



HOKKAIDO UNIVERSITY

Title	Elucidation of the murine blood-testis barrier function : Role as a "gatekeeper" regulating persistent spermatogenesis
Author(s)	千原, 正尚
Degree Grantor	北海道大学
Degree Name	博士(獣医学)
Dissertation Number	甲第11281号
Issue Date	2014-03-25
DOI	https://doi.org/10.14943/doctoral.k11281
Doc URL	https://hdl.handle.net/2115/56183
Type	doctoral thesis
File Information	Masataka_Chihara.pdf



Elucidation of the murine blood-testis barrier function

– Role as a “gatekeeper” regulating persistent spermatogenesis –

(マウス血液精巣関門の新たな機能の解明－精子発生の調節を担うゲートキーパーとしての役割－)

Masataka Chihara

Laboratory of Anatomy

Department of Biomedical Sciences

Graduate School of Veterinary Medicine

Hokkaido University

Abbreviations

Actb: actin beta

Aldh1a2: aldehyde dehydrogenase family 1, subfamily A2

Ar: androgen receptor

Amh, AMH: anti-Müllerian hormone

ANOVA: analysis of variance

BAC: bacterial artificial chromosome

BTB: blood-testis barrier

BrdU: 5-Bromo-2-deoxyuridine

Cdh1: cadherin 1

cDNA: complimentary deoxyribonucleic acid

Cldn3, CLDN3: claudin 3

Cldn4, CLDN4: claudin 4

Cldn5: claudin 5

Cldn11, CLDN11: claudin 11

Cyp26a1: cytochrome P450 family 26, subfamily a, polypeptide 1

DAB: 3,3'-diaminobenzidine tetrahydrochloride

DIG: digoxigenin

DNA: deoxyribonucleic acid

DMC1: DMC1 dosage suppressor of mck1 homolog, meiosis-specific homologous recombination

DMEM: Dulbecco modified Eagle medium

dpp: days postpartum

Egfp, EGFP: enhanced green fluorescent protein

GATA1: GATA binding protein 1

Gapdh: glyceraldehyde-3-phosphate dehydrogenase

Gnpat: glyceronephosphate O-acyltransferase

mRNA: messenger ribonucleic acid

Ocln, OCLN: occludin

orf: open reading frame

PAS-H: periodic acid-Schiff- hematoxylin

PB: phosphate buffer

PBS: phosphate-buffered saline

PCR: polymerase chain reaction

PFA: paraformaldehyde

QPCR: quantitative real-time PCR

RA: retinoic acid

RALDH: retinaldehyde dehydrogenase

RAR: retinoic acid receptor

RXR: retinoid X receptor

RT: reverse transcription

RNAi: RNA interference

SCP3, SYCP3: synaptonemal complex protein 3

SE: standard error

SSC: spermatogonial stem cell

Stra8, STRA8: stimulated by retinoic acid gene 8

TB: toluidine blue

TJ: tight junction

Tjp1: tight junction protein 1

Tjp2: tight junction protein 2

VAD: vitamin A-deficient

VAS: vitamin A-sufficient

ZO1: zonula occludens 1

ZO2: zonula occludens 2

Index

Preface	1
Figures	5
Chapter 1: Molecular dynamics of the blood-testis barrier components during murine spermatogenesis	
1) Introduction	9
2) Materials and methods	11
3) Results	16
4) Discussion	20
5) Summary	24
6) Table and figures	25
Chapter 2: Stage-specific murine expression of claudin 3 regulates progression of meiosis in early-stage spermatocytes	
1) Introduction	33
2) Materials and methods	35
3) Results	44
4) Discussion	51
5) Summary	59
6) Table and figures	60
Chapter 3: Vitamin A deprivation affects the progression of the spermatogenic wave and initial formation of the blood-testis barrier, resulting in irreversible testicular degeneration in mice	
1) Introduction	71
2) Materials and methods	75
3) Results	79
4) Discussion	87

