test followed by Scheffe's method, $p < 0.05$). The apostrophe beside the letters indicates highly significant differences compared to TF ($p < 0.01$). N = 5 ovaries (at least 50 sections/ovary).

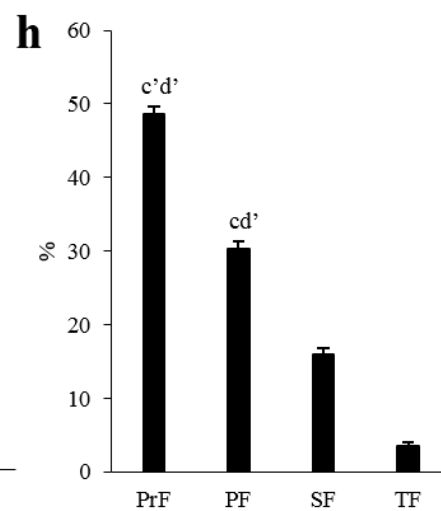
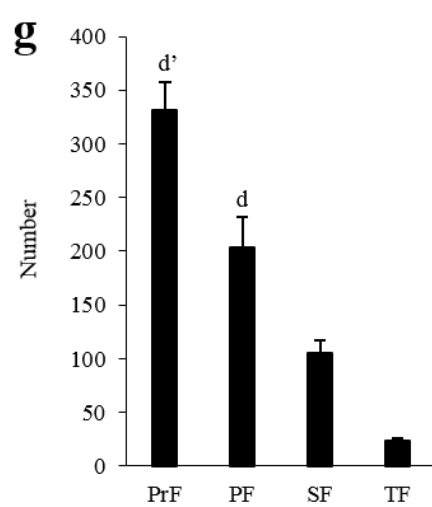
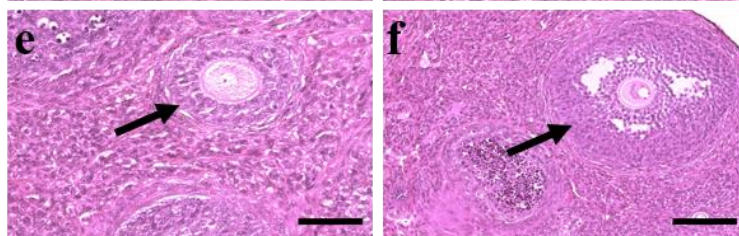
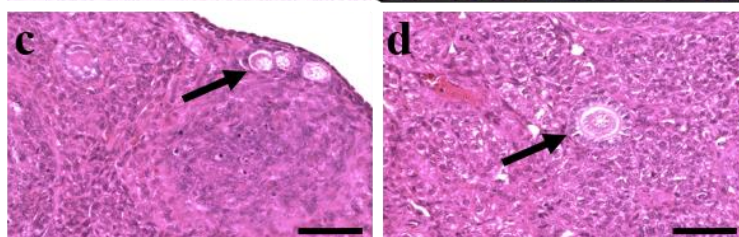
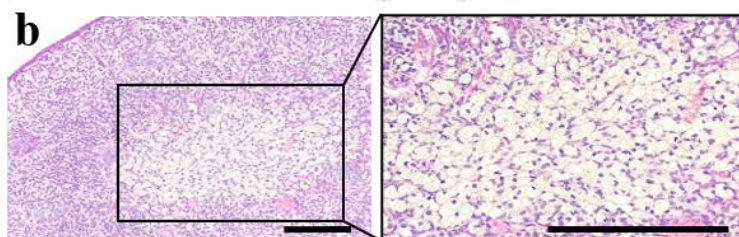
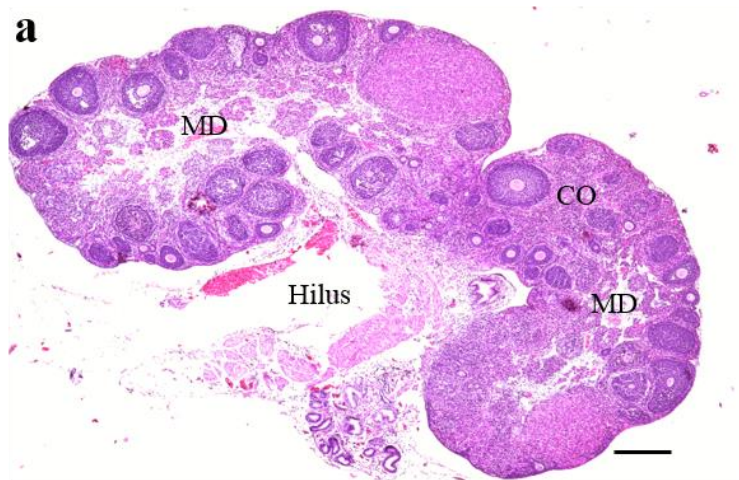


