Studies on developmental dynamics and causing factors of testicular oocyte in MRL/MpJ mice

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Abbreviations

Actb: actin, beta

ADAM-1: a disintegrin and metallopeptidase domain 1

ADAM-2: a disintegrin and metallopeptidase domain 2

AJ: A/J

AKR: AKR/N

B6: C57BL/6

BALB: BALB/c

Bmp15: bone morphogenetic protein 15

C3H: C3H/He

CD9: CD9 antigen

Cyp26b1: cytochrome P450, family 26, subfamily b, polypeptide 1

D: day

DBA: DBA/2

Dhh: desert hedgehog

Dmc1: disrupted meiotic cDNA 1 homolog

DNA: deoxyribonucleic acid

E: embryonic day

EDTA: ethylenediaminetetraacetic acid

Fgf9: fibroblast growth factor 9

Figla: folliculogenesis specific basic helix-loop-helix

Foxl2: forkhead box L2

FSH: follicle stimulating hormone

Gdf9: growth differentiation factor 9

H1oo: H1 histone family, member O, oocyte-specific

HE: hematoxylin and eosin

HMG: high mobility group

IZUMO1: izumo sperm-egg fusion 1

LH: luteinizing hormone

lpr: MRL/MpJ-*lpr*/*lpr*

M: marker

M+: MRL/MpJ-+/+

Mos: moloney sarcoma oncogene

MRL: MRL/MpJ

Nobox: NOBOX oogenesis homeobox

Nr0b1: nuclear receptor subfamily 0, group B, member 1

Ntf3: neurotrophin 3

Omt2a: oocyte maturation, alpha

PAS: periodic acid Schiff

PBS: phosphate buffered saline

PCR: polymerase chain reaction

Pdgfra: platelet derived growth factor, alpha

PIG-A: phosphatidylinositol glycan anchor biosynthesis, class A

RNA: ribonucleic acid

RT: reverse-transcriptase

Sohlh1: spermatogenesis and oogenesis specific basic helix-loop-helix 1

Sohlh2: spermatogenesis and oogenesis specific basic helix-loop-helix 2

Sox9: SRY-box containing 9

Sry: sex-determining region on Y

Sycp3: synaptonemal complex protein 3

TE: tris-EDTA

TYH: Toyoda, Yokoyama, and Hoshi

Zar1: zygote arrest 1

Zp1: zona pellucida 1

Zp2: zona pellucida 2

Zp3: zona pellucida 3

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Preface

The cells of multicelluar organisms are divided into two large lineages, somatic cells and germ cells. Somatic cells differentiate in many kinds of cells depending on tissues and end their roles at the death of individual. On the other hand, germ cells are highly specialized cells to transmit genomic information to next generation. They differentiate in either sperms or oocytes which provide survival and diversity of species.

It has been generally believed that in mammals, males produce only sperms, and oocytes are only produced in females. It is also well known that vertebrates born as either males or females never change their sex during their lifetimes except the substantial number of teleosts (Policansky, 1982; Francis, 1992). Interestingly, it has recently been demonstrated that sperm-producing germ cells in rainbow trout could be differentiated into oocytes with biotechnological modifications (Okutsu et al., 2006).

The sex of a mammal is determined when an ovum containing a haploid genome with an X chromosome is fertilized by a sperm possessing a haploid genome with either an X or Y chromosome. However, the anatomical determination of sex occurs later in development as gonads first arise as bipotential primordia with the plasticity to develop into ovaries or testes. The molecular mechanisms involved in initiating sex differentiation have begun to unravel in these decades beginning with discovery of sex-determining gene, *Sry* (sex-determining region on Y). Determination of the bipotential primordia into male gonads requires expression of *Sry*, which initiates the differentiation of Sertoli cells, as well as their structural organization into a testis cord (Koopman et al., 1991). If the primordial gonad proceeds to develop along the ovarian cascade despite being in an XY animal, this could result in sexual reversal of the animal, or the development of ovotestes. This has been observed in animals with translocations or deletions of the *Sry* gene, or animals with delayed *Sry* expression (Berta et al., 1990; Koopman et al., 1991; Bullejos and Koopman, 2005). However, it has been

reported that there are many other genes involved in sex differentiation which abnormalities also cause male to female sex reversal, such as *Sox9* (SRY-box containing gene 9) and *Fgf9* (fibroblast growth factor 9) (Foster et al., 1994; Wagner et al., 1994; Colvin et al., 2001). Another factor contributing to the plasticity of sexual determination is the fact that the differentiation of germ cells into oogonia or prospermatogonia is directed by signals from somatic cells in the gonads, not by the germ cells themselves (McLaren, 1995). These findings led the author to hypothesize that genital glands have the potential to produce differentiated germ cells of opposite sex.

The MRL/MpJ (MRL) inbred mouse strains, MRL/MpJ-+/+ (M+) and MRL/MpJ-*lpr/lpr* (*lpr*), originate from crosses initiated in the 1960s that used a number of standard inbred strains (Murphy, 1981). The MRL strains are known to have several unique characteristics in regenerative wound-healing, such as those observed in ear punch closure and in cardiomyocyte regeneration (Clark et al., 1998; Leferovich et al., 2001) and in the development of several autoimmune diseases, such as systemic lupus erythematosus, polyarteritis nodosa, rheumatoid arthritis, and systemic sclerosis. The *lpr* strain has a defect in *Fas* expression, and develops a massive generalized enlargement of lymphatic tissues, as well as these autoimmune diseases. The M+ strain also displays these phenotypes, but in milder forms. These results suggest that these phenotypes are due to the MRL genetic background (Theofilopoulos and Dixon, 1985). In addition, the testis of MRL mice is known to have at least the following unique characteristics: metaphase-specific apoptosis of meiotic spermatocytes (Kon et al., 1999; Kon and Endoh, 2000) and heat-shock resistance of spermatocytes as found in experimental cryptorchidism (Kon and Endoh, 2001). These two phenotypes are attributed to mutations in exonuclease 1, which has an important role in DNA repair (Namiki et al., 2003, Namiki et al., 2004). To investigate other characteristics of spermatogenesis in MRL mice, the differentiation process from prospermatogonia to

spermatogonia in postnatal developing mouse testes was examined. Surprisingly, the author found oocyte-like cells in the seminiferous tubules of newborn MRL male mice.

The aims of this study are to reveal the mechanism of initiating oogenesis in testis and verify the possibility of production of offspring using testicular oocyte. This thesis contains three chapters; the first chapter proves the appearance of oocytes in newborn MRL mouse testes, the second chapter examines the characteristics and function of testicular oocytes as so-called oocytes, and the third chapter discusses the cause of the appearance of testicular oocytes.

Chapter 1

Appearance of oocyte in new born MRL mouse testes

Introduction

Gonad is unique among all organs because of its bipotential nature, a testis or an ovary. In most mammals, sex determination is genetically controlled by the presence or absence of the Y chromosome. The initiation of the male pathway depends on gonadal expression of the Y-linked gene, *Sry*, which initiates the differentiation of Sertoli cells, as well as their structural organization into a testis cord (Koopman et al., 1991). Germ cells also have the plasticity to develop either oogonia or prospermatogonia; however, this sex differentiation is directed by signals from somatic cells in the gonads, not by the germ cells themselves (McLaren, 1995). These sex differentiation mechanisms advocate a universal rule 'mammalian males produce only sperms in their testes and females produce only oocytes in their ovaries'. As exceptions of this rule, some genetic abnormalities of sexual differentiation in mammals, including experimental chimeras, are known to cause the appearance of oocytes in their testes (Mystkowska and Tarkowski, 1968; McLaren, 1980; Isotani et al., 2005). Still, it has never been reported that apparently healthy and fertile male animals can produce oocytes during spermatogenesis.

Surprisingly, as a result of detail investigation on postnatal development of mouse testes, the author found oocyte-like cells in the seminiferous tubules of newborn MRL male mice. Since MRL male mice are fertile and contain XY chromosomes, the author came up with the idea that there is a mechanism somehow oocyte is produced under testicular environment with natural condition. In this chapter, the author attempted to prove the existence of testicular oocyte in MRL mice with its morphological characteristics and the expression of oocyte-specific genes in testis. The author also presents observations on the postnatal change of oocyte number per testis.

Materials and Methods

Mice

Inbred mouse strains, C57BL/6 (B6), M+, and *lpr* were used. Eight-week-old male and female mice purchased from Japan SLC were maintained with free access to food and water in our facility. In the handling of experimental animals, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals, Hokkaido University, Graduate School of Veterinary Medicine." In this chapter, newly born mice from each strain were obtained by free-breeding, and were sacrificed at 0 to 50 days afterbirth by detruncation or cervical dislocation.

Light microscopic analysis

In order to examine testicular oocytes, two types of specimens, fresh whole-mount preparations and fixed serial sections, were prepared. For whole-mount preparations, the testes of 0- to 50-day-old M+ and *lpr* mice were removed, and immediately mounted on glass-slides with cover slips. They were then crushed to make whole-mounted single tubular sheets, and were observed under a differential interference microscope (BX50F4, Olympus). For fixed preparations, the testes of 0- to 16-day-old and the ovaries of 0- to 8-day-old M+ and *lpr* mice were removed and immediately fixed with Bouin's solution for 24 hr, cut into 5 μm-thick serial paraffin sections, and stained with hematoxylin-eosin (HE) or periodic acid Schiff (PAS). Some testis sections were obtained from whole-mount preparations after microscopic observation.

Electron microscopic analysis

In order to examine the ultrastructure of testicular oocytes, testes of 4-day-old *lpr* mice were immediately fixed with 5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) at 4˚C

for 6 hr. The testes were then post-fixed with 1% osmium tetroxide in 0.1 M cacodylate buffer at room temperature for 2 hr, dehydrated with graded alcohol, and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate, and observed with an electron microscope (JEM-1210, JOEL).

Estimation of oocyte score

In order to estimate the oocyte score, the average number of oocytes per testis found at each age from days 0 to 50 afterbirth was calculated. Paraffin embedded serial sections were made from the testes of 0- to 4-day-old mice, and whole-mount preparations were made from the testes of 8- to 50-day-old mice. The number of testicular oocytes was counted in these samples.

RT-PCR for oocyte-specific genes

To examine the expression of oocyte-specific genes, total RNA was obtained from the testes and ovaries of 4- to 13-day-old B6 and *lpr* mice using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Complimentary DNA was then synthesized with ReverTra Ace (Toyobo), and PCR was carried out with ExTaq (Takara) under the following PCR conditions: 5 min at 95˚C, 35 cycles of 40 sec at 95˚C, 30 sec at 62˚C, and 1 min at 72˚C, followed by 5 min at 72˚C. The PCR primer pairs for oocyte-specific genes used in the present study are shown in Table 1-1. The zona pellucida in mice is composed of the zona pellucida proteins ZP1, ZP2, and ZP3. ZP3 and ZP2 act as primary and secondary sperm receptors respectively, while ZP1 crosslinks the filaments composed of ZP2/ZP3 dimers (Wassarman et al., 2004). Another protein, *Omt2a* (Oocyte maturation, alpha), acts after oocyte maturation and the onset of meiosis (West et al., 1996). Since these genes are produced by growing oocytes, but not by follicular epithelial cells in mice (Philpott et al.,

1987; Liang et al., 1990; Epifano et al., 1995; West et al., 1996), they were selected as oocyte-specific markers. Additionally, *Actb* (actin, beta) was chosen as internal markers. The amplification of these oocyte-specific gene transcripts were verified by sequencing with a cycle sequencing kit containing fluorescent terminators (Applied Biosystems) employing standard methods and a model 377 automatic sequencer (Applied Biosystems).

Results

Testicular oocytes in whole-mount preparations and in paraffin sections

Testicular oocytes were found in whole-mount preparations of testes obtained from M+ and *lpr* mice aged 7 to 30 days afterbirth (Fig. 1-1). They coexisted with gonocytes and spermatogonia in the seminiferous tubules, mostly in the neighborhood of the rete testis. Testicular oocytes gradually increased in diameters as age became older; however, oocyte in day 30 seemed to shrink as seen in Figure 1-1J. When there were two or more oocytes in a single seminiferous tubule, they were located close to each other (Fig. 1-2). Because of their unique size, 50 to 70 μm in diameter, they were easily distinguishable from somatic cells and sperm-producing cells. Each oocyte had an abundant cytoplasm and a large nucleus with one or two distinct nucleoli, and was surrounded by a zona pellucida, which was observed between the oocyte and the follicular epithelial cells (Fig. 1-3). The follicular epithelial cells, sometimes consisting of few layers, were clearly distinguishable at days 14 to 20.

Since no testicular oocytes were detected in whole-mount preparations of testes obtained from M+ and *lpr* mice aged 0 and 4 days, the author wondered whether paraffin-embedded serial sections of 0- to 4-day-old testes better preserved any testicular oocytes that might exist. To surprise, testicular oocytes were found as early as at birth; they were well developed, with diameters between 30 and 50 μm, and were larger than the gonocytes and somatic cells of the testes (Fig. 1-4A). The testicular oocytes contained large oval nuclei with distinct nucleoli and abundant cytoplasm stained lightly by eosin, and appeared similar to ovarian oocytes in female mice of the same age. Although, some of the testicular oocytes were surrounded by layers of squamous to cuboidal follicular epithelial cells, resembling the appearance of the primordial to secondary ovarian follicle (Fig. 1-4I), most of them obtained from 4- to 16-day-old mice were incompletely surrounded by follicular epithelial cells (Fig 1-4B-H).

Each of these follicular epithelial cells had a nucleus with an irregular shape containing distinct nucleoli similar to those in Sertoli cells. In 16-day-old or older mice, testicular oocytes with abnormal appearance were observed such as those in the process of degeneration with vacuolization of cytoplasm, disappearance of nuclear envelope, and karyolysis (Fig. 1-5 A) or containing two nuclei (Fig. 1-5B). Although the follicular epithelial cells formed a multilayer similar to that observed in early stage secondary follicles, they never a formed follicular antrum or a polar body-like structure.

The zona pellucida showed a positive reaction to the PAS stain. They were observed as discontinuous lines on days 0 and 4 afterbirth, and then as continuous lines on day 8 much like ovarian oocytes of the same age (Fig. 1-6A-F).

Ultrastructural characteristics of the testicular oocytes

Unlike spermatogonia and Sertoli cells, the testicular oocytes did not attach to the basement membrane (Fig. 1-7A). Under electron microscopy, the zona pellucida appeared discontinuous (Fig. 1-7B). Where there were gaps in the zona pellucida, follicular epithelial cells directly contacted to the oocyte (Fig. 1-7B). Where the zona pellucida was intact, the oocyte extended numerous microvilli through about half the thickness of the zona pellucida, and the follicular epithelial cells attached to the cell membrane of the oocyte with slender cytoplasmic processes penetrating the zona pellucida (Fig. 1-7C and D). In the cytoplasm of the testicular oocyte, a round, highly dense matrix bound by a single smooth membrane was found just beneath the cell membrane; these appeared similar to the cortical granules found in ovarian oocytes (Fig. 1-7D and E). These cortical granules were divided into two types by their diameters. They were located mainly in two regions. The smaller granules appeared to be synthesized in the juxta-nuclear region, and the larger granules appeared to be synthesized near the cell membrane. The testicular oocytes contained Golgi complexes

observed as multiple aggregates of small vesicles, and flattened tubules similar to those involved in the synthesis and formation of cortical granules in ovarian oocytes (Fig. 1-7F and G).

Number of testicular oocytes in postnatal development

The number of testicular oocytes per testis was counted in whole-mount preparations and in complete serial sections. The oocytes were detected in about half of the mice examined (65 of 124), and the maximum number of oocytes detected in one testis was 12. The appearance of oocytes in the testis peaked around day 14 afterbirth with an oocyte score of approximately 1.2, as shown in Fig. 1-8. No oocyte was observed on days 40 and 50 afterbirth.

Expression of oocyte-specific genes in newborn MRL testes

The expression of oocyte-specific genes in MRL testes was examined by RT-PCR and compared to the expression in B6 testes (Fig. 1-9). The *lpr* testes expressed all of the oocyte-specific genes examined in the present study; however, the intensities of expression were weaker than that found in the ovaries. Expression of the oocyte-specific genes became stronger on day 13 in comparison to day 4. The testicular expression of *Zp1* and *Omt2a* was detected only in *lpr* mice, whereas the expression of *Zp2* and *Zp3* was also observed in testes from B6 mice, and these results were confirmed by nucleotide sequencing. The expression of *Zp2* and *Zp3* in B6 mice was weaker than in *lpr* mice.

Discussion

The existence of testicular oocytes has been reported in several cases. Testicular oocytes were first found in the testes of chimeric mice in 1968 (Mystkowska and Tarkowski, 1968). Then, they were observed in the fetal testes after being transplanted under a kidney capsule of adult female hosts (Ozdzenski, 1972; Ozdzenski and Presz, 1984). Testicular oocytes also existed in the testes of XXsxr sex-reversed mice (McLaren, 1980). The most recent report of testicular oocytes was found in the testes of XX↔XY chimeric mice (Isotani et al., 2005). However, to date, this is the first report of the appearance of testicular oocytes in XY fertile males.

Although the testicular oocytes were found as early as birth in paraffin sections, they were not detected in the whole-mount preparations of testes before day 8. The detection of testicular oocytes in whole-mount preparations may have required the maturation of the zona pellucida, and the discontinuity of the zona pellucida on day 4 may explain why testicular oocytes were not detected in 4-day-old whole-mount testes.

Sertoli cells originate from the same precursors as granulosa cells (Capel, 2000), and they can transdifferentiate into follicular cells under the influence of ovarian factors (Palmer and Burgoyne, 1991). These findings suggest that the follicular epithelial cells surrounding testicular oocytes originated from Sertoli cells. However, the possibility that they are undifferentiated bipotential epithelial cells can not be excluded yet. The testicular oocytes and follicular epithelial cells appear to progress along the maturation process until the early secondary follicle stage in the testicular environment; however, their development was delayed and limited compared with oocytes and follicular cells in the ovaries. The following two reasons were suggested for this: (i) Sertoli cells mature and form tight junctions among adjacent Sertoli cells after the fetal and neonatal proliferation period (Sharpe et al., 2003), indicating that they are no longer able to transdifferentiate into follicular epithelial cells and

(ii) proliferation of follicular epithelial cells was prevented by the initiation of spermatogenesis because of the competition for space. Therefore, it was suggested that the maturation of Sertoli cells and initiation of spermatogenesis prevented the growth of follicular epithelial cells.

Some irregular morphological characteristics were observed in the testicular oocytes. The testicular oocytes without follicular epithelial cells found on day 0 already contained zona pellucida, which in the ovary, is believed to be formed only during the developmental process from primary to secondary follicles. Additionally, binuclear testicular oocytes were observed; these were thought to be a result of the resumption of meiosis, a nuclear division in an oocyte without subsequent cytokinesis. Furthermore, the oocyte score decreased over time and degenerating testicular oocytes appeared on day 16 afterbirth and later. Also, there were no testicular oocytes observed after day 30. The testicular environment changes to induce the entry of germ cells into meiosis after birth, while oocytes are arrested in the diplotene stage from birth until puberty (McLaren, 1984). It is possible that the changing testicular environment led the degeneration of testicular oocytes and induced the elimination of testicular oocytes by Sertoli cells, which have the ability to phagocytose abnormal germ cells.

Expression of *Zp1*, *Zp2*, *Zp3*, and *Omt2a* detected in *lpr* testes presented another evidence for the existence of oocytes in testes. The timing and levels of the *Zp* genes' expression coincided with the formation and completion of the zona pellucida in growing testicular oocytes as observed in PAS stained paraffin sections. The zona pellucida glycoproteins serve as sperm receptors and induce acrosome reactions (Wassarman et al., 2004), and *Omt2a* is known to have a role after the onset of meiosis (West et al., 1996). These facts indicated that testicular oocytes might have functional characteristics as an oocyte, such as ability to fuse with sperm.

Although the mechanism of the appearance of testicular oocytes is still unknown, some ideas about genetic background of this phenomenon can be cited. As both MRL strains, M+ and *lpr*, had testicular oocytes indicating that the appearance of testicular oocytes is dependent on the MRL genetic background, but not on the *Fas* gene (Yonehara et al., 1989). Thus the studies to reveal the mechanism of testicular oocyte production will provide keys to understand sex differentiation.