5) Summary	96
6) Table and figures	98
Conclusion	107
Reference	110
Acknowledgements	125
Conclusion in Japanese	126

Preface

Infertility has been recognized as a major public health problem in humans, and the World Health Organization reported that infertility affects approximately one in six couple (Hentrich et al., 2011). Infertility could be caused by several factors from females as well as males, and the causes can be attributed to the male in 46 % of cases (Hentrich et al., 2011). In veterinary medicine, especially in the area of animal science and theriogenology, only a few male animals having superior genetic traits are selected for further breeding. Therefore, male infertility in industrial animals threatens economic and genetic resources. Furthermore, in the case of endangered species, male infertility is critical for breeding program and could eventually lead them to the extinction.

Male germ cells, sperm, are produced in a cyclic and complicated process called spermatogenesis, which occurs in the seminiferous tubules in the testes. Mammalian spermatogenesis can be divided mainly into three phases: mitotic proliferation of stem spermatogonia, meiotic differentiation of spermatocytes, and transformation of spermatids into spermatozoa. Primary spermatocytes undergo prophase of meiosis I through the following six stages: preleptotene, leptotene, pachytene, zygotene, diplotene, and diakinesis, followed by entry into metaphase I. The murine spermatogenic cycle is divided into distinct (I–XII) stages, with different stage-specific sets of germ cells found in the seminiferous tubules (Oakberg, 1956; Fig. P-1). In normal adult testis, all

seminiferous stages are observed, which sequentially change along the long axis of the tubules. These characteristics contribute to the continuous sperm production over a reproductive period. Any disruption of this system could lead male infertility. Although, a key step in the investigation for male infertility is the appropriate classification of impaired spermatogenesis, it is quite hard to diagnose male infertility due to the complexity of spermatogenesis.

The differentiation of germ cells is largely supported, nurtured, and supervised by somatic Sertoli cells, which line the seminiferous tubules. In adult animals, Sertoli cells form the blood-testis barrier (BTB) near the basal lamina, which divides the seminiferous epithelium into basal and adluminal compartments. From spermatogonia up to preleptotene and leptotene spermatocytes reside in basal compartment, whereas further differentiation from zygotene spermatocyte into spermatozoa take place in adluminal compartment (Fig. P-2). The BTB is mainly composed of tight junctions (TJs) between adjacent Sertoli cells thereby restricting the entry of molecules in the interstitial space into the adluminal compartment (Cheng and Mruk, 2002; Pelletier, 2011). The BTB also segregates the antigens of differentiating meiotic and postmeiotic germ cells from the systemic circulation, thus it has been considered that BTB contributes to maintain the testicular immune privilege (Meng et al., 2011). However, to date, there is no direct evidence showing that BTB disruption provokes production of anti-sperm antibodies. Importantly, BTB abnormalities are often associated with the degeneration of

the seminiferous epithelium and germ cell loss (Gow et al., 1999; Saitou et al., 2000; Hasegawa and Saga, 2012). Furthermore, the altered expression and localization of the BTB component proteins are associated with the development of human testicular intraepithelial neoplasia (Fink et al., 2009). Although the precise function of the BTB remains to be elucidated, these reports indicate that maintenance of BTB integrity is essential for the progression of normal spermatogenesis.

The main aims of this study are to reveal the roles of the BTB in mammalian spermatogenesis, and the author analyzed the mouse testes as an experimental model. This thesis contains three chapters; the first chapter provides the dynamics of the BTB component proteins during spermatogenesis, the second chapter examines the effect of disruption of stage specific BTB regulation on spermatogenesis, and the last chapter discusses the elaborate relationships between BTB and spermatogenesis.

Contents of this research were published in the following articles.