Figure 3-2. Appearance of multi-oocyte follicles in the ovary of HIS/Hiph.

(a-d) Histological observation of periodic acid Schiff-hematoxylin (PAS-H)- stained ovary section in the HIS/Hiph at 6-8 weeks of age. Multi-oocyte follicles (MOF) are observed in the HIS/Hiph ovary, and are classified as PrF, PF, SF, and TF (panels a-d, respectively). Square area in panel a indicate the same area of the inset. Arrows in panel b-d indicate the PF, SF and TF, respectively. In panel b-d, PAS-H- positive zonae pellucidae are clearly observed. Scale bar = 100 μ m. **(e-f)** The total number and percentage of MOFs classified by the stage of developing follicle in the CR ovary at 6-8 weeks. Values are mean $\pm$ SE. The letters (a, b, c, d) denote statistical significance with primordial (PrF), primary (PF), secondary (SF), and tertiary follicles (TF), respectively (Kruskal-Wallis test followed by Scheffe's method, $p < 0.05$). The apostrophe beside the letters indicates highly significant differences compared with PrF and TF ($p < 0.01$). N = 5 ovaries (at least 50 sections/ovary).

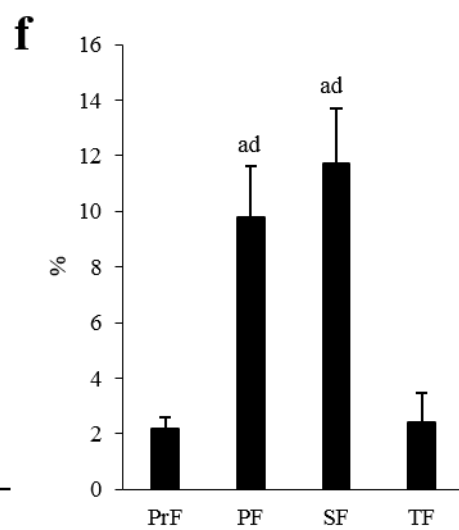
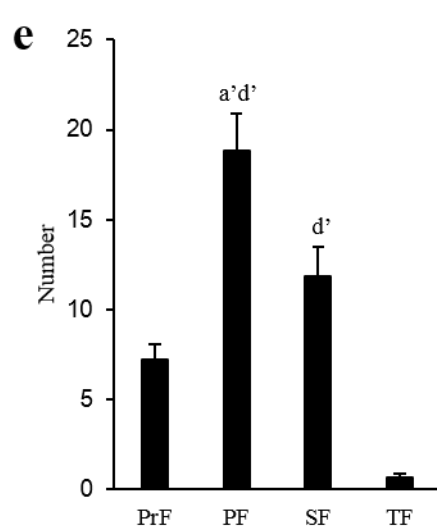
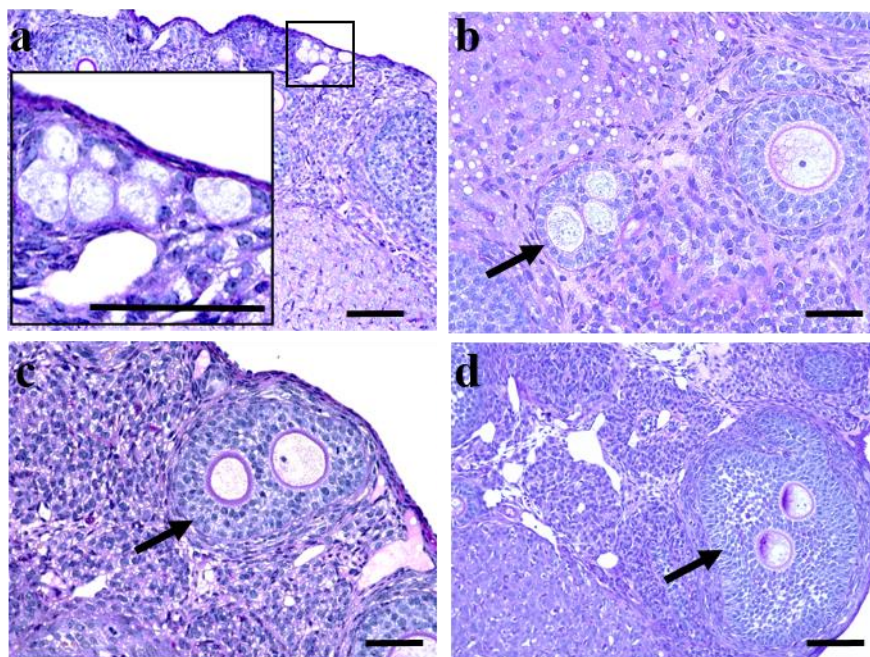