Summary

Although mammals produce either sperms or eggs depending on their sex, oocytes appeared in the testes of newborn MRL male mice. In this chapter, the author reports the morphological characteristics of testicular oocytes, the postnatal change of oocyte number per testis, and the expression of a few oocyte-specific genes in the testis of MRL mice. The testicular oocytes had a diameter of 50 to 70 μm, and were surrounded by zonae pellucidae, which were observed between oocytes and follicular epithelial cells. Ultrastructurally, the testicular oocytes contained numerous microvilli and cortical granules, receiving cytoplasmic projections from follicular epithelial cells. The testicular oocytes appeared as early as at birth and the largest number was found around day 14. The expression of the oocyte-specific genes *Zp1-3* and *Omt2a* was detected in testes from MRL mice. These results suggest that newborn male MRL mice with XY chromosomes can produce oocytes in their testes. This unique oocyte can provide more clues about the development of the reproductive system, such as the mechanisms of sex differentiation and sex-specific methylation patterns of germ cells. The author will examine and discuss the characteristics as so-called oocytes and causes of appearance of testicular oocytes in next two chapters.

Table and Figures

Table 1-1. Primer pairs for oocyte-specific genes

Gene	Accession No.	Length (bp)	Forward/Reverse (5'-3')	Amplified length (bp)
Zp1	U20448	1963	TGGCAACACAGTCACTCTCC	433
			AGTCTTATCCGTGGCAATCC	
Zp2	M34148	2201	TGGCAATGGTACAAGAGCC	500
			TCACTGTGGATAGCATGTTCC	
Zp3	M20026	1317	AGTTCACGGTGGATGTATTCC	450
			ACTTCCTGGTGACAGCAAGG	
Omt2a	S8194	734	AGGATTGCGTGGTCAGCATGATTCG	457
			CCCCACTTAAAAGCTGGACATGGCA	
Actb	M12481	1109	TGTTACCAACTGGGACGACA	165
			GGGGTGTTGAAGGTCTCAAA	

Zp1, zona pellucida 1; *Zp2*, zona pellucida 2; *Zp3*, zona pellucida 3; *Omt2a*, oocyte maturation, alpha; *Actb*, actin, beta.

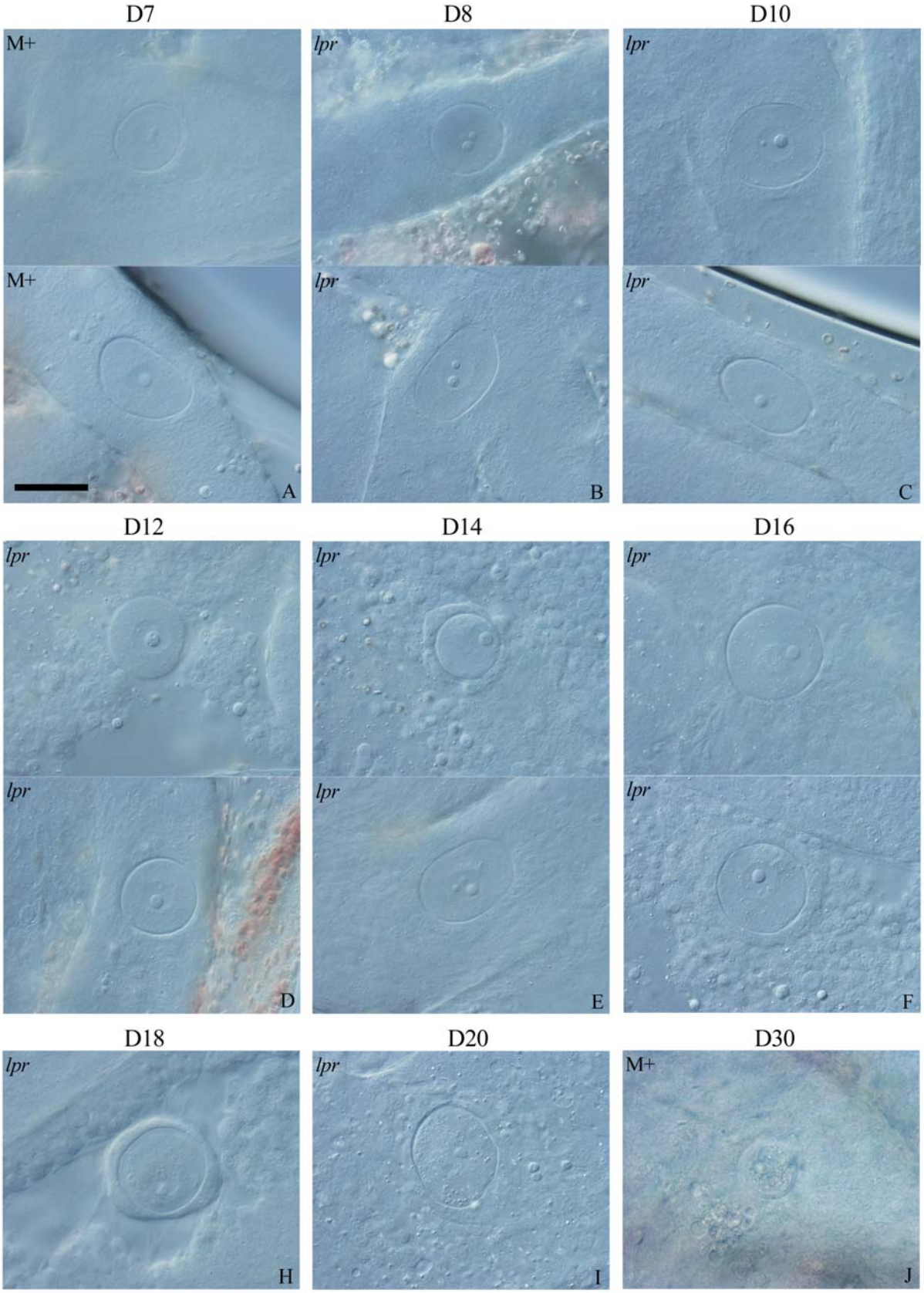

Fig. 1-1. A-J) Testicular oocytes in whole-mount testes of M+ and *lpr* mice aged 7 to 30 days under a differential interference microscope. Each oocyte has a diameter of 50 to 70 μm and a large nucleus with one or two distinct nucleoli, and was surrounded by a zona pellucida and the follicular epithelial cells. All images are at the same magnification. Bar = 50 μm.

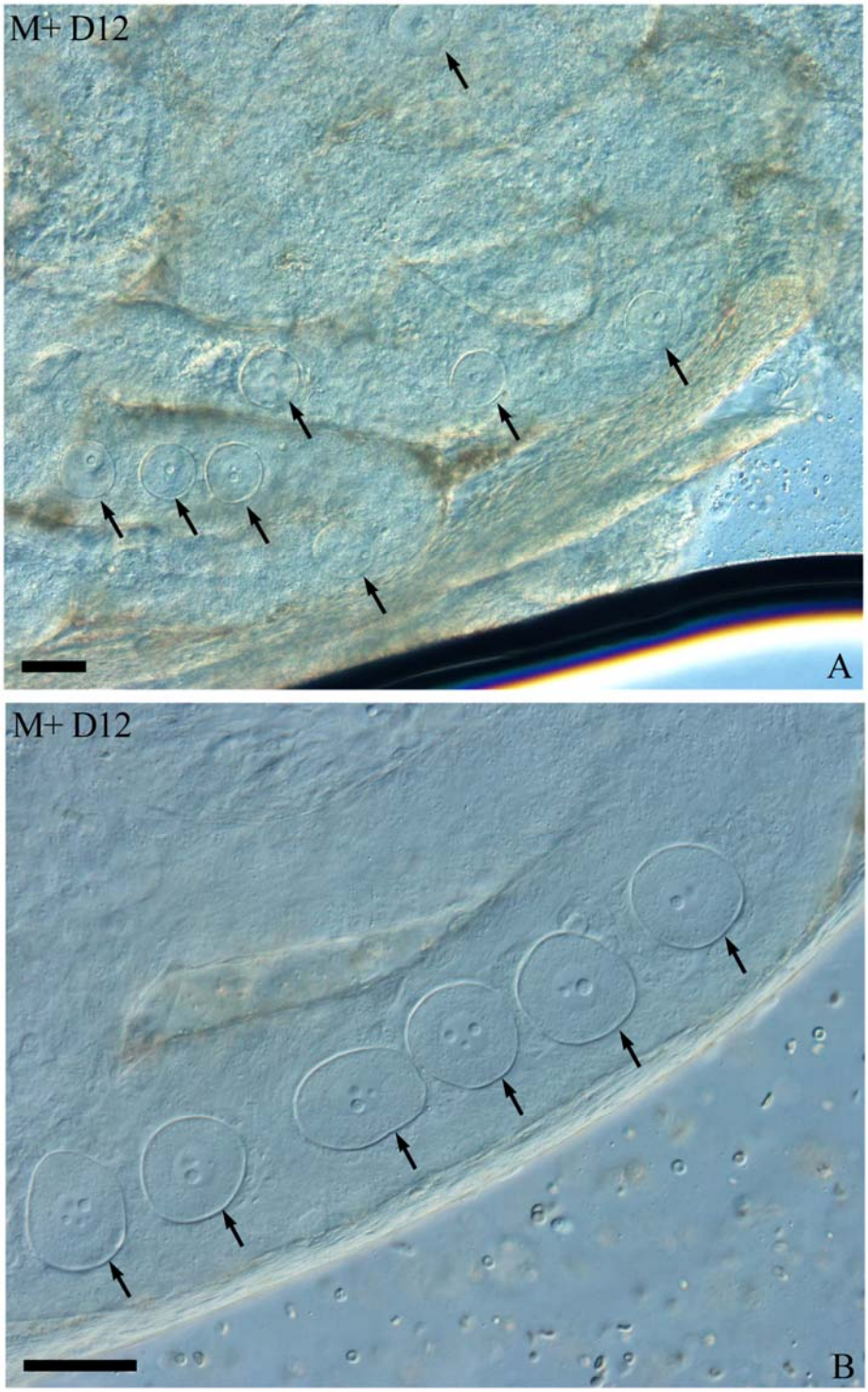

Fig. 1-2. A and B) Multiple testicular oocytes in one testis under a differential interference microscope. When there were two or more oocytes in a single seminiferous tubule, they were located close to each other. Arrows, testicular oocytes. Bars = 50 μm.

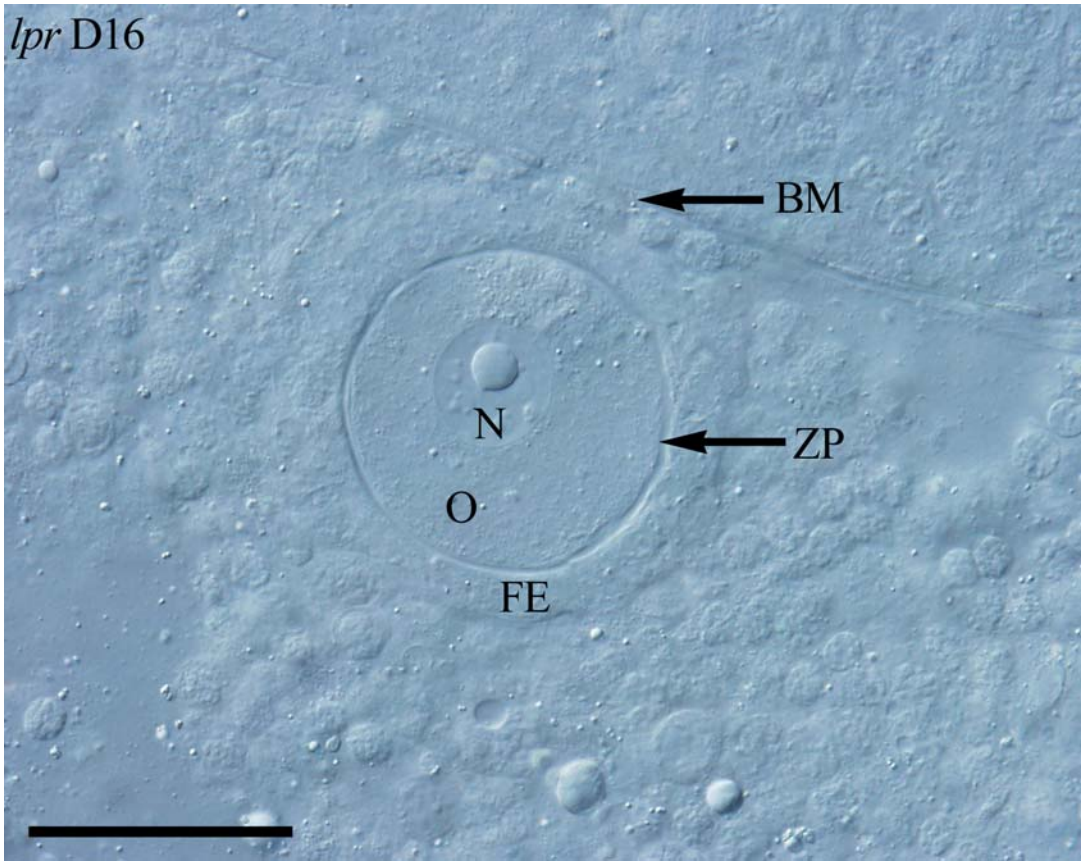

Fig. 1-3. Higher magnification of a testicular oocyte shown in Fig. 1F. Testicular oocyte had an abundant cytoplasm and a large nucleus with one or two distinct nucleoli, and each oocyte was surrounded by a zona pellucida which was observed between the oocyte and follicular epithelial cells under a differential interference microscope. BM, basement membrane; FE, follicular epithelial cell; N, nucleus; O, oocyte; ZP, zona pellucida. Bar = 50 μm.

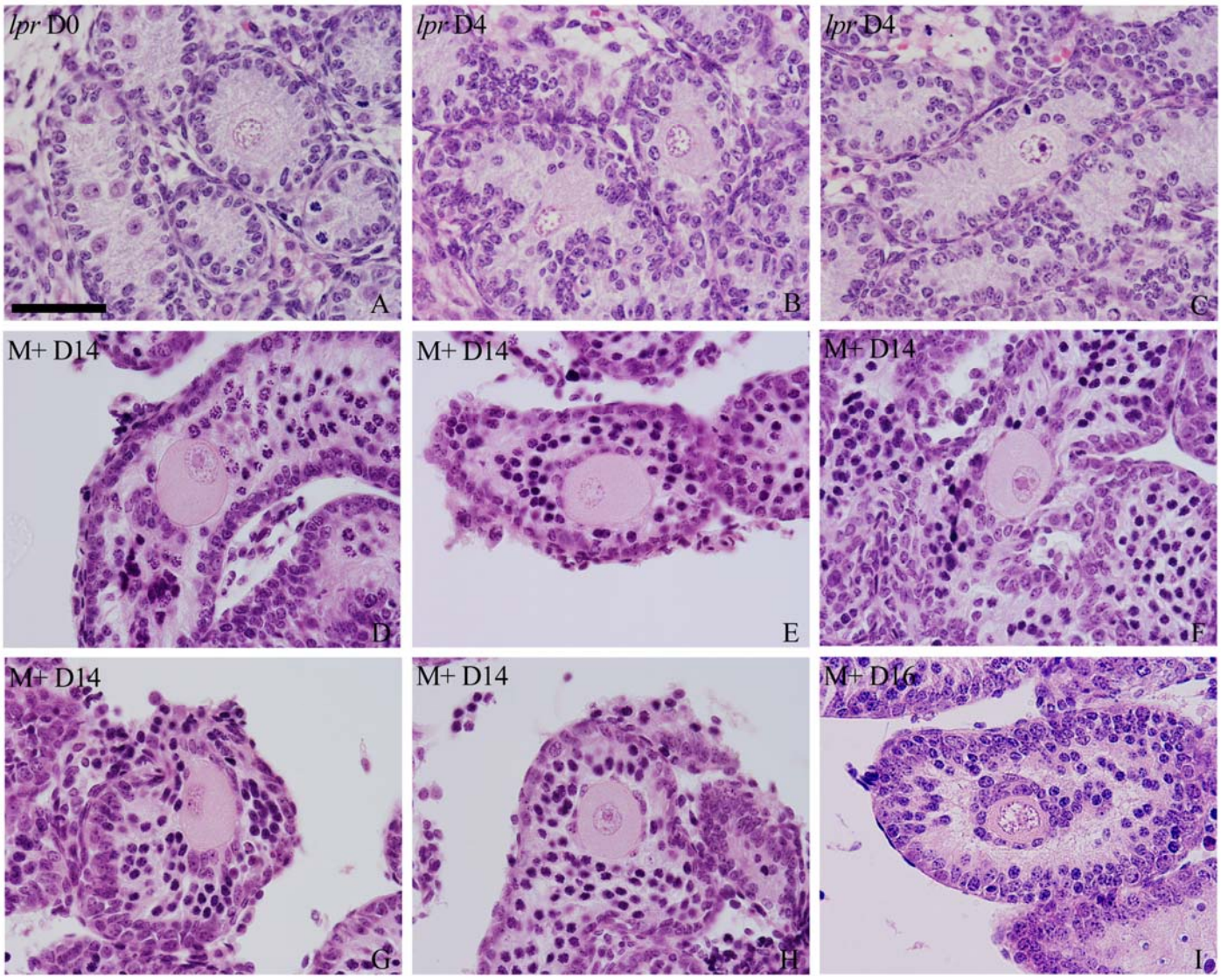

Fig. 1-4. HE stain of testicular oocytes in paraffin sections from *lpr* and M+ mice aged 0 to 16 days. Testicular oocyte development with no follicular epithelial cell (A) and with surrounding follicular epithelial cells (B-I) were observed. All images are at the same magnification. Bar = 50 μm.

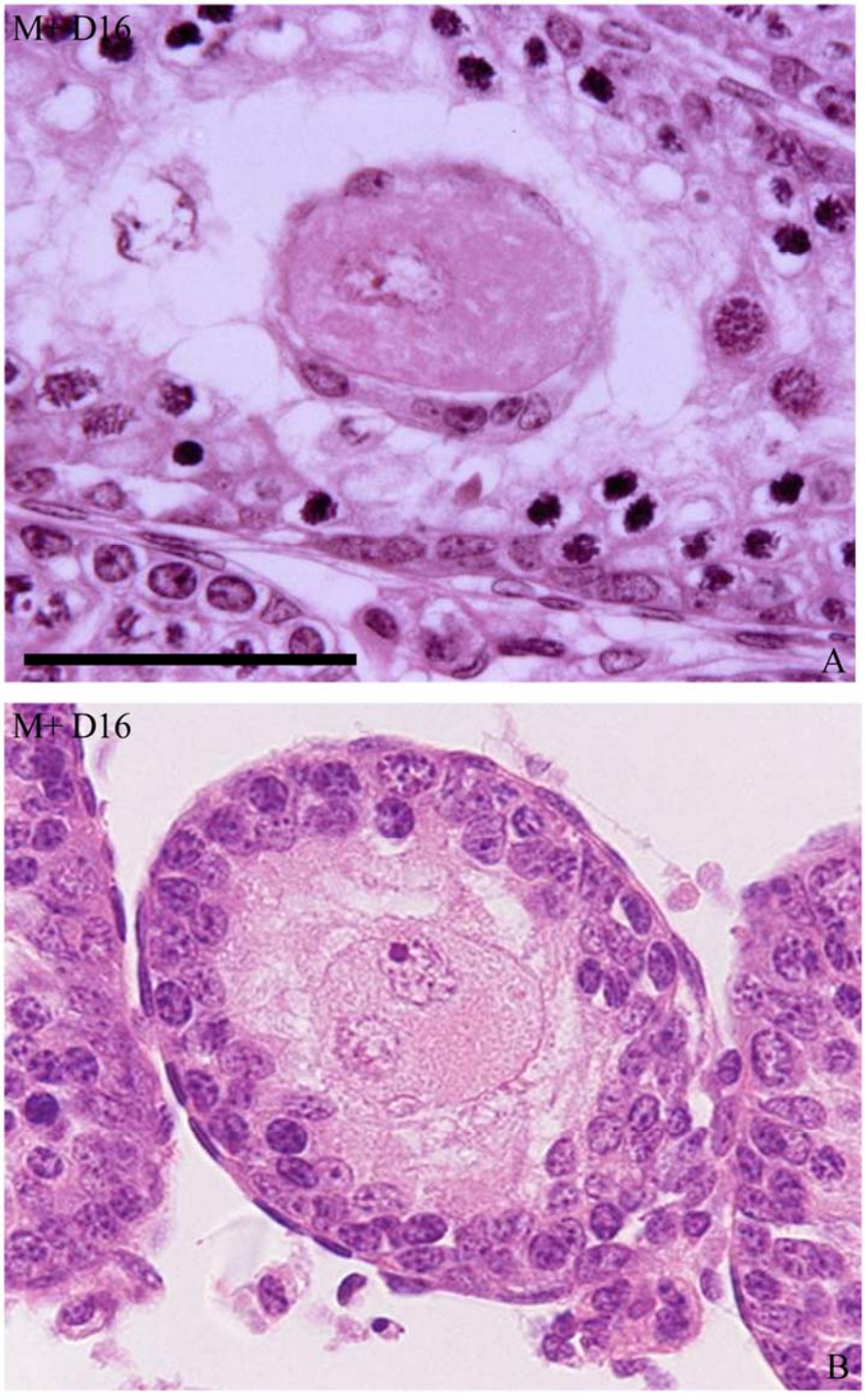

Fig. 1-5. HE stain of abnormal testicular oocytes. Testicular oocytes under degeneration (A) and with two nuclei (B) on day 16 afterbirth. All images are at the same magnification. Bar = 50 μm.