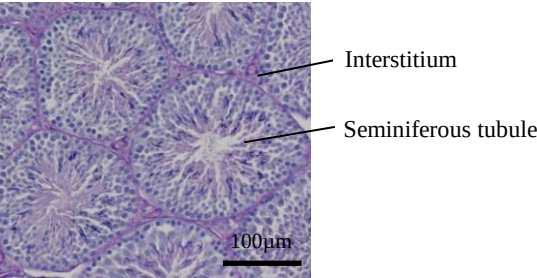
1. Chihara, M., Otsuka, S., Ichii, O., Hashimoto, Y., and Kon, Y. 2010. Molecular dynamics of the blood-testis barrier components during murine spermatogenesis. *Mol. Reprod. Dev.* 77: 630–639.
2. Chihara, M., Ikebuchi, R., Otsuka, S., Ichii, O., Hashimoto, Y., Suzuki, A., Saga, Y., and Kon, Y. 2013. Mice stage-specific claudin 3 expression regulates progression of meiosis in early stage

spermatocytes. *Biol. Reprod.* 89: 3, 1–12.

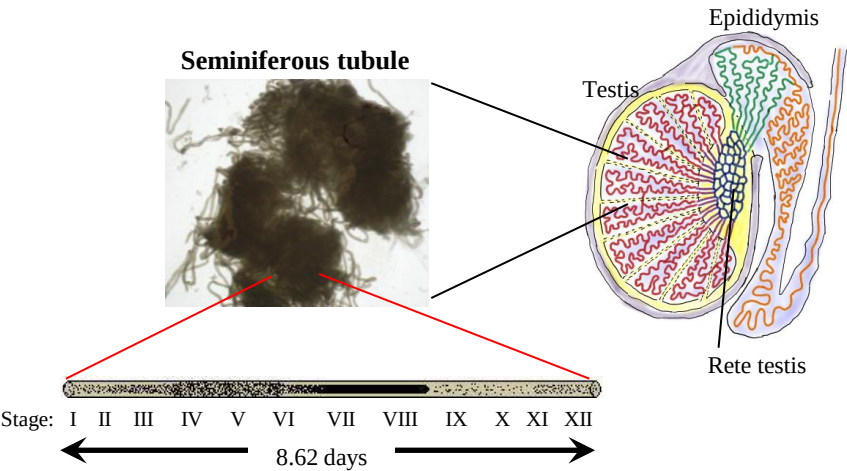
3. Chihara, M., Otsuka, S., Ichii, O., and Kon, Y. 2013. Vitamin A deprivation affects the progression of the spermatogenic wave and initial formation of the blood-testis barrier, resulting in irreversible testicular degeneration in mice. *J. Reprod. Dev.* 59: 525–535.

Figures

A



B



C

I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII
13	14	14	15	15	15	16	16				
1	2	3	4	5	6	7	8	9	10	11	12
P	P	P	P	P	P	P	P	P	P	D	2m
	In	In	In	B	preL	preL	L	L	Z	Z	Z
A	A	A	A	A	A	A	A	A	A	A	A

Figure P-1. The structure of the mouse testis.

A) The PAS-H stained cross section of a testis from an adult mouse, showing several seminiferous tubules, which are the functional units producing spermatozoa during spermatogenesis. A tubule is typified by the presence of the seminiferous tubule lumen and the seminiferous epithelium, which is constituted by Sertoli cells and germ cells at different stages of development. Each tubule is surrounded by interstitial cells, Leydig cells. **B)** The mammalian testis is composed of intertwined seminiferous tubules. The “start” and “end” of these tubules are both connected to the rete testis. In mice, spermatogenic wave is divided into distinct 12 stages (I–XII) along the seminiferous tubule. Produced immotile spermatozoa flow from the lumen of the seminiferous tubules into the epididymis via the rete testis. **C)** Standard depiction of the seminiferous epithelial stages of the mouse. Each stage is represented by a specific set of germ cells being present in the tubule at a single point. A, type A spermatogonia; In, intermediate spermatogonia; B, type B spermatogonia; preL, preleptotene spermatocytes; L, leptotene spermatocytes; P, pachytene spermatocytes; Z, zygotene spermatocytes; D, diplotene spermatocytes; 2m, secondary spermatocytes; 1–16, spermatids.