Figure 3-3. Unique morphological features of multi-oocyte follicles in the ovary of HIS/Hiph.

(a-c) Histological observation of hematoxylin eosin-stained ovary section in HIS/Hiph at 6-8 weeks of age. More than two oocytes were observed in single follicles classified as PrF, PF, or SF (panels a-c, respectively), and each oocyte was separated by follicular epithelial cells (FECs) or granulosa cells (GCs). Squares in panels a-b indicate the same area of the inset. The star in panel c indicates the common GCs contained in SF MOFs. Scale bar = 100 μ m. (d-f) Periodic acid Schiff-hematoxylin (PAS-H)-stained semi-serial sections of the ovary in HIS/Hiph at 6-8 weeks of age. Single oocyte follicles (SOFs) (arrows, panels d and e) are connected with near multi-oocyte follicle (MOF) via fine connections of GCs (arrowheads, panel f). Scale bar = 100 μ m. (g-i) Immunohistochemistry for the detection of oocyte maker in the ovary of HIS/Hiph at 6-8 weeks of age. DEAD-box helicase 4 (DDX4)-positive reactions are observed at the cytoplasm of the oocyte in the SOFs (arrowheads, panels g and i). DDX4-positive reactions are also observed in the cytoplasm of MOFs classified as PrF (square area), PF and SF (arrows) (panels g-h, respectively). Square area in panel g indicate the same area of the inset. Scale bar = 100 μ m. (j-l) Immunohistochemistry for the detection of the FECs marker in the ovary of HIS/Hiph at 6-8 weeks of age. Forkhead box L2 (FOXL2)-positive reactions are observed at the FECs in the SOFs (arrowheads, panels j and k) and MOFs (arrows, panel l). Scale bar = 100 μ m. (m-o) Periodic acid Schiff-hematoxylin (PAS-H)-stained semi-serial sections of the ovary in HIS/Hiph at 6-8 weeks of age. The thickness of the theca cell layer (double arrow heads, panels m, n and o) in SOFs (arrow heads) and MOFs (arrows). Scale bar = 100 μ m.