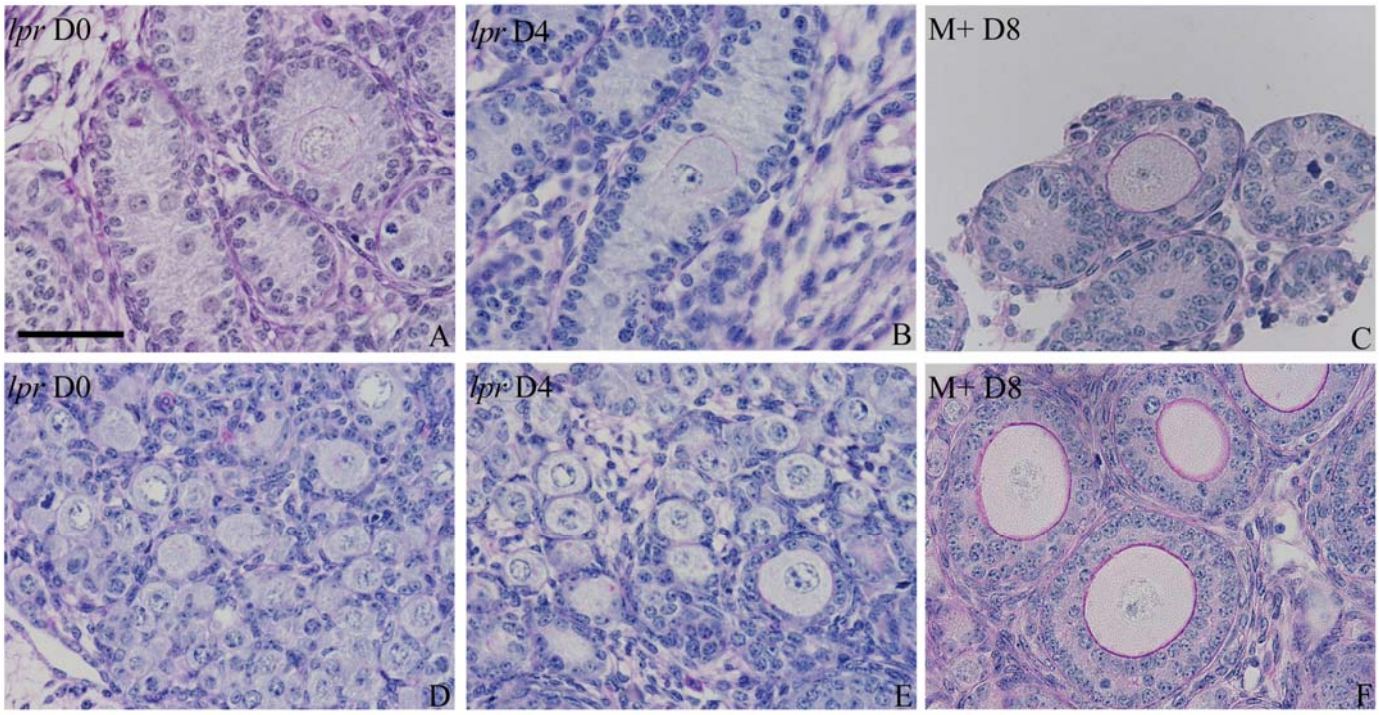

Fig. 1-6. PAS stain of testicular oocytes from days 0 (A), 4 (B), and 8 (C); ovarian oocytes from days 0 (D), 4 (E), and 8 (F). Zona pellucida of testicular oocytes showed positive reaction to PAS similar to those of ovarian oocytes. All images are at the same magnification. Bar = 50 μm.

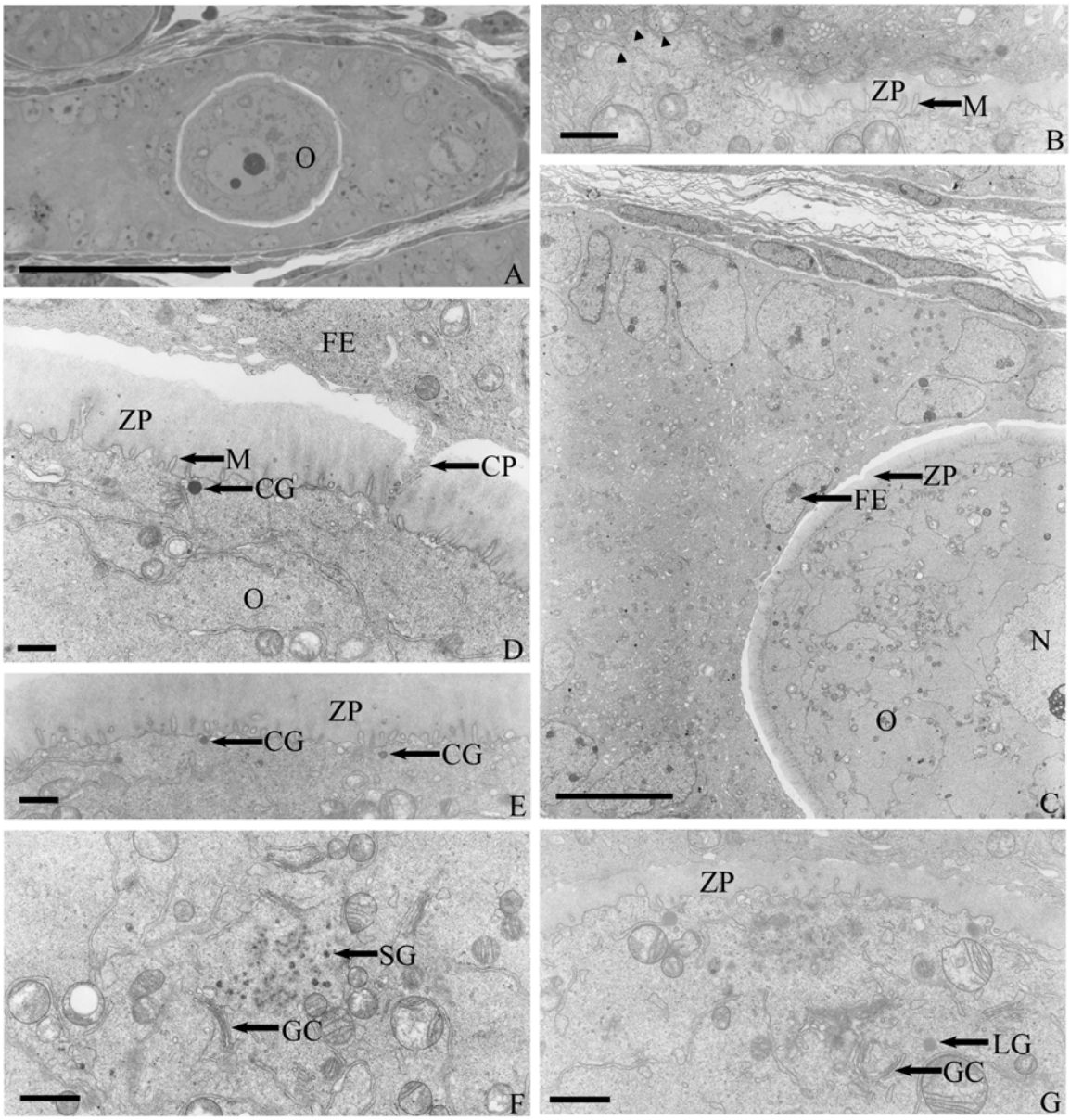

Fig. 1-7. Ultrastrucuture of testicular oocytes at day 4 afterbirth. Testicular oocyte within seminiferous tubules (A), zona pellucida and follicular epithelial cells surrounding testicular oocytes (B-D), and various organelles contained in ooplasm (E-G). O, oocyte; M, microvilli; ZP, zona pellucida; N, nucleus; CG, cortical granule; CP, cytoplasmic process; FE, follicular epithelia; GC, Golgi complex; SG, small granule; LG, large granule. Bars = 50 μm in A, 10 μm in C, and 1 μm in B and D-G.

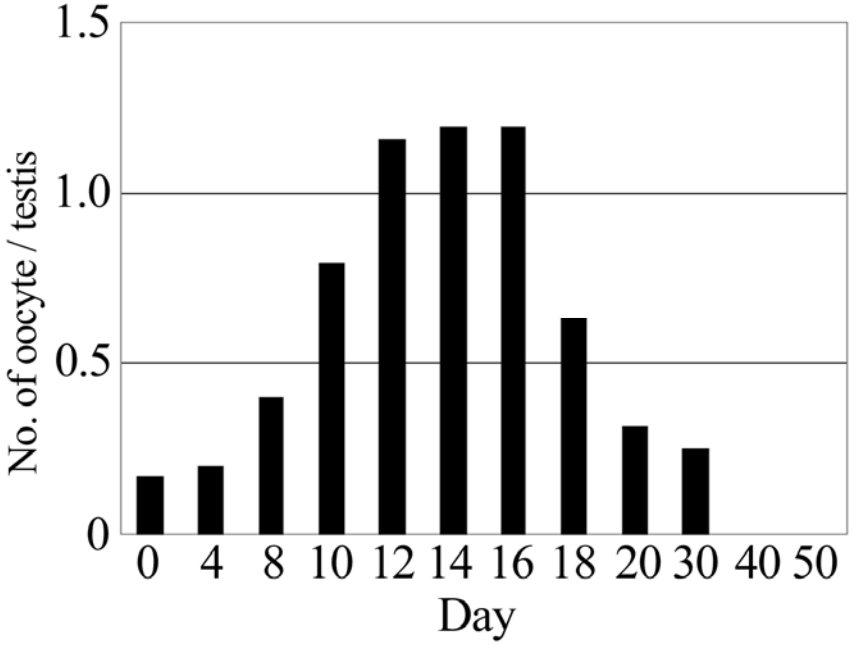

Fig. 1-8. Oocyte scores for days 0 to 30 afterbirth in MRL mouse testes. The appearance of oocytes in the testis peaked around day 14 afterbirth with an oocyte score of approximately 1.2. No oocyte was observed on days 40 and 50 afterbirth.

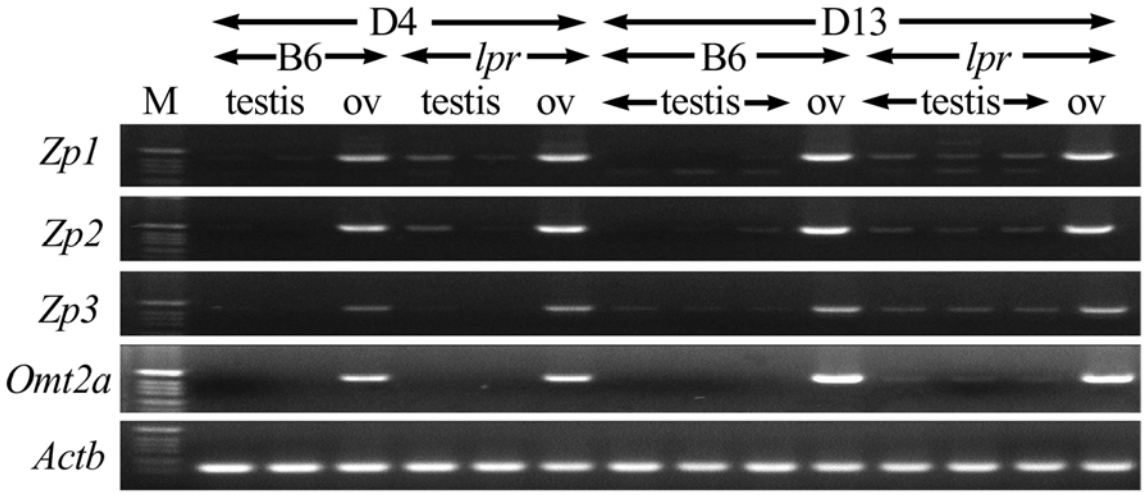

Fig. 1-9. Oocyte-specific gene expression in *lpr* mouse testes. RT-PCR products of testes and ovaries of *lpr* and B6 mice from days 4 and 13 for *Zp1*, *Zp2*, *Zp3*, *Omt2a*, and *Actb* as an internal control. The *lpr* testes expressed all of the oocyte-specific genes examined in the present study; however, the intensities of expression were weaker than that found in the ovaries. *Zp1*, zona pellucida 1; *Zp2*, zona pellucida 2; *Zp3*, zona pellucida 3; *Omt2a*, oocyte maturation, alpha; *Actb*, actin, beta; ov, ovary.

Chapter 2

Characteristics and function of testicular oocytes as so-called oocytes

Introduction

Germ cells are highly specialized cells to transmit genomic information to next generation. Meiosis is initiated at different time points between males and females in mammals and leads sex differentiation of primordial germ cells. In mice, this phenomenon occurs at E13.5, when ovarian germ cells enter meiosis and testicular germ cells are arrest in the G0/G1 of the mitotic cell cycle (McLaren, 1984). Recently, it has been reported that retinoic acid produced in mesonephros induces meiosis in fetal ovary, and CYP26B1 (cytochrome P450, family 26, subfamily b, polypeptide 1), an enzyme involved in retinoid metabolism, has a key role in preventing meiosis in the fetal testis (Bowles et al., 2006; Koubova et al., 2006). Ovarian germ cells which initiated prophase of meiosis by E13.5 are arrested in the diplotene stage of meiotic prophase I by birth until puberty (McLaren, 1984).

The ovarian germ cells also precede oogenesis other than meiosis during embryonic period. Recently, several transcription factors which regulate the early oogenesis and formation of primordial follicle, such as *Sohlh1* (spermatogenesis- and oogenesis-specific bHLH transcription factor 1), *Sohlh2* (spermatogenesis- and oogenesis-specific bHLH transcription factor 2), *Figla* (folliculogenesis specific basic helix-loop-helix), and *Nobox* (NOBOX homeobox gene) have been identified (Liang et al., 1997; Soyal et al., 2000; Suzumori et al., 2002; Rajkovic et al., 2004; Pangas et al., 2006; Choi et al., 2008). Once the follicles are formed, early stages of growth are independent of pituitary gonadotrophins and are thought primarily to involve intraovarian paracrine factors. At the antral stage, most of the follicles will undergo atresia, whereas the remaining antral follicles will survive under the influence of FSH (follicle stimulating hormone) and grow to the pre-ovulatory follicle that is capable of releasing an oocyte for fertilization, upon stimulation by LH (luteinizing hormone) (McGee and Hsueh, 2000). Additionally, during the process of oocyte maturation,

the oocyte resumes meiosis, progresses from prophase I to metaphase II, extracts the first polar body, and waits for fertilization (Jamnongjit and Hammes, 2005). Thus oocytes mature through the complicated process which is dependent on classic endocrine signaling within the hypothalamic–pituitary–gonadal axis and also the intraovarian paracrine factors.

The author has shown the existence of testicular oocyte within newborn MRL mice by morphological characteristics of testicular oocytes and expression of oocyte-specific genes in testes in chapter 1. As described in chapter 1, testicular oocytes appeared as early as at birth and their appearance rate showed peak around day 14. The testicular oocytes and follicular epithelial cells appear to progress along the maturation process until the early secondary follicle stage and disappeared after day 30. However, their derivation and functional characteristics as so-called oocytes are still unknown. In this chapter, the author reports and discusses about when and how the testicular oocytes are formed and the fusing ability with sperm.

Materials and Methods

Mice

Mice were maintained and handled as described in chapter 1. In this chapter, newly born B6, M+, and *lpr* mice were obtained by free-breeding, and were sacrificed at 8 to 14 days afterbirth by detruncation or cervical dislocation. For embryo collection, timed mating was established by housing females with males overnight. Next morning, females were checked for the presence of vaginal plugs, which denoted pregnancy, and the embryos were recorded as being in embryonic day (E) 0.5 of development. The testes and ovaries of MRL mice, and the testes of B6 mice at E13.5 to 18.5 were used as samples.

Tissue preparation

To find testicular oocytes, fresh whole-mount preparations of testes obtained from 8- to 14-day-old of M+ mice were observed under a differential interference microscope as described in chapter 1. The fetal gonads and some of the seminiferous tubules with oocytes were immediately fixed with Bouin's solution or 4% paraformaldehyde/0.1 M phosphate buffer for 24 hr, cut into 5 μm-thick serial paraffin sections, and used for immunohistochemistry.

Immunohistochemical analysis

To confirm the initiation of meiosis/oogenesis and the existence of the zona pellucida, and to determine the function of observed follicular epithelial cells, the author used immunohistochemistry to detect the presence of SYCP3 (synaptonemal complex protein 3), NOBOX, ZP3, and FOXL2 (forkhead box L2). Briefly, the deparaffinized sections were treated with antigen-unmasking solution, Target Retrieval Solution (Dako Cytomation) or with 10 mM citrate buffer, which was autoclaved for 20 min at 105˚C. The samples were

then incubated in 3% hydrogen peroxidase/methanol solution for 10 min to block endogenous peroxidase activity. The sections pretreated with in rabbit serum were incubated with goat anti-ZP3 antibody (1:100; Santa Cruz Biotechnology), and sections pretreated with normal goat serum were incubated with rabbit anti-SYCP3 antibody (1:750; GeneTex), rabbit anti-NOBOX antibody (1:500; Abcam), or rabbit anti-FOXL2 antibody (1:50; Sigma). All incubation of primary antibody was done at 4˚C overnight. Negative controls were not incubated with specific antibodies, after pretreatment of the normal sera. The sections were then treated with a secondary biotinylated anti-goat or anti-rabbit IgG antibody (Chemicon; Nichirei) for 30 min, then in streptavidin-peroxidase (Nichirei) for 30 min, and finally in 3, 3'-diaminobenzidine-H_2O_2 solution. Most of the sections were counterstained with either HE or PAS. To compare the immunolocalization of SYCP3 and NOBOX at E18.5, the serial sections were used.

Immunofluorescent analysis

The double immunofluorescence was performed to see the initiation of meiosis and oogenesis. As both anti-SYCP3 antibody and anti-NOBOX antibody were produced by rabbit, another meiosis-specific marker, DMC1 (disrupted meiotic cDNA 1 homolog), was chosen for this experiment. Briefly, the deparaffinized sections were autoclaved with 10mM citrate buffer for 20 min at 105˚C. The sections blocked in 0.25% casein/phosphate buffered saline (PBS) were incubated with mixture of antibodies containing goat anti-DMC1 antibody (1:500; Santa Cruz Biotechnology) and rabbit anti-NOBOX antibody at 4˚C overnight. The sections were then treated with FITC-conjugated donkey anti-rabbit IgG antibody (Abcam) followed by incubation with TRITC-conjugated rabbit anti-goat IgG antibody (Invitrogen). Finally, the sections were observed with confocal laser scanning microscope (Fluoview FV500, Olympus).

RT-PCR for Figla and Foxl2

In order to examine the expression of oogenesis-specific gene, *Figla*, and follicular epithelial cells-specific genes, *Foxl2*, total RNA was obtained from the fetal (E14.5 and 18.5) and postnatal (day 13) testes and ovaries of B6 and M+ mice as described in chapter 1. Complimentary DNA was then synthesized with ReverTra Ace (Toyobo), and PCR was carried out with ExTaq (Takara) under the following PCR conditions: 5 min at 95˚C, 35 cycles of 40 sec at 95˚C, 30 sec at 62˚C, and 1 min at 72˚C, followed by 5 min at 72˚C. The PCR primer pairs used to detect *Figla* and *Foxl2* are shown in Table 2-1.

Isolation of single testicular oocyte

To obtain single testicular oocyte for sperm-egg fusion assay, isolation method for testicular oocyte was established. Briefly, after microscopic observation, the whole-mount preparations were moved under dissection microscope (SZX7, Olympus). Then the oocyte-containing seminiferous tubules were transferred to PBS drops in the glass Petri dish and the oocytes were isolated from seminiferous tubules with 27 gage needles.

Sperm and oocyte fusion assay

The sperm-egg fusion assay was performed as described by Conover and Gwatkin (1988). Briefly, fresh sperms from the cauda epididymis of a B6MRLF1 male were dispersed in 0.2 ml drops of TYH (Toyoda, Yokoyama, and Hoshi) medium (Mitsubishi Chemical Medience Co.) and incubated for 1 hr to induce capacitation. The testicular and ovarian oocytes isolated from the gonads of day 12 afterbirth were treated with acidic Tyrode's solution (Sigma) for 30 sec to remove zona pellucida, washed three times with M2 medium (Invitrogen), and incubated with M2 medium for 2 hr to allow surface protein to recover. Then oocytes were incubated with 1 μg/ml Hoechst 33342 (Dojin Laboratories) for

30 min at 37˚C in 95% air/5% CO_2 and washed three times with TYH medium. The dye-loaded oocytes were co-incubated with the sperm and fixed with 2% paraformaldehyde. The transfer of Hoechst 33342 from dye-preloaded oocytes to sperm was observed with confocal laser scanning microscope and differential interference microscope.