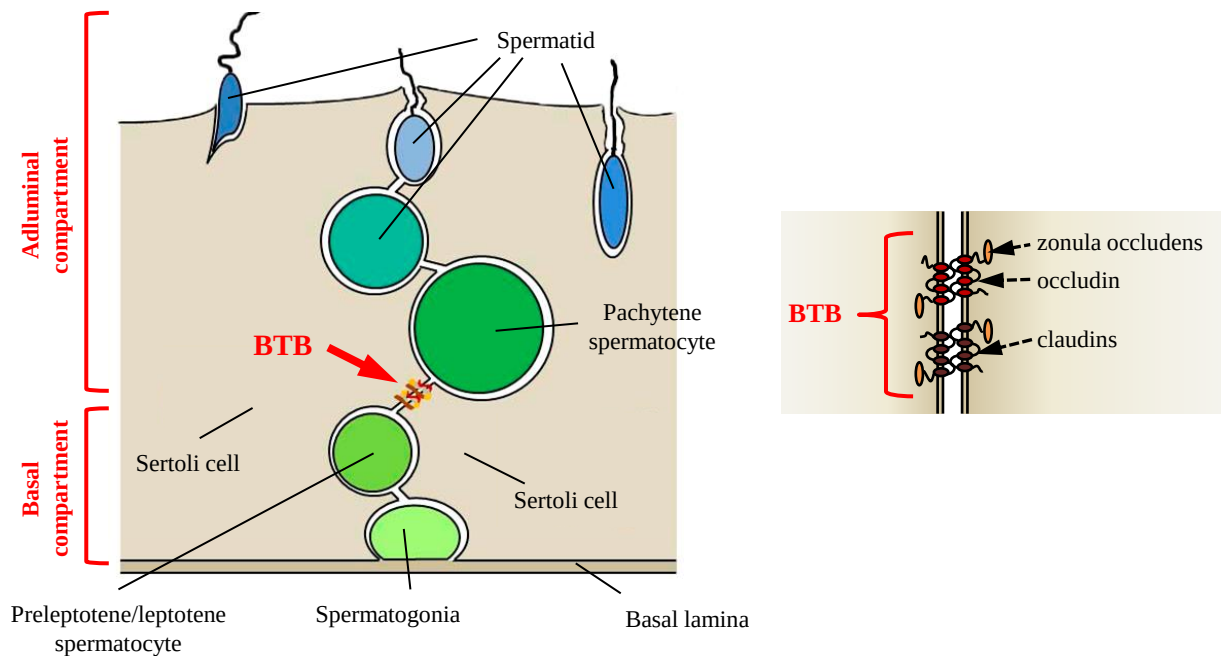


Figure P-2. Schematic drawing of the seminiferous epithelium.

Germ cells migrate towards into the tubular luminal edge differentiating from spermatogonia into fully developed spermatids (spermatozoa). The BTB physically divides the seminiferous epithelium into the basal and adluminal compartment. The BTB is composed largely by TJ proteins, such as occludin, claudins, and zonula occludens, between two adjacent Sertoli cells. Spermatogonia, preleptotene spermatocytes, and leptotene spermatocytes reside in the basal compartment, whereas further differentiation from zygotene spermatocytes takes place in the adluminal compartment.