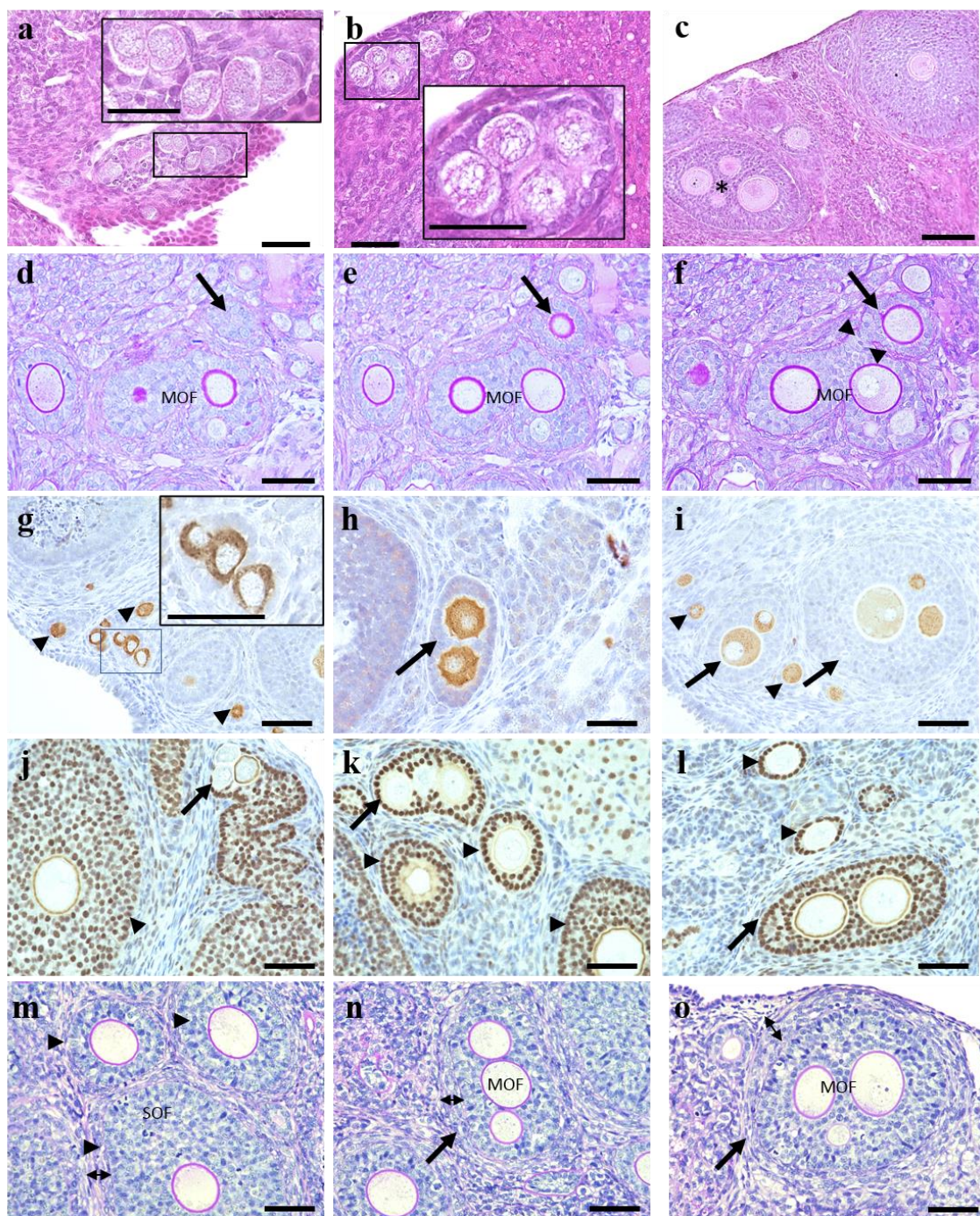


Figure 3-4. Double nucleated oocytes in the ovary of HIS/Hiph.

(a-c) Histological observation of hematoxylin eosin-stained ovary section in the HIS/Hiph at 6-8 weeks of age. Two nuclei are observed within a single oocyte in the follicles classified as PrF, PF, SF (panels a-c, respectively). Each nucleus (asterisks) shares the same cytoplasm. (d-f) Immunohistochemistry for the detection of the oocyte maker in the ovary of HIS/Hiph at 6-8 weeks of age. DEAD-box helicase 4 (DDX4)-positive reactions are observed at the cytoplasm of double nucleated oocytes (DNOs) (arrow) classified as Pr, PF, SF (panels d-f, respectively). Scale bar = 100 μ m. (g and h) The total number and percentage of DNOs) classified by the stage of follicular development in the HIS/Hiph ovary at 6-8 weeks. Values are mean $\pm$ standard error. ND: not detected. N = 5.