Results

Initiation of meiosis and oogenesis within fetal MRL testes

The immunoreactivity of SYCP3 was detected in germ cells within the testis cords of MRL testes on E13.5, 14.5, 16.5, and 18.5, similar to those detected in fetal ovarian germ cells at the same stages (Fig. 2-1 A, C, D, F, G, I, J, and L). The testis cords containing these meiotic germ cells were located mostly in the neighborhood of the rete testis or at the edge of the testis as observed with testicular oocytes after birth. Some germ cells of B6 testes at E14.5 showed weak immunopositivity to SYCP3 (Fig. 2-1E); however, there were no immunopositive cells at E13.5, 16.5, and 18.5 (Fig. 2-1B, H, and K). Immunoreactivity of NOBOX was also detected in germ cells of MRL testes and ovaries at E18.5 (Fig. 2-1M and O), but not in B6 testes (Fig. 2-1N). These immunopositive germ cells were in the neighborhood of the rete testis or at the edge of the testis just like in the case of SYCP3 (Fig. 1M). To see whether meiotic germ cells are identical to those under process of oogenesis or not, double immunofluorescence for DMC1 and NOBOX using MRL testes and ovaries was performed. As a result, germ cells with positive reactions to both proteins were observed in MRL testes and ovaries at E18.5 (Fig. 2-2 arrows). Additionally, mRNA expression of another specific marker for oogenesis, *Figla*, was also detected in MRL fetal testes and ovaries of E14.5 and 18.5, but not in the same stage of B6 testes (Fig. 2-3).

Zona pellucida and follicular epithelial cells in testicular oocytes

Zona pellucida of testicular oocyte was shown to express ZP3 by immunostaining (Fig. 2-4A) at the same level as that of ovarian oocytes (Fig. 2-4B). Additionally, testicular oocyte also contains NOBOX in its nucleus just like fetal testicular and ovarian oocytes (Fig. 2-4C and D). On the other hand, although *Foxl2* mRNA expression was observed in MRL testes at day 13 afterbirth by RT-PCR (Fig. 2-5A), the follicular epithelial cells of testicular

oocytes were not immunostained with FOXL2 (Fig. 2-5B and C), while those of ovarian oocytes were stained (Fig. 2-5D).

Ability of testicular oocyte to fuse with sperm

In sperm-egg fusion assay, sperms invaded in pre-loading oocyte incorporate the fluorochrome and fluoresce upon fusion with the oocyte plasma membrane. As a result of this assay, fluoresced sperms were observed in both testicular and ovarian oocytes (Fig. 2-6A and B arrows). The invaded sperms were sometimes also visible under the differential interference microscope (Fig. 2-6C arrow).

Discussion

One of the first steps of oogenesis in mammalian germ cells is the initiation of meiosis during embryonic period. In contrast, as a result of sequestration within testis cords, mouse testicular germ cells are arrested in G0/G1 of the mitotic cell cycle by E13.5, and they do not enter meiosis until after birth (McLaren, 1984). However, meiotic germ cells were observed in the testes of E13.5, 14.5, 16.5, and 18.5 MRL mice, and in the testes of E14.5 B6 mice; these cells were located near the rete testis. Retinoic acid is produced and released by the mesonephros (Bowles et al., 2006), indicating that the rete testis might be the place with the highest concentration of retinoic acid, which causes the initiation of meiosis in neighboring germ cells. Temporal existence of meiotic germ cells observed in B6 fetal testes has been also reported in CD-1 mice embryos (Di Carlo et al., 2000). Since male germ cells that prematurely enter meiosis were known to follow apoptotic fates (MacLean et al., 2007), the meiotic germ cells in B6 mice might be eliminated by a meiosis-preventing mechanism present in the fetal testis. Additionally, testicular oocytes of MRL mice always coexisted with normal spermatogonia that could undergo spermatogenesis after birth, indicating that the testicular cords have the ability to prevent germ cells from entering meiosis during the embryonic period. Taking all of the above into consideration, the following possibilities could explain the presence of germ cells that enter meiosis in MRL fetal testis: (i) genetic mutations in genes such as *Sry* and *Cyp26b1*; (ii) the existence of germ cells with high sensitivity to retinoic acid; (iii) the influence of unknown factors that impede the meiosis-preventing mechanism; (iv) defects in the mechanism eliminating meiotic germ cells; or (v) a combination of the above. In any cases, a unique meiotic progressing mechanism exists in the fetal testes of MRL mice that may be involved in the appearance of testicular oocytes.

As similar to SYCP3, NOBOX positive germ cells were observed near the rete testis in

MRL testes at E18.5. Furthermore, double immunofluorescence for DMC1 and NOBOX revealed that germ cells which entered meiosis were identical to those initiated oogenesis. Moreover, *Figla* was expressed in MRL testes as early as E14.5. *Figla* and *Nobox* are known to act as the earliest transcription factors in oogenesis which regulate other oocyte-specific genes (Soyal et al., 2000; Rajkovic et al., 2004). It has been reported that *Figla* regulates *Zp3* and *Nobox* regulates *Gdf9* (growth differentiation factor 9), *Bmp15* (bone morphogenetic protein 15), *H1oo* (H1 histone family, member O, oocyte-specific), *Zar1* (zygote arrest 1), and *Mos* (moloney sarcoma oncogene), and the deficiency of one of these transcription factors disrupts early folliculogenesis (Choi and Rajkovic, 2006; Pangas and Rajkovic, 2006). The evidence of the initiation of meiosis and oogenesis in fetal MRL testes indicated that testicular oocytes are derived from bipotential primordial germ cells which pursued female pathway.

Mainly two complex events are involved in the fusion between the sperm and the oocyte. The first event is the adhesion between the sperm plasma membrane and the zona pellucida, and the second event is the binding of sperm to the oocyte plasma membrane. As a result of immunohistochemistry for ZP3, testicular oocytes were shown to have zona pellucida containing ZP3 which serves as a primary sperm receptor and induces acrosome reactions (Wassarman et al., 2004). The existence of ZP3 proteins and expression of *Zp* genes indicated that the testicular oocytes might have the ability to success the first step of fertilization, adhesion between the sperm plasma membrane and the zona pellucida. Additionally, sperm-egg fusion assay showed the ability of testicular oocyte to fuse with sperm. Although, in normal fertilization, the oocyte will not meet the sperm until ovulation of an 80 μm diameter oocyte that is arrested in metaphase II, oocyte at a diameter of about 20 μm which is still arrested in prophase of first meiotic division can fuse with sperm (Zuccotti et al., 1994). The molecular mechanisms of the interactions between the sperm and oocyte

plasma membranes are not well understood yet; some essential proteins have been identified such as CD9 (CD9 antigen) and PIG-A (phosphatidylinositol glycan anchor biosynthesis, class A) on oocyte (Chen et al., 1999; Miyado et al., 2000; Alfieri et al., 2003) and ADAM-1 and 2 (a disintegrin and metallopeptidase domain 1 and 2), and IZUMO1 (izumo sperm-egg fusion 1) on sperm (Cho et al., 1998; Nishimura et al., 2001; Inoue et al., 2005). The success of the sperm invasion showed that testicular oocyte was recognized by sperm as a so-called oocyte and contained all essences necessary for membrane fusion.

In order to produce next generation, oocytes must complete oogenesis properly. However, testicular oocytes showed some abnormal development during oogenesis as discussed in chapter 1. For example, the testicular oocytes without follicular epithelial cells found on day 0 already contained zona pellucida and the follicular development of testicular oocyte was limited to early stage of secondary follicles. Additionally the binuclear and degenerated testicular oocytes were also found. Furthermore, *Foxl2*, an important factor for ovarian somatic cell differentiation, follicular development, and maintenance (Schmidt et al., 2004; Uhlenhaut and Treier, 2006), was only detected in gene level, but not in protein level in MRL testes. Although this contradiction might be caused by the detection ability of antibody used in this study, the abnormalities found in testicular oocytes indicated that the follicular epithelial cells do not have the abilities to regulate oocyte growth the way those in the ovary do.

Even though it is difficult to expect normal development of oocyte within testicular environment at this point, there is still a possibility of healthy growth by rescue and culture *in vitro*. If the testicular oocytes can be made to mature properly *in vitro*, it may be possible to obtain an embryo with a genome derived entirely from male mice by *in vitro* fertilization and gamete intrafallopian transfer techniques. Because of their morphological similarities to ovarian oocytes, it is speculated that the testicular oocytes would become haploids with an X

chromosome after maturation. This means that the sex ratio of the embryos obtained after *in vitro* fertilization will become one male to one female, and the existence of females with a genome originating only from males could be like deriving ‘‘Eve from Adam’’. Furthermore, if we obtain oocytes from one side of the testes and sperm from the other side of the epididymis, an embryo with a genome derived from only one male will be produced. Thus testicular oocyte contains potential keys for development of reproductive biology.

Summary

Meiosis is initiated at different points of time between male and female and leads sex differentiation of primordial germ cells. In mice, this phenomenon is known to occur at E13.5. In MRL fetal testes, some germ cells underwent meiosis and oogenesis just like those observed in fetal ovary. The zona pellucida of testicular oocyte contained ZP3 proteins, while follicular epithelial cells lacked FOXL2. Additionally, testicular oocyte had ability to fuse with sperms. These results suggest that testicular oocytes are derived from primordial germ cells at about E13.5 under the same process with ovarian oocytes. Additionally, testicular oocytes contain the characteristics as oocytes such as the ability to fuse with sperms. However, follicular epithelial cells lacking FOXL2 might be involved in the limitation of testicular oocyte development. For normal follicular development, meiosis completion, and further experiments such as *in vitro* fertilization, it is necessary to establish to rescue and culture the testicular oocytes. The author will examine and discuss the causes of appearance of testicular oocytes in next chapter.

Table and Figures

Table 2-1. Primer pairs used in this chapter

Gene	Accession No.	Length (bp)	Forward/Reverse (5'-3')	Amplified length (bp)	
Figla	U91840	759	ATCTGTAGGCTCAAGCGCCT	546	
			TCTTGGCAATGCTTTTTGAA		
Foxl2	BC137812	1128	GGAATTCCAAGCCCCCGTACTCGTACG	288	*
			GCTCTAGAGCGTAGTTGCCCTTCTCGAACA		

* Crisponi et al., 2001

Figla, folliculogenesis specific basic helix-loop-helix; *Foxl2*, forkhead box L2.

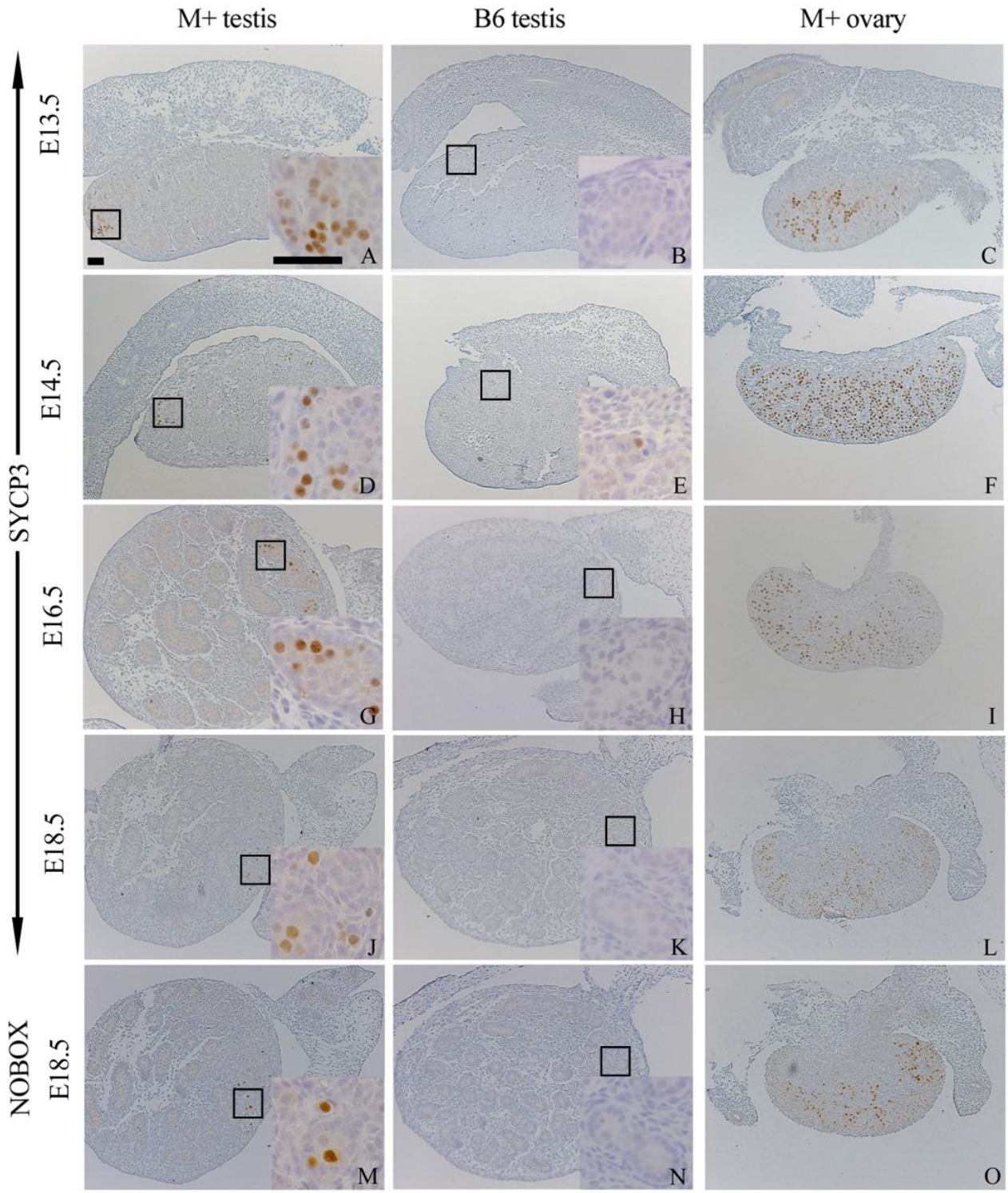

Fig. 2-1. Initiation of meiosis and oogenesis in MRL fetal testes. SYCP3 immunostaining of MRL testes from E13.5 (A), E14.5 (D), 16.5 (G), and 18.5 (J); B6 testes from E13.5 (B), E14.5 (E), 16.5 (H), and 18.5 (K); and MRL ovary from E13.5 (C), E14.5 (F), 16.5 (I), and 18.5 (L) as positive controls. NOBOX immunolocalization of E18.5 MRL testis (M), B6 testes (N), and MRL ovary (O) as positive control. The germ cells in MRL gonads show positive reaction to SYCP3 from E13.5 (A, C, D, F, G, I, J, and L), while there was no positive reaction in B6 testes except E14.5. NOBOX immunostaining was only detected in MRL gonads at E18.5 (M and O). The immunohistochemistry of SYCP3 and NOBOX at E18.5 were serial sections. All images in A-O and insets are at the same magnification, respectively. Bars = 50 μm.

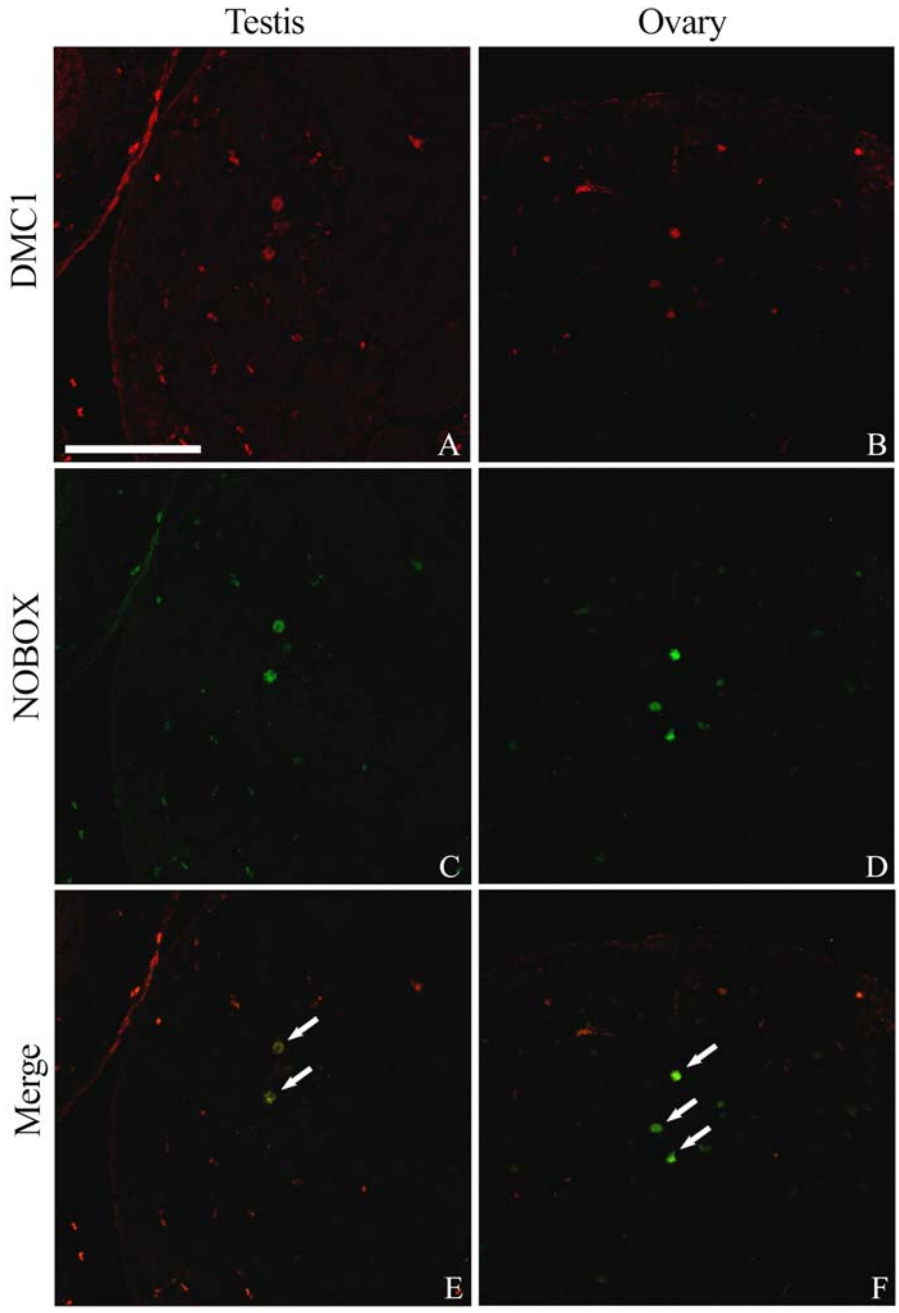

Fig. 2-2. Double immunofluorescent staining of DMC1 and NOBOX in MRL testis and ovary at E18.5. MRL testis and ovary at immunohistochemistry of DMC1 (A and B), NOBOX immunostaining (C and D), and merged (E and F). Arrows, Germ cells with positive reactions to both proteins. All images are at the same magnification. Bar = 50 μm.

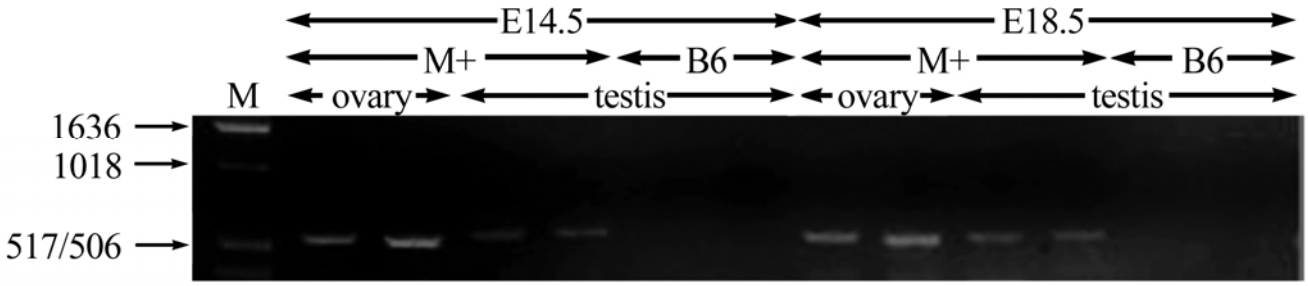

Fig. 2-3. *Figla* expression in MRL fetal testes. RT-PCR products of testes and ovaries of M+ and B6 mice from E14.5 and 18.5 for *Figla*. The mRNA expression of *Figla* is detected in MRL fetal testes of E14.5 and 18.5, but not in the same stage of B6 testes. M, marker.