Chapter 1

Molecular dynamics of the blood-testis barrier components during murine spermatogenesis

Introduction

In the adult testes, the BTB is localized between adjacent Sertoli cells. The principal components of the BTB are TJ proteins such as claudins, occludin (OCLN), and zonula occludens (ZO). Claudins and OCLN are integral membrane proteins localized at the TJ strands. Claudins and OCLN consist of 4 transmembrane domains and their N- and C-terminal ends are located in the cytoplasm. The claudin family is formed by at least 24 members and the expression pattern of them varies considerably among tissues (for review, see Tsukita et al., 2001). ZOs are known to interact with the C-terminus of claudins and OCLN, and they also connect to actin at their C-terminus (for review, see Mruk and Cheng, 2004). Recent studies have reported that *Ocln* or claudin 11 (*Cldn11*) null mice are sterile (Saitou et al., 2000; Gow et al., 1999). Furthermore, it was suggested that changes in the expression and localization of CLDN11 in the BTB are associated with the development of human testicular intraepithelial neoplasia (Fink et al., 2009). These reports indicated that the maintenance of the integrity of the BTB is essential for normal spermatogenesis.

During spermatogenesis, preleptotene/leptotene spermatocytes migrate across the BTB from the basal to the adluminal compartment for further development. In rodents, the migration of germ cells occurs from late stage VIII to early stage IX of the seminiferous epithelial cycle (Russell, 1977). To accommodate the passage of germ cells, it was postulated that the BTB is required to be transiently

“OPEN” from late stage VIII to early stage IX. In cultured Sertoli cells, it has been shown that several cytokines, such as tumor necrosis factor α and transforming growth factor- β , produced by Sertoli and germ cells can reversibly perturb the TJ-permeability barrier (for review, see Xia et al., 2005). However, it is difficult to investigate the regulatory mechanisms of the BTB by using *in vivo* models, since the migration of germ cells across the BTB occurs briefly during spermatogenesis.

In this chapter, to elucidate the dynamics of the BTB component proteins, the author investigated the expression and localization of TJ proteins including claudins, OCLN, and ZO₂ in the testes of mice by performing immunohistological and electron microscopic analyses. The results indicated that claudin 3 (CLDN3) plays a crucial role in regulating the integrity of the BTB during germ cell migration.

Materials and Methods

Animals

Male C57BL/6N mice aged 3 months were purchased from Japan SLC (Hamamatsu, Japan).

Mice were maintained and used according to the Guide for the Care and Use of Laboratory Animals of Hokkaido University, Graduate School of Veterinary Medicine (approved by the Association for Assessment and Accreditation of Laboratory Animal Care International).

Microdissection of staged segments in the seminiferous tubules

Testes were isolated from C57BL/6N mice and the tunica albuginea were removed. To digest the connective tissue, decapsulated testes were incubated in a collagenase solution (0.5 mg/ml in Ham's F-12 nutrient mixture/Dulbecco's modified Eagle's medium, 1:1, v/v) for 20–30 min, shaking at 80 oscillations/min at 35 °C. The seminiferous tubules were washed 3 times in PBS and suspended in ice-cold phosphate-buffered saline (PBS). Isolation of staged tubules was performed under a stereoscopic microscope (SZX7; Olympus, Tokyo, Japan). The spermatogenic wave was defined by the light absorption pattern of seminiferous tubules (Kotaja et al., 2004), and dissected tubules were divided into the following 3 groups: Early stage (stages II–VI), Middle stage (stages VII–VIII), and Late stage (stages IX–I). Total RNA was extracted from the isolated tubules using RNAqueous kit

(Ambion, Foster City, USA).

Reverse transcription (RT) and quantitative real-time polymerase chain reaction (PCR)

Total RNA was extracted from testes in 0–30-day-old mice using the TRIzol reagent (Life Technologies, Carlsbad, CA, USA) following the manufacturer's protocol. Purified total RNAs were treated with Turbo DNase (Ambion) for DNA digestion and complimentary DNAs (cDNAs) were synthesized by the RT reaction using the ReverTra Ace reverse transcriptase enzyme (Toyobo, Osaka, Japan) and oligo-dT primers (Life Technologies). Quantitative real-time PCR (QPCR) analysis was performed using the Brilliant II SYBER Green QPCR Master Mix (Agilent Technologies, Santa Clara, CA, USA) and a real-time thermal cycler (MX 3000P, Agilent Technologies) according to the manufacturer's instructions. The expression levels of the genes were normalized to the expression of actin, beta (*Actb*). The details of the specific primers used for each gene are provided in Table 1-1.