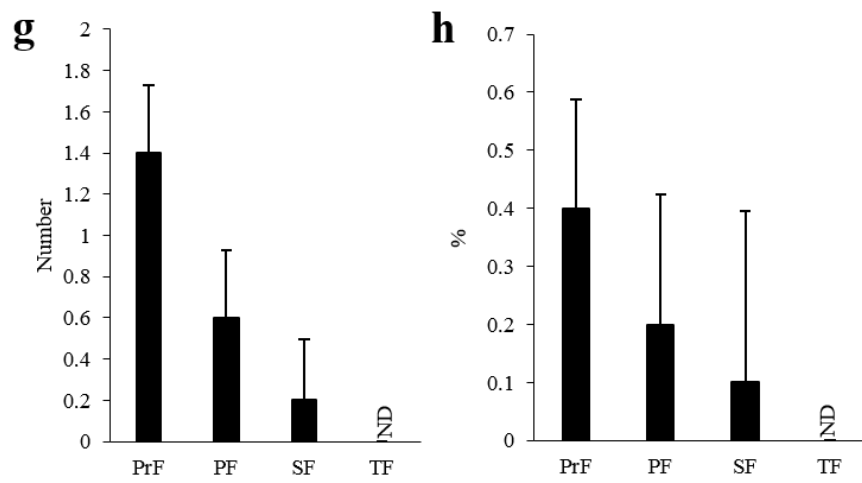
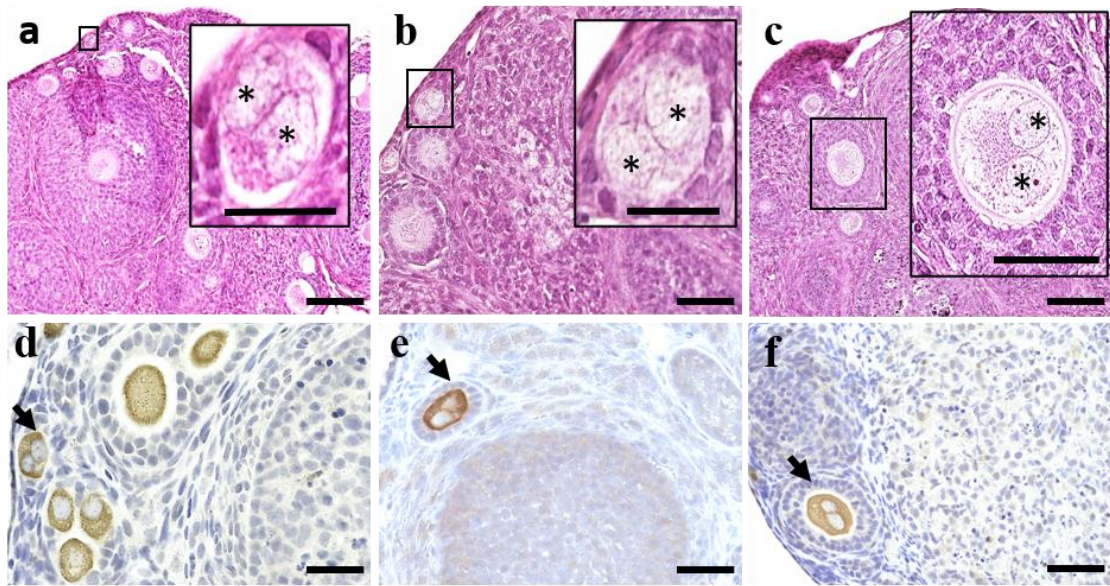