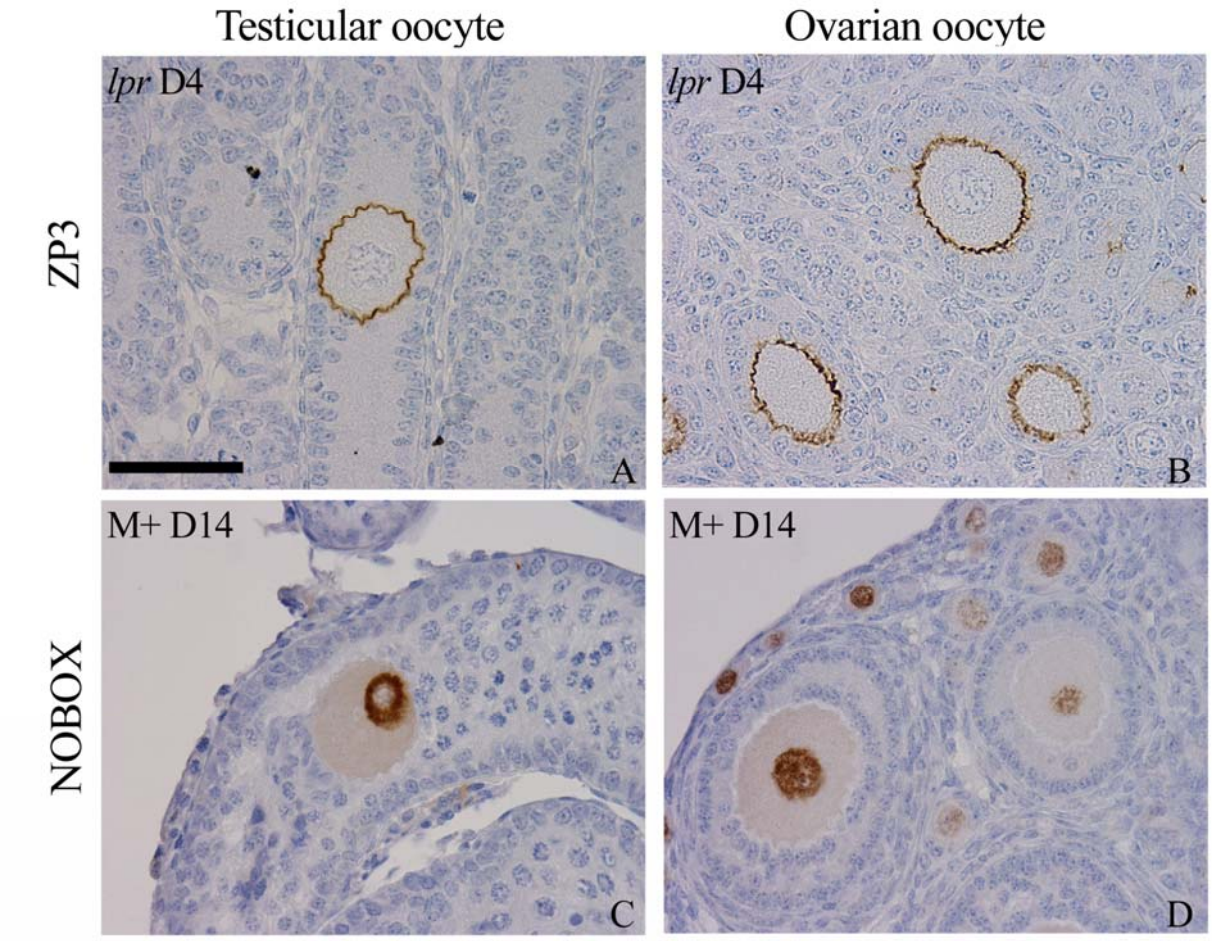

Fig. 2-4. Oocyte-specific proteins in testicular oocytes. Immunohistochemistry of ZP3 of testicular (A) and ovarian oocytes (B) from day 4 and immunolocalization of NOBOX of testicular (C) and ovarian oocyte (D) from day 14 afterbirth. Testicular oocyte contains ZP3 in its zona pellucida and NOBOX in its nucleus at the same level as those in ovarian oocytes, respectively. Zona pellucida of each testicular and ovarian oocyte deformed because of autoclave treatment. All images are at the same magnification. Bar = 50 μm.

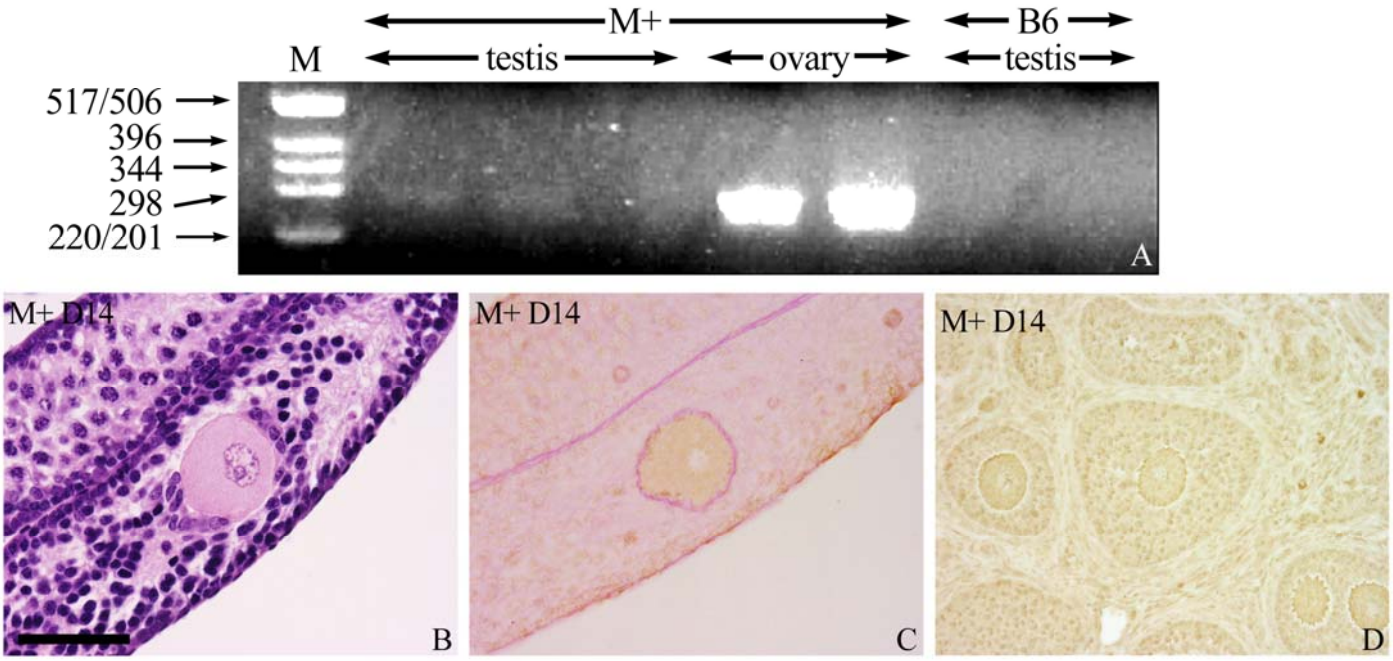

Fig. 2-5. Detection of *Foxl2* at gene and protein level. RT-PCR products of testes and ovaries of M+ and B6 mice from day 13 for *Foxl2* (A). FOXL2 immunostaining of testicular oocyte with PAS counterstaining (C) and ovarian (D) oocytes from day 14 afterbirth. The weak *Foxl2* mRNA expression was observed in MRL testes at day13 afterbirth by RT-PCR, but not in B6 testes. The follicular epithelial cells of testicular oocytes were not immunostained with FOXL2, while those in ovaries were stained. A serial section of C with HE stain is also shown (B). The images in B-D are at the same magnification. Bar = 50 μm. M, marker.

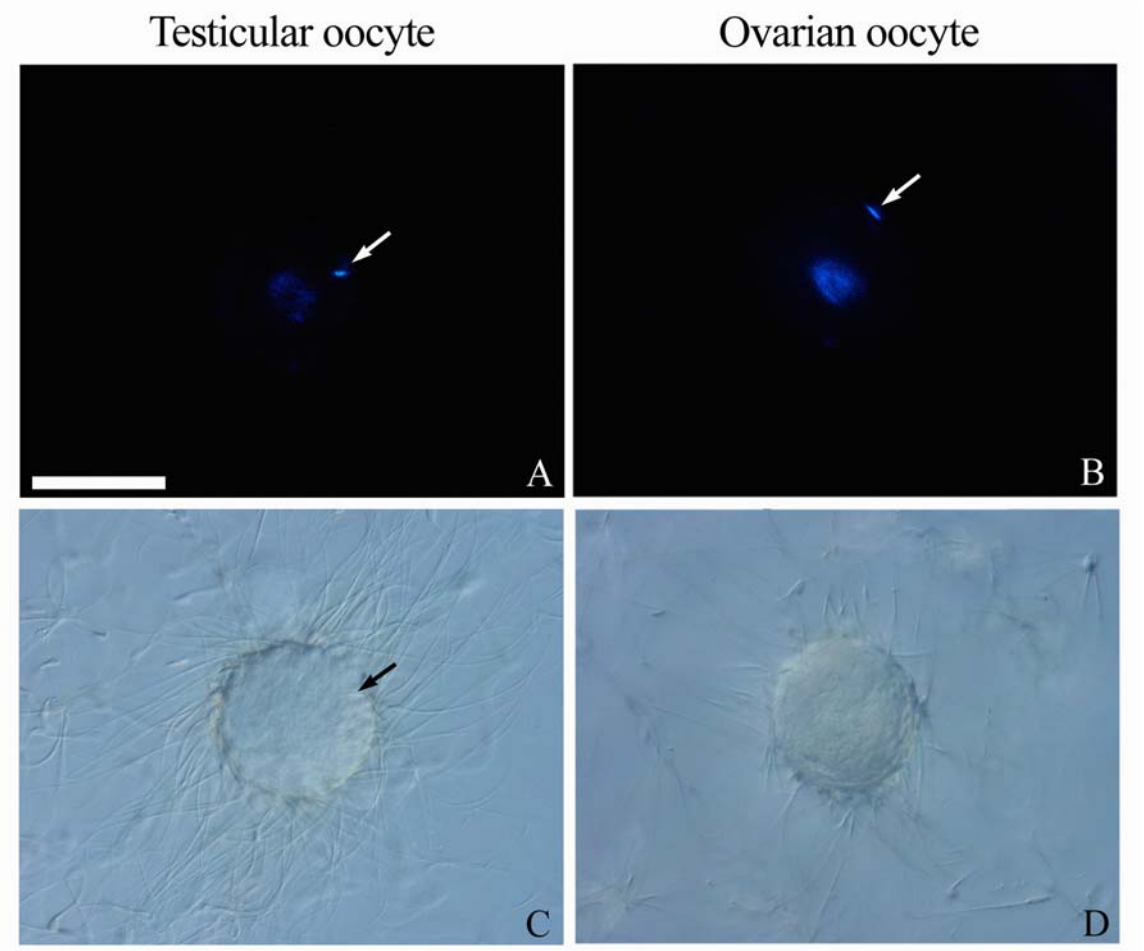

Fig. 2-6. Sperm-egg fusion assay. Testicular and ovarian oocytes under confocal laser scanning microscope (A and B) and differential interference microscope (C and D). Testicular oocyte shows its ability to fuse with sperms. Arrows, heads of invading sperms. All images are at the same magnification. Bar = 50 μm.

Chapter 3

Cause of the appearance of testicular oocytes

Introduction

It has been generally believed that in mammals, males produce only sperms, and oocytes are only produced in females. The sex of a mammal is determined when an ovum containing a haploid genome with an X chromosome is fertilized by a sperm possessing a haploid genome with either an X or Y chromosome. However, the anatomical determination of sex occurs later in development as gonads first arise as bipotential primordia with plasticity to develop into ovaries or testes. Determination of the bipotential primordia into male gonads requires expression of *Sry*, which initiates the differentiation of Sertoli cells, as well as their structural organization into a testis cord (Koopman et al., 1991). If the primordial gonad proceeds to develop along the ovarian cascade despite being in an XY animal, this could result in sexual reversal of the animal, or the development of ovotestes. This has been observed in animals with translocations or deletions of the *Sry* gene or animals with delayed *Sry* expression (Berta et al., 1990; Koopman et al., 1991; Bullejos and Koopman, 2005). As another factor contributing to the plasticity of sexual determination, it is reported that the differentiation of germ cells into oogonia or prospermatogonia is directed by signals from somatic cells in the gonads, not by the germ cells themselves (McLaren, 1995).

In mice, SRY contains the high-mobility group (HMG) box at its N-terminus (3-82 amino acids) and a glutamine (CAG in nucleotide sequence) repeat region interspersed by a sequence FHDHHH (Phe-His-Asp-His-His-His) or similar sequences at the caudal half region (144-367 amino acids) (Fig. 3-1). The HMG box region has been known as DNA-binding domain suggesting that it is predominantly responsible for the role of SRY protein as a transcription factor (Nasrin et al., 1991; Dubin and Ostrer, 1994). On the other hand, the glutamine repeat region has been thought to mediate interaction with other proteins and to have an essential role in sex determination (Coward et al., 1994; Lau and Zhang, 1998; Bowles et al., 1999; Zhang et al., 1999). Although some genetic abnormalities of sexual

differentiation in mammals, including experimental chimeras, can cause the appearance of oocytes in the testes or the development of ovotestis, it has never been reported that fertile male animals can produce oocytes during spermatogenesis.

However, the author found oocytes within testes of newborn MRL mice. The MRL inbred mouse strains, M+ and *lpr*, originate from a series of crosses with strains B6 (0.3%), C3H/He (C3H) (12.1%), AKR/J (AKR) (12.6%), and LG/J (75%) and then followed by inbreeding initiated (Murphy, 1981). As discussed in chapters 1 and 2, the testicular oocytes appeared in MRL mice are derived from primordial germ cells at embryonic period, and have some of the morphological and functional characteristics as so-called oocytes such as the existence of zona pellucida and follicular epithelial cells, expression of oocyte-specific genes, and ability to fuse with sperm.

In this chapter, to identify the cause of the appearance of testicular oocyte, the author examined the existence of testicular oocyte in inbred strains including B6, C3H, and AKR, which are contained in the MRL background and F1 generations between MRL and B6. Additionally, the author focused on the *Sry* to reveal its relevance to the appearance of testicular oocytes by comparison of its nucleotide sequences among MRL and other inbred strains.

Materials and Methods

Mice

Several inbred mouse strains and F1, AKR, BALB/c (BALB), B6, C3H, DBA/2 (DBA), A/J (AJ), M+, *lpr*, B6MRLF1 (F1 between female B6 and male MRL), and MRLB6F1 (F1 between female MRL and male B6) were used in this chapter. These mice were maintained and handled as described in chapter 1. Newly born mice from each strain were obtained by free-breeding, and were sacrificed at 14 days afterbirth by cervical dislocation. The B6MRLF1 and MRLB6F1 strains were also examined to determine whether this phenotype is recessive or dominant.

Light microscopic analysis

To find testicular oocytes, fresh whole-mount preparations of testes obtained from 14-day-old of each strain were observed under a differential interference microscope as described in chapter 1. The number of oocytes per testis (number of oocyte/number of testis) was calculated as an oocyte score.

Genomic DNA extraction and Sry PCR and nucleotide sequencing

To determine the relationship between *Sry* from the MRL Y chromosome and the appearance of testicular oocyte, MRL *Sry* nucleotide sequence was compared with those of other inbred strains. Since *Sry* cDNA contains only one exon, genomic DNA was used for *Sry* nucleotide sequencing (AC_000043.1) (Fig. 3-1). Genomic DNA was prepared from the tail with length of approximately 5 mm for each of the inbred strains. These samples were incubated in lysis buffer containing 50 mM Tris-HCl, 100 mM NaCl, 20 mM EDTA, 1% sodium dodecyl sulfate, and 100 μg/ml proteinase K, overnight at 56˚C, and then treated with phenol extraction. Finally, genomic DNA was purified by ethanol precipitation and resolved

in 500 μl of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

PCR was carried out with ExTaq (Takara) under the following PCR conditions: 5 min at 95˚C, 35 cycles of 40 sec at 95˚C, 30 sec at 62˚C, and 1 min at 72˚C, followed by 5 min at 72˚C. The proper combinations of forward and reverse PCR primer pairs listed in Table 3-1 and Figure 3-1 were used. After the amplified samples were electrophoresed with 2% agarose gel containing ethidium bromide, bands were cut and PCR products were purified with the GENECLEAN II Kit (Funakoshi). Extracted PCR products were labeled with a cycle sequencing kit containing fluorescent terminators employing standard methods (Applied Biosystems) using the listed primer pairs in Table 3-1, and finally analyzed with a model 310 automatic sequencer (Applied Biosystems).

Additionally, in order to compare *Sry* nucleotide sequence at the polymorphic CAG repeat, a two-step PCR method was created as the following protocol. The first PCR products were obtained and purified by procedures written above with primers, mSry-934F and mSry1219R. Then extracted PCR products were diluted by TE to 1:20,000 and used as templates for the second PCR. The author targeted on the sequence FHDHHH which interspersed the glutamine repeat region and created a forward primer as 5’-TTCCATGACCACCACCAC-3’. For the reverse primer, mSry1155R as listed in Table 3-1 was used. The second PCR was carried out with *Taq* DNA polymerase (Promega) under the following PCR conditions: 5 min at 95˚C, 40 cycles of 20 sec at 95˚C, 20 sec at 64˚C, and 30 sec at 72˚C, followed by 5 min at 72˚C. Finally, amplified samples were electrophoresed with 2% NuSieve 3:1 Agarose gel (Cambrex) containing ethidium bromide.

Furthermore, to compare *Sry* nucleotide sequence at the polymorphic HMG box, genomic DNA was amplified with following primer pairs: forward, 5’-TTGTCTAGAGAGCATGGAGG-3’, reverse, 5’-GCTGGTTTTTGGAGTACAGG-3’. The PCR products (398 bp) were digested overnight with the restriction enzyme *Mbo*I

(Takara), and restricted samples were electrophoresed with 2% NuSieve 3:1 Agarose gel containing ethidium bromide.

Results

Number of testicular oocytes in various mouse strains and F1 progenies

Testicular oocytes were found in M+, *lpr*, AKR, and B6MRLF1, but not in other inbred strains including MRLB6F1 mice (Table 3-2 and Fig. 3-2). The oocyte scores of M+ and *lpr* were almost the same (M+: 1.23, *lpr*: 1.27); however, those of AKR and B6MRLF1 were less than the MRL strains (AKR: 0.15, B6MRLF1: 0.29). Morphologically, each testicular oocyte found in B6MRLF1 and AKR contained large nucleus with nucleoli, zona pellucida, and follicular epithelial cells that were similar to those of MRL. Apparently, the follicular epithelial cells in AKR mice were less developed than those of MRL and B6MRLF1.

Comparison of Sry nucleotide sequences and corresponding amino acids among M+, B6, and AKR

Initially, the whole nucleotide sequence of *Sry* between M+ and B6 were compared. The open reading frames of *Sry* and corresponding amino acid sequences are shown in Figure 3-3. The open reading frame of B6 *Sry* encodes a protein of 395 amino acids, while MRL *Sry* contains 389 amino acids, and both of the nucleotide sequences and amino acids homology of these two strains were approximately 97%. There were only three single-base changes, which caused amino acid alternations within upper half of *Sry* sequences between B6 and MRL, Ile63Thr in HMG box and Trp133Thr and Leu143Pro in the junctional site between HMG box and CAG repeats. In contrast, there were many substitutions within the glutamine repeat region such as deletion of glutamine at codon 153 and 154, His169Gln, insertion of histidine at codon 275 and 297, Pro286Gln, Lys289Gln, Pro296His, and His307Gln. Strikingly, Gln351His and the following deletion of six CAG repeat causing shortened glutamine repeat was observed in MRL SRY. There was no difference between B6 and MRL at the downstream of the glutamine repeat region at C-terminal. As a result of

comparison of the whole nucleotide sequences and corresponding amino acid sequences of *Sry* between M+ and AKR, only two differences were found, Gln284Pro and Gln305His (Fig. 3-4).