Histological and immunohistochemical analyses of the BTB component proteins

The mice were killed by cervical dislocation, and the testes were immediately removed and fixed with 4% (w/v) paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB) at 4 °C overnight. Then, 2–4- μ m-thick serial paraffin sections were prepared. A number of paraffin sections were stained with

the periodic acid-Schiff-hematoxylin (PAS-H) reagent to distinguish the seminiferous epithelial cycle as previously described (Oakberg, 1956), and the other sections were immunostained using the following procedure. For antigen retrieval, sections were incubated in buffered citrate (pH 6.0) for 15 min at 105 °C for CLDN3 or 0.05% trypsin/0.01M PBS (pH 7.4) for 5–10 min at 37 °C for CLDN11, OCLN, and ZO1. The samples were then soaked in methanol containing 0.3% H₂O₂ to remove internal peroxidase activity. Sections blocked in 10% normal goat serum for 30 min at room temperature were incubated with rabbit anti-CLDN3 (1:200; Life Technologies), anti-CLDN11 (1:100; Life Technologies), anti-OCLN (1:150; Life Technologies), or anti-ZO1 (1:100; Life Technologies) at 4 °C overnight. The sections were subsequently treated with biotin-conjugated goat anti-rabbit IgG (Nichirei, Tokyo, Japan) for 30 min at room temperature, and then in Vectastain Elite ABC Reagent (Vector Laboratories, Burlingame, USA) for 30 min at room temperature. The sections were incubated with 3,3'-diaminobenzidine tetrahydrochloride (DAB) solution containing 0.006% H₂O₂ until the stain developed, and then slightly counterstained with hematoxylin.

Histoplanimetric analysis of the BTB component proteins in the seminiferous epithelial cycle

To assess whether TJ proteins have stage-dependent expression patterns, the author evaluated the immunoreactivity of CLDN3, CLDN11, OCLN, and ZO1 at the basal part of the seminiferous

tubules using a quantitative method. Briefly, the stages of seminiferous tubules were determined from serial sections stained with PAS-H. Firstly, a digital image of the seminiferous tubules in each stage was prepared, and immunoreactivity (expressed as IntDen) of the binary image was measured using Image J 1.36b (NIH, Bethesda, MD, USA). The area of each seminiferous tubule was measured, and the immunoreactivity of TJ proteins at the basal area of the seminiferous tubules was expressed as IntDen/mm².

Immunoelectron microscopy

The author performed a pre-embedding protocol. Briefly, after deep anesthesia with isoflurane and flushing of blood, mice were perfused with 4% PFA at 4 °C via the left ventricle. The testes were removed and fixed with 2.5% glutaraldehyde in 0.1 M PB (pH 7.4) containing 2% PFA and 20 mg/ml sucrose at 4 °C for 4 h. The testes were sequentially placed in 10, 15, and 20% sucrose in PB for 4 h. The testes were frozen in liquid nitrogen after treatment using an OCT compound embedding medium (Tissue-Tek; Sakura Finetek, Torrance, CA, USA) and stored at –80 °C until use.

Immunohistochemical analysis using immunoelectron microscopy was performed as previously described (Kuriyama et al., 2004). Briefly, 5-μm-thick cryosections of the testes were immunostained with rabbit anti-CLDN11 (1:100), anti-OCN (1:50), or anti-ZO1 (1:50) antibodies as described above.