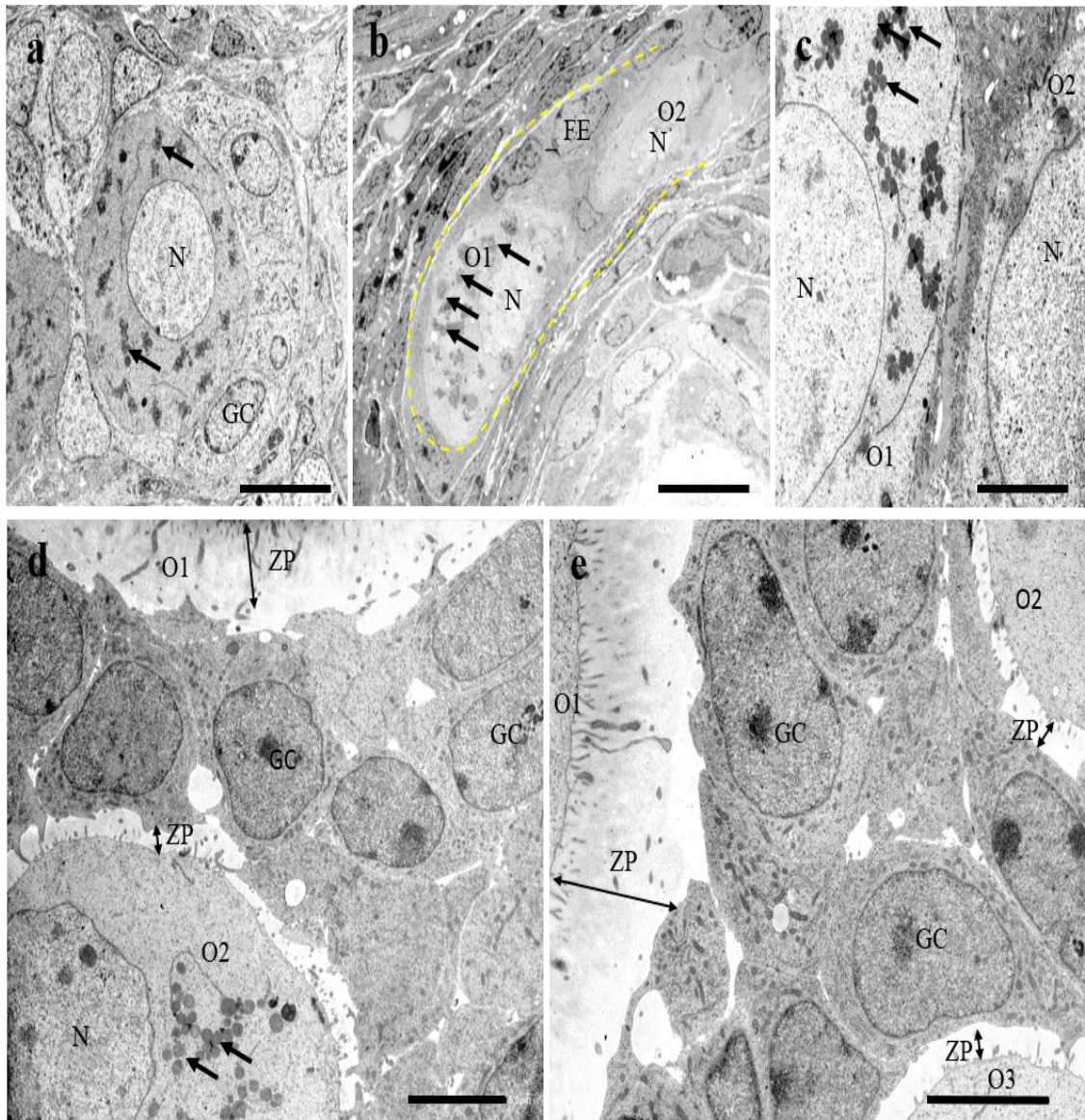