Comparison of SRY glutamine repeats among representative inbred strains

Since polymorphism among MRL, B6, and AKR was concentrated in the glutamine repeat region, the glutamine regions between testicular oocyte-producing strains (MRL and AKR) and other non-producing inbred strains (BALB, B6, C3H, DBA, and AJ) were compared. Only 282-381 amino acids are shown in Figure 3-5. As a result of the comparison, polymorphism common in MRL and AKR but not in other strains was only found in two regions. One was at 294-302 amino acids in which DHHHQQQQQ was in MRL and AKR, while DHQ-QQQQQ, DHP-QQQQQ, and DHH-QQQQQ were in AJ, B6, and other strains, respectively. The other was the substitution of His351Gln and the deletion of six glutamine repeat at 351-357 in MRL and AKR.

In order to prove the shortened glutamine repeat, the polymorphic glutamine repeat with a two-step PCR method using the forward primer corresponding to the region FHDHHH was compared. From a deduced sequence (Fig. 3-6A), SRY of the inbred strains except MRL and AKR contained three FHDHHH regions at 202-207, 279-284, and 333-338 amino acids which should result as PCR product of 552, 411, and 255 bp sizes, respectively. On the other hand, four amplified products should be observed in MRL because of the substitution or insertion of histidines at codons 296, 297, and 307 resulting in the existence of FHDHHH regions at 202-207, 279-284, 292-297, and 376-381 with products of 537, 396, 270, and 108 bp. In AKR mice, since there was no substitution of histidine at codon 307, five PCR products should be obtained at 537, 396, 270, 237, and 108 bp. The results of PCR shown in Figure 3-6B corresponded to the expectation, especially the presence of the 108 bp band

provided evidence of the shortened glutamine repeat in MRL and AKR.

Additionally, the author tried to detect polymorphism found in HMG box by using restricted enzyme, *Mbo*I (Fig. 3-7A). This restricted enzyme recognizes the sequences GATC and digests DNA at cis side. The DNA sequence of *Sry* HMG box of the inbred strains except MRL and AKR contained two GATCs at 102-105 and 186-189, which should result as PCR product of 114, 84, and 200 bp, respectively. On the other hand, only two restricted products should be obtained in MRL and AKR mice, because of the substitution of thymine to cytosine at 188 resulting in only one GATC. The results of DNA digestion shown in Figure 7B correspond to the expectation, two bands, 114 and 284bp in AKR and MRL and three bands, 200, 114, and 84bp in other inbred strains.

Discussion

The existence of testicular oocytes has been reported in several cases such as chimeric mice, XXsxr sex-reversed mice, and the fetal testis transplanted under an adult female kidney capsule (Mystkowska and Tarkowski, 1968; Ozdzenski, 1972; McLaren, 1980; Ozdzenski and Presz, 1984; Isotani et al., 2005). Thus, to date, testicular oocyte in MRL mice was the only report of the appearance of testicular oocytes in XY fertile males. In this study, the testicular oocytes were also found in newborn AKR mice. As AKR is one of the MRL ancestor strains, it is suggested that the appearance of testicular oocyte was genetically derived in part from AKR. Since both MRL strains, M+ and *lpr*, showed similar oocyte score, the development of testicular oocytes appears to be dependent on the MRL genetic background, but not on the *Fas* gene (Theofilopoulos and Dixon, 1985). Based on the observation that testicular oocytes were found only in F1 mice that had an MRL father, it was suggested that this phenotype was dominant as long as the Y chromosome was derived from the MRL background, meaning that one of the genes responsible for the appearance of testicular oocytes is on the Y chromosome. However, AKR including B6MRLF1 showed much lower appearance rates than MRL, indicating that multiple genes, probably on the autosomes, are also required for the development of this phenotype.

As a result of comparison, three amino acid alternations within the upper half of SRY sequences between B6 and MRL, Ile63Thr in HMG box and Trp133Thr and Leu143Pro in the junctional site which also observed in AKR were found. Unlike the junctional site between HMG box and glutamine repeat region, the importance of HMG box in SRY function is well known as the mutations in this DNA binding domain cause sex reversal in humans (Harley et al., 2003). Since many of the *Mus musculus domesticus* contain threonine at positions 63 and 133 and proline at position 143 like MRL and AKR (Albrecht and Eicher, 1997; Washburn et al., 2001), these alternations do not seem to be important for the appearance of

testicular oocyte. However, the alternation in SRY HMG box is important to know evolutional origin of Y chromosome in mice (Kunieda and Toyoda, 1992; Nagamine et al., 1994). The method to detect Ile63Thr in HMG box by using restricted enzyme, *Mbo*I, can be a useful tool to trace paternal ancestry in mice.

At the glutamine repeat region, there were two regions of polymorphism common in MRL and AKR, but not in other strains, codons 294-302 and the substitution and deletion of seven glutamines at 351-357. The 294-302 amino acids contained DHHHQQQQQ in MRL and AKR, and DHQ-QQQQQ, DHP-QQQQQ, or DHH-QQQQQ in other strains. However, this substitution and insertion of histidine in MRL and AKR seemed less likely to have a relationship with the appearance of testicular oocyte as substitutions varied among strains. By contrast, the substitution and deletion of seven glutamines at 351-357 amino acids was only found in MRL and AKR, and is suggested as one of the candidate factors for the appearance of testicular oocyte.

Polymorphism of the glutamine repeat has been known to cause many diseases in humans. Many of these diseases, such as Huntington disease, spinal bulbar muscular atrophy, several spinocerebellar ataxias, and dentatorubral-pallidoluysian atrophy are results of the extension of glutamine repeat (Shao and Diamond, 2007; Stack and Ferrante, 2007; Walker, 2007). Conversely, there are only few reports about the effect of shortening glutamine, mostly about the relationship between androgen receptor gene and risk for cancer (Yu et al., 2000; De Abreu et al., 2007). Several mechanisms causing these diseases have been identified such as protein aggregation, mitochondrial dysfunction, oxidative stress, transcriptional dysregulation, aberrant apoptosis, altered proteosomal function, and excitotoxicity (Rubinsztein and Carmichael, 2003; Shao and Diamond, 2007; Stack and Ferrante, 2007; Katsuno et al., 2008).

The importance of glutamine repeat has also been reported in mice (*Mus musculus*) SRY.

When the Y chromosome of a *Mus musculus domesticus poschiavinus* strain is placed onto the C57BL/6J genetic background, the XY (B6.Y^{DOM}) progeny show sex reversal (Eicher et al., 1982). This phenomenon has been known to correlates with polymorphism of a glutamine repeat within SRY; however, these mice possess only sex reversal or ovotestis, but never testicular oocyte (Coward et al., 1994; Bullejos and Koopman, 2005). Additionally, even though SRY alone induce testes in XX gonads in mice, SRY constructs without the glutamine repeat region could not induce testis formation in XX embryos. (Bowles et al., 1999). Although the exact roles of this structure within mouse SRY protein functions have not been identified yet, it is suggested that they contribute to the biological functions of mouse SRY through protein-protein interactive roles (Lau and Zhang, 1998; Zhang et al., 1999). Hence the glutamine repeat region of mouse SRY is thought to have an essential role in sex determination.

Sex differentiation of mouse primordial germ cell occurs at E13.5 when female germ cells enter meiosis while male germ cells are arrested in G0/G1 of mitosis. The meiosis-inhibiting mechanism found in fetal testes can be mainly divided into two events, formation of testis cords and prevention of retinoic acid known as a meiosis-inducing factor. The formation of testis cords occurs at E12.0-12.5 as a result of interaction of many genes, which are thought as downstream targets of *Sry* such as *Nr0b1* (nuclear receptor subfamily 0, group B, member 1), *Dhh* (desert hedgehog), *Pdfgra* (platelet-derived growth factor receptor, alpha), and *Ntf3* (neurotrophin 3) (Levine et al., 2000; Pierucci-Alves et al., 2001; Cupp et al., 2002 and 2003; Yao and Capel, 2002; Meeks et al., 2003; Brennan et al., 2003; Brennan and Capel, 2004; Smith et al., 2005). Additionally, testicular somatic cells express *Cyp26b1*, an enzyme involved in retinoid metabolism, under the regulation of *Sry* (Bowles et al., 2006; Koubova et al., 2006). Thus, by being sequestrated within testis cords, germ cells are prevented to enter meiosis in the XY gonad with normal *Sry* expression. In spite of the

existence of the meiosis-inhibiting mechanism, it was noted that meiosis prematurely progresses in MRL fetal testes starting as early as E13.5 as shown in chapter 2. Taking all of the above into consideration, it is indicated that MRL fetal testes possess a unique meiotic progression mechanism, which might be the result of the shortened glutamine repeats in SRY and involved in the appearance of testicular oocytes. Additionally, the two-step PCR method that the author used to compare CAG repeats can be an easy way to find mouse strains with the possibility to produce testicular oocytes.

Since the glutamine repeat in SRY is unique in mice (Whitfield et al., 1993), it is suggested that the testicular oocyte can appear only in mice. Although it is necessary to identify the complicity genes, clarification of the relationship between SRY with shortened glutamine repeat and testicular oocyte can provide more clues concerning the development of the reproductive system in mice, such as the mechanisms of sex differentiation and the mechanism that prevents entry into meiosis in male embryos.

Summary

Although mammals produce either sperms or eggs depending on their sex, newborn MRL male mice contain oocytes within their testes. In the previous chapters, the author showed that the testicular oocytes had many morphological and functional characteristics as so-called oocytes such as existence of zona pellucida, follicular epithelial cells, and fusing ability with sperm. In this chapter, the testicular oocytes were also found in newborn AKR mice and B6MRLF1. Based on the observation of F1, one of the genes causing the appearance of testicular oocyte exists on the Y chromosome. Additionally, the analysis on the *Sry* genes from several inbred mouse strains revealed a shortened glutamine repeat near the C-terminal region was unique to MRL and AKR. These findings suggest that polymorphism of glutamine repeat within SRY correlates with the appearance of testicular oocyte and this phenotype is derived from AKR, one of the original strains of MRL mice.

Tables and Figures

Table 3-1. Primer pairs used for *Sry* sequencing

	Name	5' - 3'	Position
Forward	mSry-934F	TGCATGGTGATGCTGTAAGG	-934 ~ -915
	mSry-634F	TACTAATGATAGGTTCCATGC	-634 ~ -614
	mSry-56F	TCTTAAACTCTGAAGAAGAGAC	-56 ~ -35
	mSry53F	GTGAGAGGCACAAGTTGGC	53 ~ 71
	mSry270F	GCCTGCAGTTGCCTCAACAA	270 ~ 289
	mSry389F	GCTTTTATTGGCAGCCTGTTG	389 ~ 409
	mSry566F	AGCAGCAGTTTCATGACCAC	566 ~ 585
Reverse	mSry198R	CTCTGTGTAGGATCTTCAATC	198 ~ 179
	mSry1155R	GTGCTCCTGGTATGCTGTAT	1155 ~ 1136
	mSry1219R	GTGTTGGCATAGGTAGGAGA	1219 ~ 1200

The first base of *Sry* nucleotide sequence, adenine, is set as position one. The sequences locate upstream of this adenine is shown with minus symbols.

Table 3-2. Comparison of oocyte socre

Strain	No. of testis	No. of oocyte	Oocyte score
M+	98	121	1.23
lpr	30	38	1.27
AKR	78	12	0.15
AJ	24	0	0
BALB	20	0	0
B6	50	0	0
C3H	46	0	0
DBA	20	0	0
B6MRLF1	68	20	0.29
MRLB6F1	68	0	0

Testicular oocytes were found in M+, *lpr*, AKR, and B6MRLF1, but not in other inbred strains including MRLB6F1 mice. The oocyte scores of M+ and *lpr* were almost the same; however, those of AKR and B6MRLF1 were less than the MRL strains.

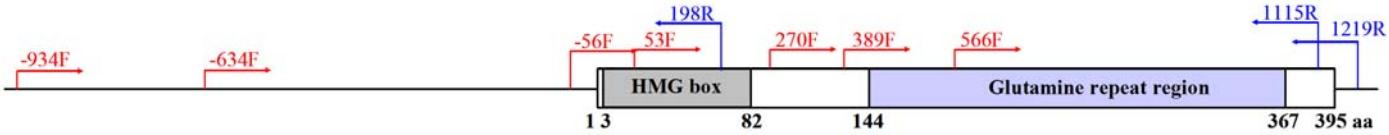

Fig. 3-1. Mouse SRY and the position of primer pairs used for sequencing. Mouse SRY is composed of only one exon and contains HMG box (gray) and glutamine repeat region (light blue). Red arrows and letters, forward primers; Blue arrows and letters, reverse primers; -934F, mSry-934F; -634F, mSry-634F: -56F, mSry-56F; 53F, mSry53F, 270F, mSry270F; 389F, mSry389F; 566F, mSry566F; 198R, mSry198R; 1155R, mSry1155R; 1219R, mSry1219R.

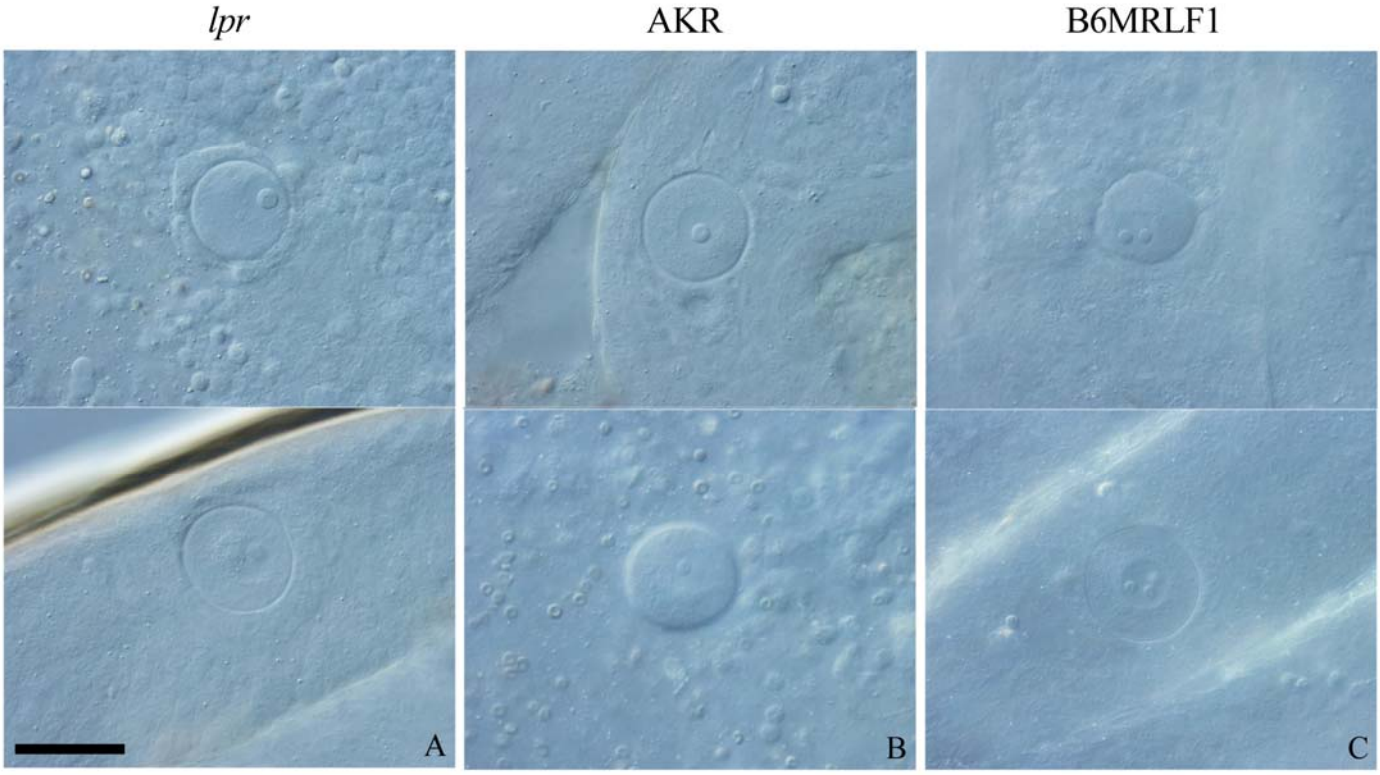

Fig. 3-2. Testicular oocytes in *lpr*, AKR, and B6MRLF1 aged 14 days a differential interference microscope. Testicular oocytes in whole mount testis of *lpr* (A), AKR (B), and B6MRLF1 (C). Morphologically, the testicular oocytes in B6MRLF1 and AKR contained large nuclei with nucleoli, zona pellucida, and follicular epithelial cells that were similar to those of MRL. Apparently, the follicular epithelial cells in AKR mice were less developed than those of MRL and B6MRLF1. All images are at the same magnification. Bar = 50 μm.

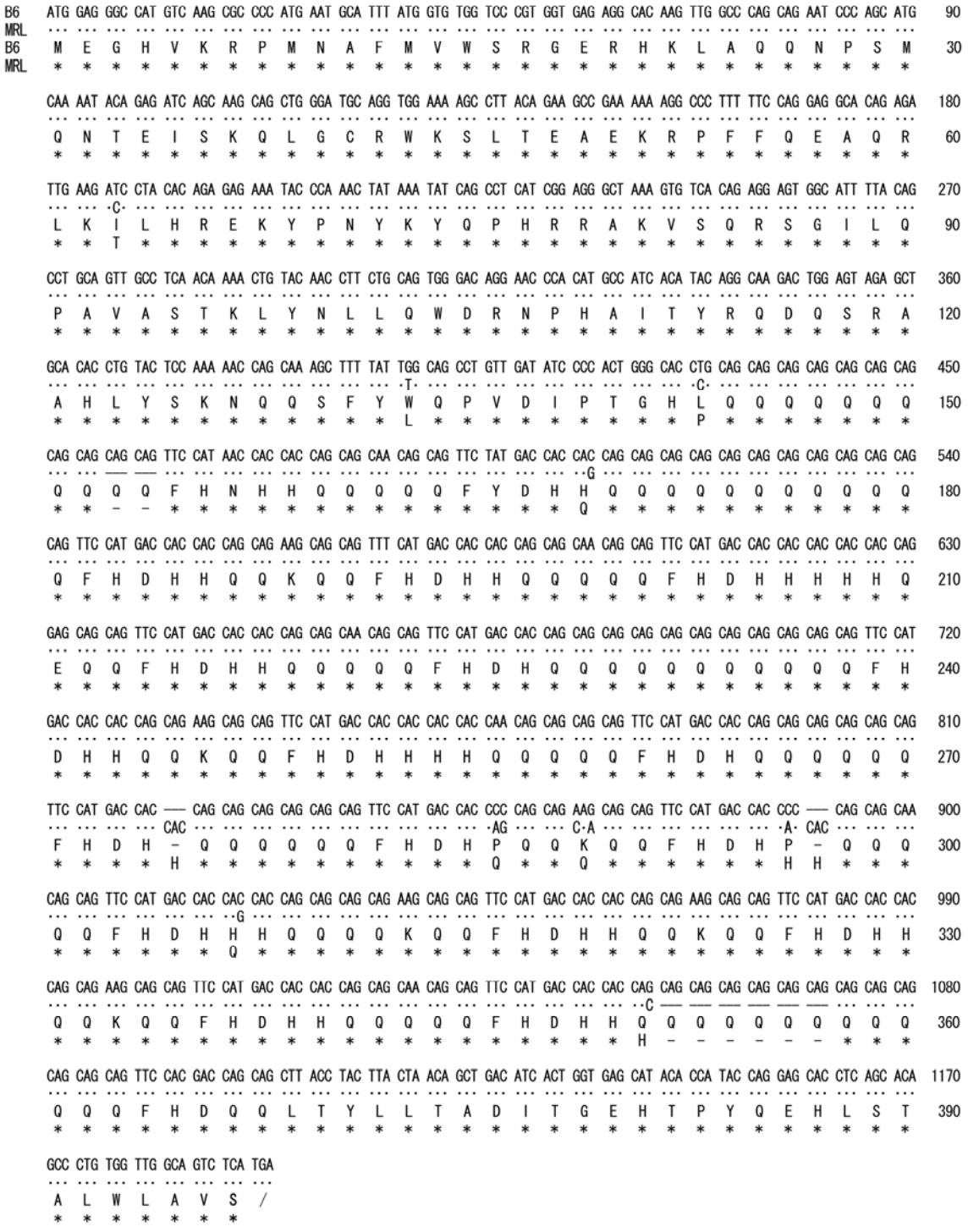

Fig. 3-3. Alignment of B6 and MRL *Sry* sequences. First line and second lines show DNA sequences and third and fourth lines show amino acid sequences, respectively. Dot (.), identical DNA sequences; hyphen (-), deletion; asterisk (*), identical amino acids; slash (/), stop codon.