After incubation with a secondary antibody for 6 h at 4 °C, the sections were washed in PBS at 4 °C, and then immersed in 1% glutaraldehyde for 5 min. After washing in PBS at 4 °C, the sections were immersed in a DAB solution without H₂O₂ for 30 min. The sections were then incubated in a DAB solution containing 0.01% H₂O₂ until the stain developed. After washing, the sections were post-fixed with 2% osmium tetroxide in 0.1 M PB for 90 min. After washing, the sections were dehydrated in graded alcohol and embedded in Quetol-filled gelatin capsules using the conventional method. Ultrathin (80-nm-thick) sections were collected from the surface of the embedded sections. Ultrathin sections were stained using lead citrate and examined with an electron microscope (JEM-1210; JEOL, Tokyo, Japan).

Statistical analysis

Results were expressed as the mean $\pm$ SEM and analyzed by non-parametric statistical methods. The Kruskal-Wallis test was used to compare the numerical results, and multiple comparisons were performed using Scheffe's method when significant differences were observed ($P < 0.05$).

Results

Changes in the mRNA levels of the BTB component genes in staged seminiferous tubules

To examine whether the mRNA levels of the BTB component genes change during spermatogenesis, the author isolated seminiferous tubules by stereomicroscopy and divided them into the 3 following groups: Early stage (stages II–VI), Middle stage (stages VII–VIII), and Late stage (stages IX–I). The mRNA levels of the BTB component genes in these groups were compared by QPCR (Fig. 1-1). The mRNAs of *Cldn3* and *Cldn11* decreased from the Early stage to the Late stage, and significant differences were observed for *Cldn3* between the Early and Late stages. In contrast to *Cldn3*, *Ocln* showed the opposite trend. The mRNAs of tight junction protein 1 (*Tjp1*, encoding ZO1) and tight junction protein 2 (*Tjp2*, encoding ZO2) also decreased from the Early stage to the Late stage, and significant differences were observed between the Early stage and the Late stage. Conversely, the mRNA for cadherin 1 (*Cdh1*, encoding E-cadherin) was significantly induced during the Middle stage.

Expression and localization of CLDN11, OCLN, ZO1, and CLDN3 in the adult mouse testis

To assess the expression and localization of the BTB component proteins during the seminiferous epithelial cycle in the adult mouse, immunohistochemical analysis for CLDN3, CLDN11, OCLN, and ZO1 were performed. The seminiferous stages were judged by the acrosomes of

spermatids stained with PAS-H (data not shown). At all stages, a filamentous-positive reaction to CLDN11, OCLN, and ZO1 was predominantly observed at the basal part of the seminiferous tubules and formed the continuous belts (Fig. 1-2A; a–c, e–g, and i–k). The immunoreactive level of CLDN11 was at its highest during stages V–VII (Fig. 1-2B; ●). In contrast to the expression pattern of CLDN11, the immunoreactive level of OCLN was at its lowest during stages II–XI (Fig. 1-2B; ● vs. ■). Conversely, the author detected no stage-specific immunoreactive change in the levels of ZO1 at all stages (Fig. 1-2B; ▲). CLDN3 was localized to the basal part of the seminiferous tubules only during stages VI–IX (Fig. 1-2A; d, h, and l) and its levels dramatically increased during stages VI–VIII (Fig. 1-2B; ◆).

BTB localization during the migration of spermatocytes across the BTB

To investigate whether there was any change of the BTB localization during stages VIII–IX, when the germ cells cross the BTB, the author performed immunohistochemical analyses for CLDN3, CLDN11, and ZO1 using serial sections (Fig. 1-3A). At stage VII, just before the migration of germ cells, ZO1 and CLDN11 were localized to the luminal side of the preleptotene spermatocytes (Fig. 1-3A; e and f, large arrowheads), while CLDN3 surrounded the preleptotene spermatocytes (Fig. 1-3A; d, arrow). At stage X, just after the migration of germ cells, ZO1 and CLDN11 were localized to

the basal side of the spermatocytes (Fig. 1-3A; e and f, small arrowheads), while there was no immunoreactivity for CLDN3 (Fig. 1-3A; d). Interestingly, at stage IX, when the migration of germ cells occurs, ZO1 and CLDN11 were present at both of the basal and luminal sides of the leptotene spermatocytes (Fig. 1-3A; k and l, large arrowheads). During this stage, immunoreactivity for CLDN3 became indistinct, but it still surrounded the leptotene spermatocytes (Fig. 1-3A; j, arrow). Like ZO1 and CLDN11, OCLN was observed at both of the basal and luminal sides of the leptotene spermatocytes during stage IX (Fig. 1-3B; b, large arrowheads).