Figure 3-5. Ultrastructural features of multi-oocyte follicles in the ovary of HIS/Hiph.

Panels are transmission electron microscopy images of the ovary section of HIS/Hiph at 6-8 weeks of age. Panel a display a representative PrF having a single oocyte with a clear round-shaped nucleus (N) and clouded mitochondria (arrows) distributed throughout the cytoplasm. Panel b shows a MOF classified as PrF. Oocytes (O1 and O2) are separated by follicular epithelial cells (FECs). Mitochondrial clouds (arrows) aggregated in the MOF (panels b-c), in contrast with the follicles having a single oocyte (panel a), and they tended to localize to one side of the cytoplasm. Panel d and e shows the more developed MOF, and oocytes are separated by granulosa cells (GC). The border structure separating each oocyte is unclear. Oocytes of MOFs in panel d and e possess a clear zona pellucida (ZP), and their thickness differs among oocytes. Scale bar = 5 μm in panels a, b, and c., and 10 μm in panels d and e.



Conclusions

The CR is commonly used as an experimental animal model in biomedical research, especially infectious disease-related experiments. Besides, the morphology of its urogenital and gastrointestinal organs, such as the kidney and uterus, and intestine, is known to have a characteristic phenotype, but there is no information on the morphology of the ovary. In this study, the ovarian morphology, especially folliculogenesis, of CR inbred strains, HIS/Hiph (from the Hokkaido Institute of Public Health) and HIS/Mz (from the University of Miyazaki), was intimately examined and their characteristics were clarified.

In Chapter 1, the author histologically examined the neonatal HIS/Hiph ovaries at PND0, 4, and 7. The follicles were developed up to secondary stages at these ages while MOFs and DNOs were also found. DNOs were observed at PND0 whereas MOFs started to develop from PND4. With ages, the follicle population was decreasing by cell death. Subsequently, with ages, DNOs were also decreased, but MOFs increase with the progression of the follicle development. Like SOF, MOFs expressed the oocyte marker DDX4, and fewer HIS/Hiph oocytes were positive TUNEL analysis than ICR mice, and GCs surrounding MOFs and DNOs showed proliferative activity. Moreover, like SOF, MCs assembly was observed throughout the cytoplasm of MOFs and DNOs. These results suggest that nest breakdown in HIS/Hiph, a decrease in the number of cell deaths associated with follicle closure, and changes in follicle structure due to proliferation and infiltration of GCs layer may affect the appearance of MOFs and DNOs. Although MOFs and polynucleated oocytes have been reported in several mammals, their significance remains unclear. The author findings on the ovarian phenotype of HIS/Hiph

may lead to a better understanding of their development.

In Chapter 2, the author examined the 4-week-old age as the time when the first estrus and spring onset of CR are observed. Thus, the author clarified the strain differences in the CRs, used HIS/Hiph and HIS/Mz and compared their pup numbers and ovarian morphology at 4 weeks of age. There was no difference in the number of pups or the sex ratio of litters between the two strains, but the follicle population of HIS/Mz was significantly lower than that of HIS/Hiph, almost half. The frequency of MOFs in HIS/Hiph was 2-6 times higher than that in HIS/Mz. Like neonate ages, MCs assembly was observed throughout the cytoplasm of all oocytes. Proliferative activities (PCNA/Ki67) tended to be stronger in HIS/Hiph but cell death (p63) expression were normal in both strains. In this chapter, the author found that the neonate phenotype of HIS/Hiph ovaries was also observed at 4 weeks of age and was partially common among another strains, while the number of follicles and the appearance of MOFs and DNOs depended on the genetic background of CR.

In Chapter 3, the ovaries of CRs (HIS/Hiph, 6-8 weeks old) that had reached the fertile stage were scrutinized. MOFs was found in all follicular stages and DNOs also found except tertiary follicles. Like neonate ages, MOFs and DNOs were maintained the expression of DDX4 but MCs assembly was polarized in the cytoplasm of oocytes. These results indicate that the characteristic phenotypes of CR ovaries, especially the appearance of MOFs and DNOs, are maintained from the neonatal period to the fertile period.

Therefore, the analysis of unique phenotypes of the CR ovary might contribute to the understanding of mammalian folliculogenesis, oocyte formation, and follicular disorders, indicating that CRs might be useful as an animal model in these areas.

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Conclusions in Japanese

コットンラット (*Sigmodon hispidus*) の卵胞形成にみられる特異な形態機能

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エムディー ラシェドウル イスラム

コットンラット (*Sigmodon hispidus*, CR) は感染実験等に汎用される実験げっ歯目である。加えて、その腎臓や子宮等、尿生殖器の形態には特徴的な表現型がみられることも知られているが、卵巣の形態に関する情報は無い。本研究では、CRの近交系であるHIS/Hiph (北海道立衛生研究所由来) とHIS/Mz (宮崎大学由来) の卵巣形態、特に卵胞形成を精査し、その特徴を明らかにした。