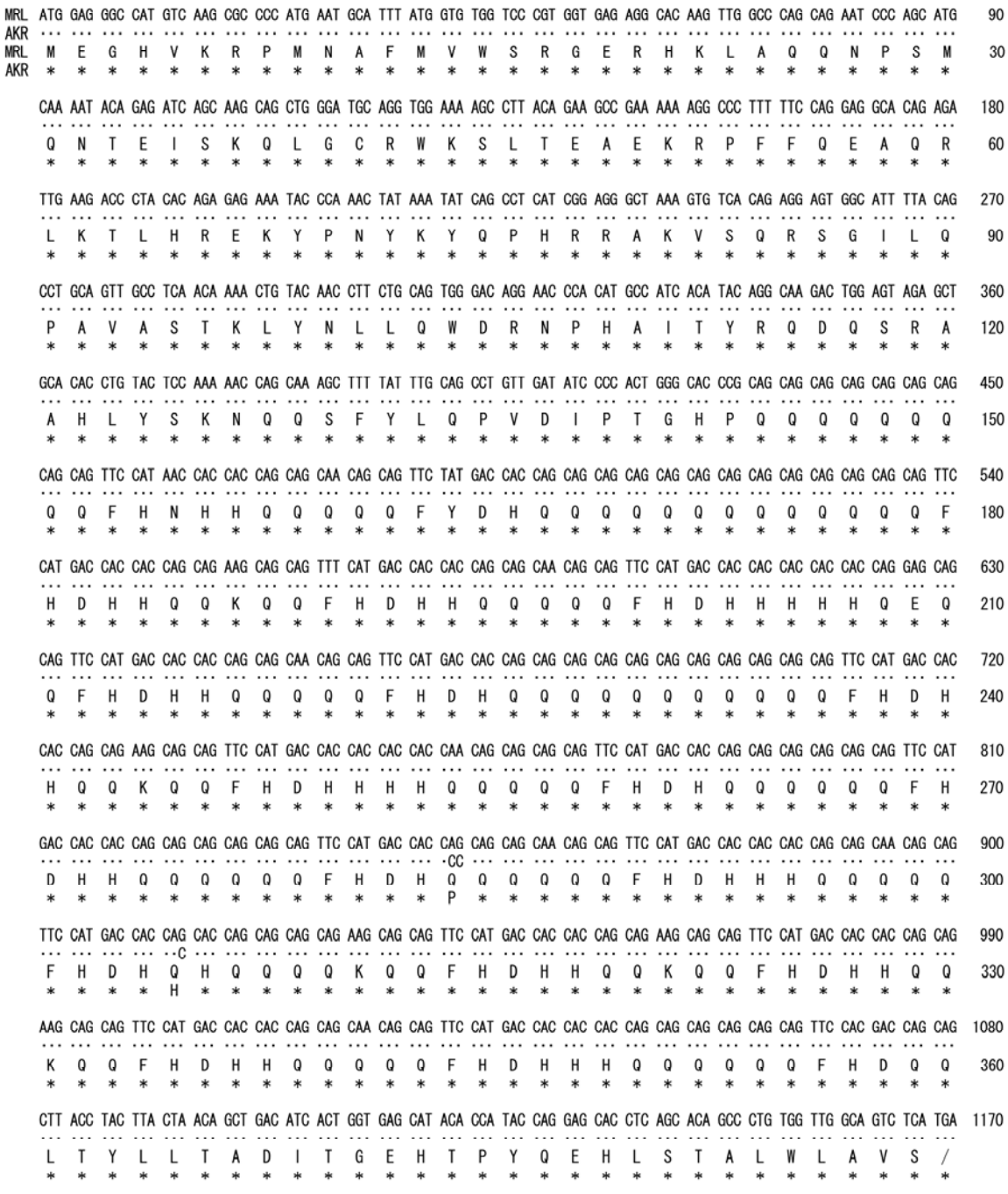

Fig. 3-4. Alignment of MRL and AKR *Sry* sequences. First line and second lines show DNA sequences and third and fourth lines show amino acid sequences, respectively. There are only two differences in the whole nucleotide sequence and corresponding amino acid sequence of *Sry* between M+ and AKR, Gln284Pro and Gln305His. Dot (.), identical DNA sequences; hyphen (-), deletion; asterisk (*), identical amino acids; slash (/), stop codon.

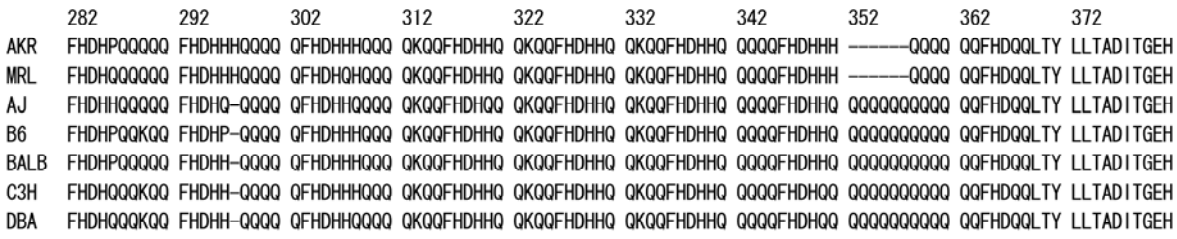

Fig. 3-5. Comparison of glutamine repeat region (282-381) of SRY among inbred strains. The substitution of His351Gln and the deletion of six glutamine repeat at 351-357 were only found in MRL and AKR. -, deletion.

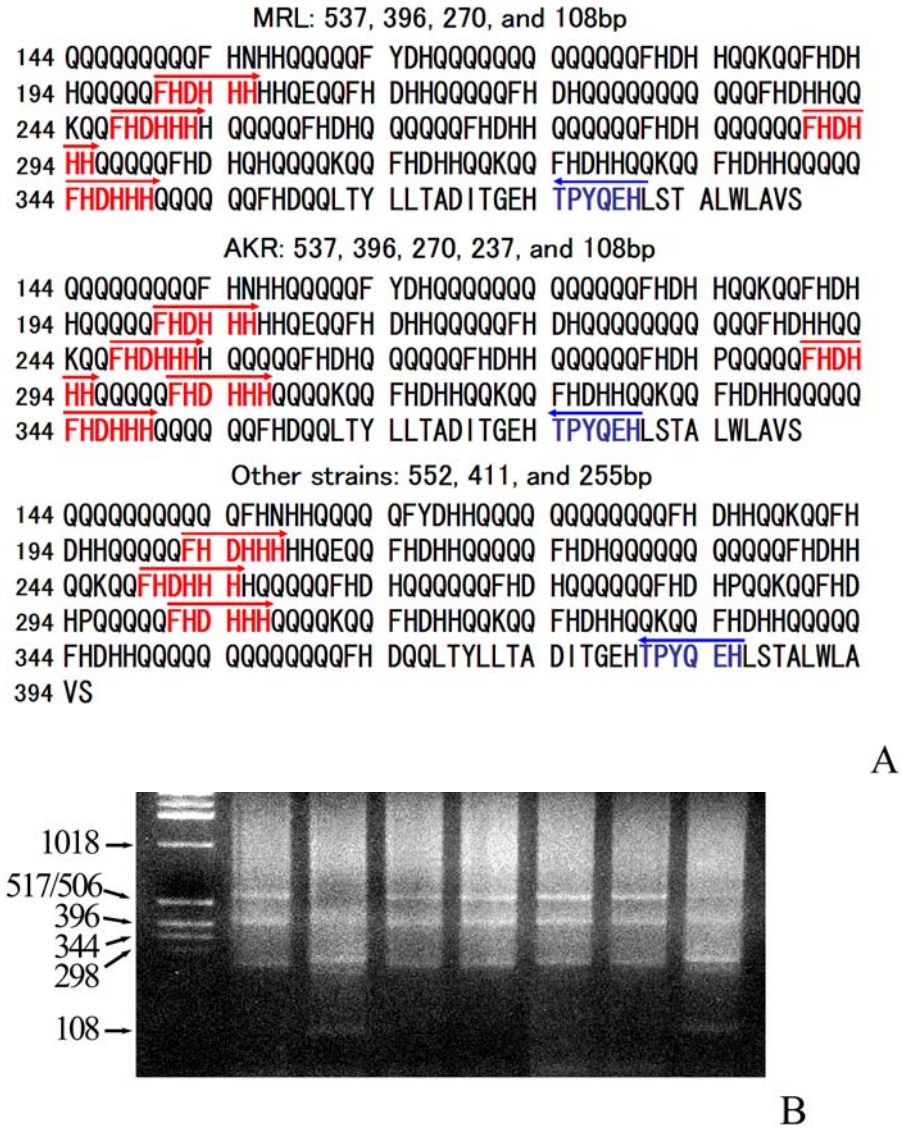

Fig. 3-6. Comparison of *Sry* glutamine repeats among inbred strains. Two-step PCR method using the forward primer corresponding to the region FHDHHH (A). Red arrows and letters, the sequence of forward primers; Blue arrows and letters, the sequence of reverse primers. Comparison of *Sry* electrophoresis patterns (B). Note that distinctive bands at 108 bp found only in MRL and AKR strains.

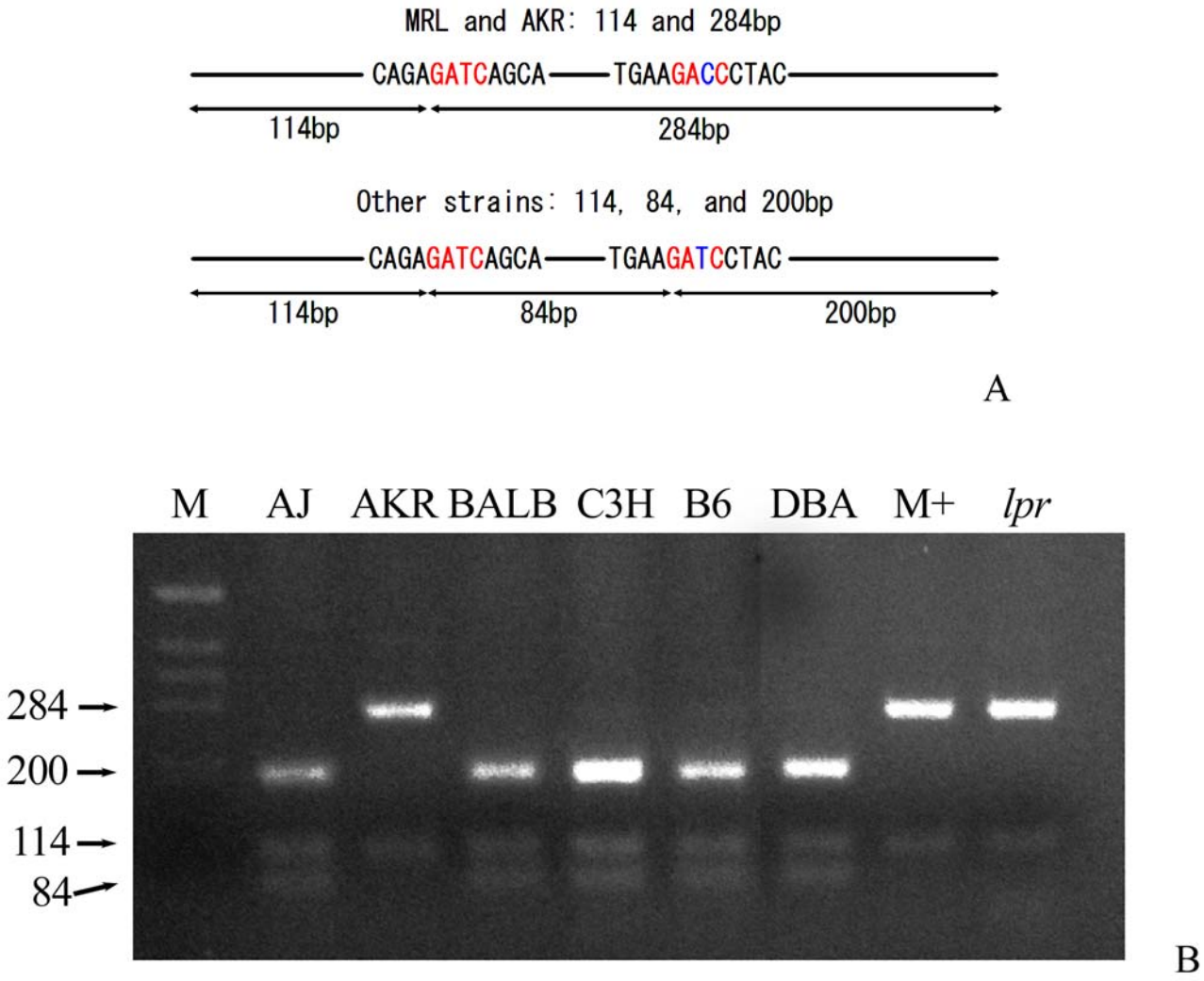

Fig. 3-7. Comparison of *Sry* electrophoresis patterns with digestion by *Mbo*I. The comparison of *Sry* HMG box and *Mbo*I recognition sequences among strains (A). Red letters, *Mbo*I recognition sequences; Blue letters, the substitution among MRL, AKR, and other strains. The results of DNA digestion correspond to the expectation, two bands, 114 and 284bp, in AKR and MRL and three bands, 200, 114, and 84bp, in other inbred strains (B).

Conclusion

Gonad is unique among all organs because of its bipotential nature, a testis or an ovary. In most mammals, sex determination is genetically controlled by the presence or absence of the Y chromosome. Germ cells also have the plasticity to develop either oogonia or prospermatogonia. The sex of primordial germ cell is determined by the time points of meiosis initiation which regulated by gonadal somatic cells. In mice, this phenomenon occurs at E13.5, when ovarian germ cells enter meiosis and testicular germ cells are arrest in the G0/G1 of the mitotic cell cycle. These sex differentiation mechanisms advocate a universal rule 'mammalian males produce only sperms in their testes and females produce only oocytes in their ovaries'. However, as an exception to this rule, the author found oocytes in the testes of newborn MRL male mice. In this thesis, the author attempted to reveal the mechanism of initiating oogenesis in testis and verify the possibility of production of offspring using testicular oocyte.

At first, the author proved the existence of oocytes in testis by their morphological characteristics and the expression of some oocyte-specific genes in the testis of MRL mice. The testicular oocytes had a diameter of 50 to 70 μm, and were surrounded by zona pellucida observed between oocytes and follicular epithelial cells. Although the follicular epithelial cells formed a multilayer similar to that observed in early stage secondary follicles, they never a formed follicular antrum or a polar body-like structure. Ultrastructurally, the testicular oocytes contained numerous microvilli and cortical granules, receiving cytoplasmic projections from follicular epithelial cells. The testicular oocytes appeared from 0 to 30 days afterbirth and the largest number was found around day 14. The expression of the oocyte-specific genes *Zp1-3* and *Omt2a* was detected in testes from MRL mice. These morphological characteristics as an oocyte and the expression of oocyte-specific genes indicate that newborn MRL male mice evidently have the ability to produce oocytes in their

testes.

The author next examined the derivation of testicular oocyte and verified the characteristics as an oocyte. In MRL fetal testes, some germ cells underwent meiosis and oogenesis just like fetal ovarian germ cells at same stages. Additionally, the zona pellucida of testicular oocyte contained ZP3, while follicular epithelial cells lacked FOXL2. Furthermore, the testicular oocyte had the ability to fuse with sperms. These results suggest that testicular oocytes are derived from primordial germ cells at about E13.5 under the same process with ovarian oocytes and they contain the characteristics as so-called oocytes such as the ability to fuse with sperms. However, follicular epithelial cells lacking FOXL2 might be involved in the limitation of testicular oocyte development.

Finally, the author attempted to identify the causing genes of appearance of testicular oocyte. As a result of examination of testes from several inbred strains and F1 produced between MRL and B6, testicular oocytes were also found within newborn AKR mice and B6MRLF1 other than MRL mice. Based on the observation of F1, one of the genes causing the appearance of testicular oocyte existed on the Y chromosome. Then the author analyzed the *Sry* genes from several inbred mouse strains and identified a shortened glutamine repeat near the C-terminal region that was unique to MRL and AKR. These findings suggest that polymorphism of glutamine repeat within SRY correlates with the appearance of testicular oocyte and this phenotype is derived from AKR, one of the original strains of MRL mice.

As conclusion, testicular oocytes found in newborn male MRL mice are derived from primordial germ cells during embryonic period and their appearance correlates with polymorphism of SRY glutamine repeat. Although it is difficult to expect normal development of oocytes within testicular environment at this point, there is still a possibility of healthy growth by rescue and culture *in vitro*. Thus testicular oocytes contain potential keys to open new doors in reproductive biology and provide more clues concerning the

development of the reproductive system in mice.

References

1. Albrecht, K.H. and Eicher, E.M. 1997. DNA sequence analysis of *Sry* alleles (subgenus Mus) implicates misregulation as the cause of C57BL/6J-Y^{POS} sex reversal and defines the SRY functional unit. Genetics 147: 1267-1277.
2. Alfieri, J.A., Martin, A.D., Takeda, J., Kondoh, G., Myles, D.G. and Primakoff, P. 2003. Infertility in female mice with an oocyte-specific knockout of GPI-anchored proteins. J. Cell Sci. 116: 2149-2155.
3. Berta, P., Hawkins, J.R., Sinclair, A.H., Taylor, A., Griffiths, B.L., Goodfellow, P.N. and Fellous, M. 1990. Genetic evidence equating SRY and the testis-determining factor. Nature 348: 448-450.
4. Bowles, J., Cooper, L., Berkman, J. and Koopman, P. 1999. *Sry* requires a CAG repeat domain for male sex determination in *Mus musculus*. Nat. Genet. 22: 405-408.
5. Bowles, J., Knight, D., Smith, C., Wilhelm, D., Richman, J., Mamiya, S., Yashiro, K., Chawengsaksophak, K., Wilson, M.J., Rossant, J., Hamada, H. and Koopman, P. 2006. Retinoid signaling determines germ cell fate in mice. Science 312: 596-600.
6. Brennan, J. and Capel, B. 2004. One tissue, two fates: molecular genetic events that underlie testis versus ovary development. Nat. Rev. Genet. 5: 509-521.
7. Brennan, J., Tilmann, C. and Capel, B. 2003. *Pdgfr-α* mediates testis cord organization and fetal Leydig cell development in the XY gonad. Genes. Dev. 17: 800-810.
8. Bullejos, M. and Koopman, P. 2005. Delayed *Sry* and *Sox9* expression in developing mouse gonads underlies B6-Y^{DOM} sex reversal. Dev. Biol. 278: 473-481.
9. Capel, B. 2000. The battle of the sexes. Mech. Dev. 92: 89-103.
10. Chen, M.S., Tung, K.S., Coonrod, S.A., Takahashi, Y., Bigler, D., Chang, A., Yamashita, Y., Kincade, P.W., Herr, J.C. and White, J.M. 1999. Role of the integrin-associated

protein CD9 in binding between sperm ADAM 2 and the egg integrin α6β1: implications for murine fertilization. Proc. Natl. Acad. Sci. U.S.A. 96: 11830-11835.

11. Cho, C., Bunch, D.O., Faure, J.E., Goulding, E.H., Eddy, E.M., Primakoff, P. and Myles, D.G. 1998. Fertilization defects in sperm from mice lacking fertilin beta. Science 281: 1857-1859.
12. Choi, Y. and Rajkovic, A. 2006. Genetics of early mammalian folliculogenesis. Cell Mol. Life Sci. 63: 579-590.
13. Choi, Y., Yuan, D. and Rajkovic, A. 2008. Germ cell-specific transcriptional regulator *sohlh2* is essential for early mouse folliculogenesis and oocyte-specific gene expression. Biol. Reprod. 79: 1176-1182.
14. Clark, L.D., Clark, R.K. and Heber-Katz, E. 1998. A new murine model for mammalian wound repair and regeneration. Clin. Immunol. Immunopathol. 88: 35-45.
15. Colvin, J.S., Green, R.P., Schmahl, J., Capel, B. and Ornitz, D. M. 2001. Male-to-female sex reversal in mice lacking fibroblast growth factor 9. Cell 104: 875-889.
16. Conover, J.C. and Gwatkin, R.B. 1988. Pre-loading of mouse oocytes with DNA-specific fluorochrome (Hoechst 33342) permits rapid detection of sperm-oocyte fusion. J. Reprod. Fertil. 82: 681-690.
17. Coward, P., Nagai, K., Chen, D., Thomas, H.D., Nagamine, C.M. and Lau, Y.F. 1994. Polymorphism of a CAG trinucleotide repeat within Sry correlates with B6.YDom sex reversal. Nat. Genet. 6: 245-250.
18. Crisponi, L., Deiana, M., Loi, A., Chiappe, F., Uda, M., Amati, P., Bisceglia, L., Zelante, L., Nagaraja, R., Porcu, S., Ristaldi, M.S., Marzella, R., Rocchi, M., Nicolino, M., Lienhardt-Roussie, A., Nivelon, A., Verloes, A., Schlessinger, D., Gasparini, P., Bonneau, D., Cao, A. and Pilia, G. 2001. The putative forkhead transcription factor *FOXL2* is

mutated in blepharophimosis/ptosis/epicanthus inversus syndrome. Nat. Genet. 27: 159-166.