第1章では、生後0、4、7日目 (PND0、4、7) の新生子期HIS/Hiph卵巣を組織学的に解析した。これらの日齢では、原始卵胞、一次卵胞、二次卵胞が観察されたが、三次卵胞はなかった。CR卵巣の特徴的として、多卵細胞性卵胞 (MOF) と二核卵細胞 (DNO) が出現した。MOFはPND4から出現し、日齢および卵胞の発達と共に増加する傾向にあった。一方、DNOは全ての日齢で出現したが、卵胞の発達と共に減少した。単卵細胞性卵胞 (SOF) と同様、MOFの卵細胞は卵細胞マーカーDEAD - box helicase 4 (DDX4)を発現した。またCR では、Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) 解析で陽性を示す卵細胞がICRマウスに比べて少なかった。SOFと同様、MOFやDNOを囲む顆粒層細胞は増殖活性を示した。また、SOF卵細胞と同様、ミトコンドリアの集合がMOF卵細胞やDNOの細胞質全体に観察された。これらの結果より、新生子期のCR卵巣におけるnest breakdownや卵胞閉鎖に伴う細胞死数の低下、顆粒層細胞の増殖・浸潤による卵胞構造の変化等がMOFやDNO出現に影響を与えている可能性が示唆された。MOFや多核卵細胞はいくつかの動物種でも報告されているが、その存在意義は未だ不明な点が多く、CRの卵

巣表現型に関する知見はそれらの出現の理解に繋がると考えられた。

第2章では、CRの初回発情や春期発動がみられる時期として、生後4週齢に着目した。また、CRの系統差を明らかにするため、HIS/HiphとHIS/Mzを用い、それらの産子数や生後4週齢の卵巢形態を比較した。2系統の産子数や産子の雌雄比に違いは見られなかったが、HIS/Mz卵胞数はHIS/Hiphよりも有意に少なく、ほぼ半数であった。さらに、HIS/HiphのMOF出現頻度はHIS/Mzの2～6倍程度であった。HIS/Hiphでは全ての卵胞ステージで、HIS/Mzでは三次卵胞を除くステージでMOFが認められた。HIS/HiphのDNOは新生子期同様、卵胞の発達と共に減少したが、HIS/MzではDNOが欠如していた。SOF卵細胞同様、両系統のMOF卵細胞はp63陽性を示し、卵細胞死抑制が示唆された。また、細胞増殖解析の特徴的な結果として、HIS/HiphのSOF卵細胞、MOF卵細胞およびDNOにおけるproliferating cell nuclear antigen(PCNA)の陽性反応は、HIS/MzのSOF卵細胞やMOF卵細胞のそれらに比べて強い傾向にあった。また、新生子期同様、ミトコンドリアの集合がMOF卵細胞やDNOの細胞質全体に観察された。本章では、CR卵巢の新生子期表現型は生後4週齢でも維持され、他系統間でも一部共通する一方、卵胞数、MOFやDNOの出現はCRの遺伝学的背景によって変化することを明らかにした。また、卵細胞の死や増殖に関連する機構が本表現型の形成や系統差に影響を与える可能性を考えた。

第3章では、妊娠可能期に達したCR（HIS/Hiph、6～8週齢）の卵巢を精査した。全ての卵胞ステージでMOFがみられた。また、DNOは三次卵胞を除く卵胞ステージでみられた。新生子期同様、MOF卵細胞やDNOは卵細胞マーカーDDX4の発現を維持した。新生子期や生後4週齢ではミトコンドリアの集合が卵細胞の細胞質全体に分布していたのに対し、解析週齢では卵母細胞の細胞質において極性を持って分布した。以上、CR卵巢の特徴的な表現型、特にMOFとDNOの出現は新生子期から妊娠可能期まで維持されていることが明らかとなった。

本研究において、著者は実験動物CRの卵巢に特異な表現型を見出した。特に新生子期から妊娠可能期の卵胞形成過程で出現するMOFやDNOは特徴的だった。その出現は、新生

子期のnest breakdownや卵胞閉鎖に伴う卵細胞死の抑制、顆粒層細胞の増殖・浸潤、さらには卵細胞自体の死または増殖の程度に影響を受け、遺伝学的背景も本表現型を変化させることが明らかとなった。CR卵巣にみられるMOFおよびDNOの精査は、卵母細胞発達、卵胞形成およびそれらの病理発生の理解に繋がり、CRは当該分野のモデル動物として有用である可能性が示された。