19. Cupp, A.S., Tessarollo, L. and Skinner, M.K. 2002. Testis developmental phenotypes in neurotropin receptor *trkA* and *trkC* null mutations: role in formation of seminiferous cords and germ cell survival. Biol. Reprod. 66: 1838-1845.
20. Cupp, A.S., Uzumcu, M. and Skinner, M.K. 2003. Chemotactic role of neurotropin 3 in the embryonic testis that facilitates male sex determination. Biol. Reprod. 68: 2033-2037.
21. De Abreu, F.B., Pirolo, L.J., Canevari Rde, A., Rosa, F.E., Moraes Neto, F.A., Caldeira, J.R., Rainho, C.A. and Rogatto, S.R. 2007. Shorter CAG repeat in the AR gene is associated with atypical hyperplasia and breast carcinoma. Anticancer Res. 27: 1199-1205.
22. Di Carlo, A.D., Travia, G. and De Felici, M. 2000. The meiotic specific synaptonemal complex protein SCP3 is expressed by female and male primordial germ cells of the mouse embryo. Int. J. Dev. Biol. 44: 241-244.
23. Dubin, R.A. and Ostrer, H. 1994. Sry is a transcriptional activator. Mol. Endocrinol. 8: 1182-1192.
24. Eicher, E.M., Washburn, L.L, Whitney, J.B., and Morrow, K.E. 1982. *Mus poschiavinus* Y chromosome in the C57BL/6 murine genome causes sex reversal. Science 217: 535-537.
25. Epifano, O., Liang, L.F., Familari, M., Moos, M.C. and Dean, J. 1995. Coordinate expression of the three zona pellucida genes during mouse oogenesis. Development 121: 1947-1956.
26. Foster, J.W., Dominguez-Steglich, M.A., Guioli, S., Kowk, G., Weller, P.A., Stevanović, M., Weissenbach, J., Mansour, S., Young, I.D., Goodfellow, P.N., Brook, J.D. and

Schafer, A.J. 1994. Campomelic dysplasia and autosomal sex reversal caused by mutations in an *SRY*-related gene. Nature 372: 525-530.

27. Francis, R.C. 1992. Sexual lability in teleosts: developmental factors. Q. Rev. Biol. 67: 1-18.
28. Harley, V.R., Clarkson, M.J. and Argentaro, A. 2003. The molecular action and regulation of the testis-determining factors, SRY (sex-determining region on the Y chromosome) and SOX9 [SRY-related high-mobility group (HMG) box 9]. Endocr. Rev. 24: 466-487.
29. Inoue, N., Ikawa, M., Isotani, A. and Okabe, M. 2005. The immunoglobulin superfamily protein Izumo is required for sperm to fuse with eggs. Nature 434: 234-238.
30. Isotani, A. Nakanishi, T., Kobayashi, S., Lee, J., Chuma, S., Nakatsuji, N., Ishino, F. and Okabe, M. 2005. Genomic imprinting of XX spermatogonia and XX oocytes recovered from XX↔XY chimeric testes. Proc. Natl. Acad. Sci. U.S.A. 102: 4039-4044.
31. Jamnongjit, M. and Hammes, S.R. 2005. Oocyte maturation: the coming of age of a germ cell. Semin. Reprod. Med. 23: 234-241.
32. Katsuno, M., Banno, H., Suzuki, K., Takeuchi, Y., Kawashima, M., Tanaka, F., Adachi, H. and Sobue, G. 2008. Molecular genetics and biomarkers of polyglutamine diseases. Curr. Mol. Med. 8: 221-234.
33. Kon, Y. and Endoh, D. 2000. Morphological study of metaphase-specific apoptosis in MRL mouse testis. Anat. Histol. Embryol. 29: 313-319.
34. Kon, Y. and Endoh, D. 2001. Heat-shock resistance in experimental cryptorchid testis of mice. Mol. Reprod. Dev. 58: 216-222.
35. Kon, Y., Horikoshi, H. and Endoh, D. 1999. Metaphase-specific cell death in meiotic spermatocytes in mice. Cell Tissue Res. 296: 359-369.
36. Koopman, P., Gubbay, J., Vivian, N., Goodfellow, P. and Lovell-Badge, R. 1991. Male

development of chromosomally female mice transgenic for *Sry*. Nature 351: 117-121.

37. Koubova, J., Menke, D.B., Zhou, Q., Capel, B., Griswold, M.D. and Page, D.C. 2006. Retinoic acid regulates sex-specific timing of meiotic initiation in mice. Proc. Natl. Acad. Sci. U.S.A. 103: 2474-2479.
38. Kunieda, T. and Toyoda, Y. 1992. Nucleotide sequence of mouse Sry gene is different between Y chromosome originating from *Mus musculus musculus* and *Mus musculus domesticus*. Genomics 13: 236-237.
39. Lau, Y.F. and Zhang, J. 1998. Sry interactive proteins: implication for the mechanisms of sex determination. Cytogenet. Cell Genet. 80: 128-132.
40. Leferovich, J.M., Bedelbaeva, K., Samulewicz, S., Zhang, X.M., Zwas, D., Lankford, E.B. and Heber-Katz, E. 2001. Heart regeneration in adult MRL mice. Proc. Natl. Acad. Sci. U.S.A. 98: 9830-9835.
41. Levine, E., Cupp, A.S. and Skinner, M.K. 2000. Role of neurotropins in rat embryonic testis morphogenesis (cord formation). Biol. Reprod. 62: 132-142.
42. Liang, L.F., Chamow, S.M. and Dean, J. 1990. Oocyte-specific expression of mouse Zp-2: developmental regulation of the zona pellucida genes. Mol. Cell Biol. 10: 1507-1515.
43. Liang, L.F., Soyal, S.M. and Dean, J. 1997. FIGα, a germ cell specific transcription factor involved in the coordinate expression of the zona pellucida genes. Development 124: 4939-4947.
44. McGee, E.A. and Hsueh, A.J. 2000. Initial and cyclic recruitment of ovarian follicles. Endocr. Rev. 21: 200-214.
45. MacLean, G., Li, H., Metzger, D., Chambon, P. and Petkovich, M. 2007. Apoptotic extinction of germ cells in testes of *Cyp26b1* knockout mice. Endocrinology 148: 4560-4567.

46. McLaren, A. 1980. Oocytes in the testis. Nature 283: 688-689.

47. McLaren, A. 1984. Meiosis and differentiation of mouse germ cells. Symp. Soc. Exp. Biol. 38: 7-23.

48. McLaren, A. 1995. Germ cells and germ cell sex. Philos. Trans. R. Soc. Lond. B Biol. Sci. 350: 229-233.

49. Meeks, J.J., Crawford, S.E., Russell, T.A., Morohashi, K., Weiss, J. and Jameson, J.L. 2003. Dax1 regulates testis cord organization during gonadal differentiation. Development 130: 1029-1036.

50. Miyado, K., Yamada, G., Yamada, S., Hasuwa, H., Nakamura, Y., Ryu, F., Suzuki, K., Kosai, K., Inoue, K., Ogura, A., Okabe, M. and Mekada, E. 2000. Requirement of CD9 on the egg plasma membrane for fertilization. Science 287: 321-324.

51. Murphy, E.D. 1981. Lymphoproliferation (*lpr*) and other single-locus models for murine pulus. In: Gershwin ME, Merchant B, editors. Immunologic defects in laboratory animals. New York: Plenum Press. pp.143-173.

52. Mystkowska, E.T. and Tarkowski, A.K. 1968. Observations on CBA/-p/CBA-T6T6 mouse chimeras. J. Embryol. Exp. Morphol. 20: 33-52.

53. Nagamine, C. M., Shiroishi, T., Miyashita, N., Tsuchiya, K., Ikeda, H., Takao, N., Wu, X. L., Jin, M.L., Wang, F.S., Kryukov, A.P., Akbarzade, J. and Moriwaki, K. 1994. Distribution of the molossinus allele of *Sry*, the testis-determining gene, in wild mice. Mol. Biol. Evol. 11: 864-874.

54. Namiki, Y., Endoh, D. and Kon, Y. 2003. Genetic mutation associated with meiotic metaphase-specific apoptosis in MRL/MpJ mice. Mol. Reprod. Dev. 64: 179-188.

55. Namiki, Y., Kon, Y., Sasaki, N., Agui, T. and Endoh, D. 2004. Exon skipping of exonuclease 1 in MRL/MpJ mice is caused by a nucleotide substitution of the branchpoint sequence in intron eight. Jpn. J. Vet. Res. 52: 125-134.

56. Nasrin, N., Buggs, C., Kong, X.F., Carnazza, J., Goebl, M. and Alexander-Bridges, M. 1991. DNA-binding properties of the product of the testis-determining gene and a related protein. Nature 354: 317-320.

57. Nishimura, H., Cho, C., Branciforte, D.R., Myles, D.G. and Primakoff, P. 2001. Analysis of loss of adhesive function in sperm lacking cyritestin or fertilin β. Dev. Biol. 233: 204-213.

58. Okutsu, T., Suzuki, K., Takeuchi, Y., Takeuchi, T. and Yoshizaki, G. 2006. Testicular germ cells can colonize sexually undifferentiated embryonic gonad and produce functional eggs in fish. Proc. Natl. Acad. Sci. U.S.A. 103: 2725-2729.

59. Ozdzenski, W. 1972. Differentiation of the genital ridges of mouse embryos in the kidney of adult mice. Arch. Anat. Microsc. Morphol. Exp. 61: 267-278.

60. Ozdzenski, W. and Presz, M. 1984. Precocious initiation of meiosis by male germ cells of the mouse. Arch. Anat. Microsc. Morphol. Exp. 73: 1-7

61. Palmer, S.J. and Burgoyne, P.S. 1991. XY follicle cells in the ovaries of XO/XY and XO/XY/XYY mosaic mice. Development 111: 1017-1019.

62. Pangas, S.A. and Rajkovic, A. 2006. Transcriptional regulation of early oogenesis: in search of masters. Hum. Reprod. Update 12: 65-76.

63. Pangas, S.A., Choi, Y., Ballow, D.J., Zhao, Y., Westphal, H., Matzuk, M.M. and Rajkovic, A. 2006. Oogenesis requires germ cell-specific transcriptional regulators *Sohlh1* and *Lhx8*. Proc. Natl. Acad. Sci. U.S.A. 103: 8090-8095.

64. Philpott, C.C., Ringuette, M.J. and Dean, J. 1987. Oocyte-specific expression and developmental regulation of ZP3, the sperm receptor of the mouse zona pellucida. Dev. Biol. 121: 568-575.

65. Pierucci-Alves, F., Clark, A.M. and Russell, L.D. 2001. A developmental study of the Desert hedgehog-null mouse testis. Biol. Reprod. 65: 1392-1402.

66. Policansky, D. 1982. Sex change in plants and animals. Ann. Rev. Ecol. Syst. 13: 471-495.

67. Rajkovic, A., Pangas, S. A., Ballow, D., Suzumori, N. and Matzuk, M.M. 2004. NOBOX deficiency disrupts early folliculogenesis and oocyte-specific gene expression. Science 305: 1157-1159.

68. Rubinsztein, D.C. and Carmichael J. 2003. Huntington's disease: molecular basis of neurodegeneration. Expert. Rev. Mol. Med. 5: 1-21.

69. Schmidt, D., Ovitt, C.E., Anlag, K., Fehsenfeld, S., Gredsted, L., Treier, A.C. and Treier, M. 2004. The murine winged-helix transcription factor Foxl2 is required for granulosa cell differentiation and ovary maintenance. Development 131: 933-942.

70. Shao, J. and Diamond, M.I. 2007. Polyglutamine diseases: emerging concepts in pathogenesis and therapy. Hum. Mol. Genet. 16: R115-123.

71. Sharpe, R.M., McKinnell, C., Kivlin, C. and Fisher, J.S. 2003. Proliferation and functional maturation of Sertoli cells, and their relevance to disorders of testis function in adulthood. Reproduction 125: 769-784.

72. Smith, C.A., McClive, P.J., Hudson, Q. and Sinclair, A.H. 2005. Male-specific cell migration into the developing gonad is a conserved process involving PDGF signaling. Dev. Biol. 284: 337-350.

73. Soyal, S.M., Amleh, A. and Dean, J. 2000. FIGα, a germ cell-specific transcription factor required for ovarian follicle formation. Development 127: 4645-4654.

74. Stack, E.C. and Ferrante, R.J. 2007. Huntington's disease: progress and potential in the field. Expert. Opin. Investig. Drugs 16: 1933-1953.

75. Suzumori, N., Yan, C., Matzuk, M.M. and Rajkovic, A. 2002. *Nobox* is a homeobox-encoding gene preferentially expressed in primordial and growing oocytes. Mech. Dev. 111: 137-1341.

76. Theofilopoulos, A.N. and Dixon, F.J. 1985. Murine models of systemic lupus erythematosus. Adv. Immunol. 37: 269-390.

77. Uhlenhaut, N.H. and Treier, M. 2006. *Foxl2* function in ovarian development. Mol. Genet. Metab. 88: 225-234.

78. Wagner, T., Wirth, J., Meyer, J., Zabel, B., Held, M,. Zimmer, J., Pasantes, J., Bricarelli, F.D., Keutel, J., Hustert, E., Wolf, U., Tommerup, N., Schempp, W. and Scherer, G. 1994. Autosomal sex reversal and campomelic dysplasia are caused by mutations in and around the *SRY*-related gene *SOX9*. Cell 79: 1111-1120.

79. Walker, F. O. 2007. Huntington's disease. Lancet 369: 218-228.

80. Washburn, L.L., Albrecht, K.H. and Eicher, E.M. 2001. C57BL/6J-*T*-associated sex reversal in mice is caused by reduced expression of a *Mus domesticus Sry* allele. Genetics 158: 1675-1681.

81. Wassarman, P.M., Jovine, L. and Litscher, E.S. 2004. Mouse zona pellucida genes and glycoproteins. Cytogenet. Genome Res. 105: 228-234.

82. West, M.F., Verrotti, A.C., Sallés, F.J., Tsirka, S.E. and Strickland, S. 1996. Isolation and characterization of two novel, cytoplasmically polyadenylated, oocyte-specific, mouse maternal RNAs. Dev. Biol. 175: 132-141.

83. Whitfield, L.S., Lovell-Badge, R. and Goodfellow, P.N. 1993. Rapid sequence evolution of the mammalian sex-determining gene *SRY*. Nature 364: 713-715.

84. Yao, H.H. and Capel, B. 2002. Disruption of testis cords by cyclopamine or forskolin reveals independent cellular pathways in testis organogenesis. Dev. Biol. 246: 356-365.

85. Yonehara, S., Ishii, A. and Yonehara, M. 1989. A cell-killing monoclonal antibody (anti-Fas) to a cell surface antigen co-downregulated with the receptor of tumor necrosis factor. J. Exp. Med. 169: 1747-1756.

86. Yu, H., Bharaj, B., Vassilikos, E.J., Giai, M. and Diamandis, E.P. 2000. Shorter CAG repeat length in the androgen receptor gene is associated with more aggressive forms of breast cancer. Breast Cancer Res. Treat. 59: 153-161.

87. Zhang, J., Coward, P., Xian, M. and Lau, Y.F. 1999. *In vitro* binding and expression studies demonstrate a role for the mouse Sry Q-rich domain in sex determination. Int. J. Dev. Biol. 43: 219-227.

88. Zuccotti, M., Piccinelli, A., Marziliano, N., Mascheretti, S. and Redi, C.A. 1994. Development and loss of the ability of mouse oolemma to fuse with spermatozoa. Zygote 2: 333-339.

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Conclusion in Japanese

MRL/MpJ マウスにおける精巣内卵細胞の発生動態と責任因子に関する解析

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多細胞生物を構成する細胞系列は、大きく体細胞系列と生殖細胞系列とに分けられる。体細胞系列は、個体の発生と共に様々な組織に分化し、その死と共に役割を終える。一方、生殖細胞系列は雌雄に分かれ、有性生殖を行うことで、種の存続と遺伝的多様性を生み出し、生命の連続性を担う。

哺乳類の個体の性は、常に X 染色体をもつ卵細胞と X あるいは Y 染色体をもつ精子によって受精時に決まるが、生殖腺、および生殖細胞は本来、雄にも雌にもなれる両性性を有する。生殖腺の性分化には Y 染色体上の *Sry*（Sex determining region on Y）が重要な役割を有しており、雄では個体発生初期に *Sry* が発現することによって精巣が形成される。一方、始原生殖細胞はそれ自身の性染色体組成ではなく、周囲の体細胞の性によって決まる。すなわち、始原生殖細胞はその染色体組成に関わらず、胎子期に減数分裂に移行して卵細胞となる資質を有するが、雄では生殖腺内の体細胞が Y 染色体上の *Sry* を発現し、始原生殖細胞の減数分裂移行を阻害、精子形成細胞へと分化させる。いずれにしても、いったん雄として生まれた個体が卵細胞を有することは、人為的操作を行わない限り決してあり得ない。

しかし、生後間もない MRL/MpJ マウス精巣について詳細な解析を行った結果、精細管内に卵細胞が発見された。本マウスの精巣内卵細胞を解析することによって、卵細胞分化の新たなモデルを提供することが出来る。本研究の目的は、MRL マウスに出現する精巣内卵細胞の発生動態を形態学的に解析するとともにその責任因子の一つを分子生物学的に明らかにすることである。

まず、MRL マウスにおける精巣内卵細胞を経時的に検出し、その形態的特徴を観察した。その結果、精巣内卵細胞は精巣網付近の精細管内に存在し、透明帯や卵胞上皮細胞を有する直径約 50μm の細胞として観察された。卵胞上皮の軽度重層化（初期二次卵胞）まで成長しているものが観察されたが、卵胞腔を有するものは観察されなかった。電子顕微鏡下では、卵細胞は多数の微絨毛と皮質顆粒を有し、卵胞上皮細胞から透明帯を貫通して卵細胞に連絡する細胞質突起が観察された。また、精巣内卵細胞は生後 0 から 30 日の間に出現し、1 精巣あたり約 1.2 個が観察された生後 14 日目を中心に出現頻度のピークが見られ、その後徐々に減少した。さらに、MRL マウス精巣では *Zp* や *Omt2a* などの卵細胞特異的遺伝子の発現が認められた。これらのことから、出生直後の MRL マウス精巣内には卵細胞が出現することが証明された。

次に、精巣内卵細胞の発生動態、およびその機能について、卵巣内卵細胞のものと比較検証した。MRL マウスの胎子精巣では減数分裂特異的マーカーである DMC1、および卵細胞形成マーカーである NOBOX の双方に陽性反応を示す細胞が認められ、これらの細胞は精巣の辺縁あるいは、中腎との結合部に集合していた。このことから、精巣内卵細胞は胎子期に始原生殖細胞から派生することが示唆された。また、精巣内卵細胞は透明帯に透明帯タンパクである ZP3 を含んでいた。しかしながら、精巣内卵細胞の卵胞上皮細胞は、その機能に重要である FOXL2 を欠いていた。さらに Sperm-egg fusion assay により、精巣内卵細胞への精子の侵入が観察され、精巣内卵細胞が精子受容能力を有することが明らかになった。

最後に、精巣内卵細胞出現の要因を明らかにするため、MRL/MpJ-+/+、MRL/MpJ-*lpr/lpr*、C57BL/6、BALB/c、C3H/He、DBA/2、A/J、AKR/N の近交系マウスと、MRL マウスと C57BL/6 の間の F1 である B6MRLF1 と MRLB6F1 の精巣を検索した。その結果、精巣内卵細胞は 2 系統の MRL マウスに加えて AKR/N と B6MRLF1 にも検出された。F1 での解析から、精巣内卵細胞出現の責任遺伝子の 1 つが Y 染色体上にあることが明らかになった。Y 染色体上の性決定因子である *Sry* に注目し、近交系マウス間でその遺伝子配列を比較した結果、MRL マウスと AKR/N に共通して

Sry の CAG リピートの一部欠失が見られた。また、F1 及び AKR における精巣内卵細胞の出現頻度は MRL のものに比べると低く、常染色体上にも責任遺伝子の存在が示唆された。

上述の結果は、精巣内卵細胞が卵巣内卵細胞と類似した発生動態、形態ならびに機能を有することを示す。また、精巣内卵細胞出現の責任因子の一つとして *Sry* と強い関連性を有することが示唆された。これらは、マウス精巣において生殖細胞が潜在的に卵細胞となる能力を有することを証明するもので、新たな生殖生物学を拓く重要な知見を提供する。