Ultrastructural localization of CLDN11, OCLN and ZO1 in the adult mouse testis

The exact ultrastructural localization of the BTB component proteins was further examined using immunoelectron microscopy (Fig. 1-4). The immunoreactivity of CLDN11 was observed in the sheets of plasma membranes of adjacent Sertoli cells (Fig. 1-4A; arrowheads). A typical BTB is formed at the attached regions of apposing Sertoli cells and is sandwiched between the endoplasmic reticulum (for review, see Lee and Cheng, 2004). Hence, the magnified view of the boxed area in Fig. 1-4A indicated the existence of CLDN11 at the BTB (Fig. 1-4B). No signal was detected in the negative control which was incubated with normal rabbit serum instead of the primary antibody (Fig. 1-4C). The BTB at both of the basal and luminal sides of the leptotene spermatocytes were also

observed by immunoelectron microscopy for ZO1 (Fig. 1-4D–F) and the other TJ proteins (data not shown). Taken collectively, these results confirmed that the immunoreactive signals of TJ proteins found at the basal part of the seminiferous tubules by immunohistochemical analysis were located at the BTB.

Discussion

Integrity of the BTB is maintained over the cycle of the seminiferous epithelium

The migration of preleptotene/leptotene spermatocytes across the BTB occurs during stages VIII–IX of the seminiferous epithelial cycle in the adult mouse testis (Russell, 1977). However, the mechanisms regulating the migration of germ cells remain obscure. In the present study, the author provide for the first time the changes in the mRNA levels of the BTB component genes, *Cldn3*, *Cldn11*, *Ocln*, *Tjp1* (encoding ZO1), and *Tjp2* (encoding ZO2), during spermatogenesis *in vivo*. Relatively high levels of OCLN have been observed in murine testicular endothelial cells (Moroi et al., 1998). The collagenase method performed in isolation of staged seminiferous tubules was considered to be useful for reducing the effects of the expression of TJs in interstitial cells and microvessels of testis by removing these endothelial cells. The success of my dissection method was confirmed by the stage-specific expression pattern of *Cdh1* (encoding E-cadherin) mRNA, which was consistent with a previous report (Cheng et al., 2003).

The author's results demonstrated that the mRNAs of *Cldn3*, *Cldn11*, *Ocln*, *Tjp1*, and *Tjp2* were expressed at all stages; however, they did not change consistently. In cultured Sertoli cells, *Cldn11* mRNA expression was shown to correlate with inter-Sertoli TJ assembly (Kaitu'u-Lino et al., 2007). Therefore, it was considered that the BTB exists at all stages.

In immunohistochemical and immunoelectron microscopy analyses, CLDN11, OCLN, and ZO1 proteins were localized at the BTB at all stages. However, the author showed that the immunoreactive level of CLDN11 was opposite to the level of OCLN throughout spermatogenesis, suggesting that CLDN11 and OCLN act complementarily in the mouse testis. Conversely, the immunoreactive level of ZO1 stayed relatively consistent throughout all of the stages. ZO1 functions as a cross-linker between TJ strands and actin filaments (Itoh et al., 1997). In addition, CLDNs and OCLN directly bind to ZO1 (Itoh et al., 1999). Thus, it is plausible that ZO1 is retained at the BTB over the cycle of the seminiferous epithelium, because of the complementary expression levels of CLDN11 and OCLN. However, there was no significant decline in the immunoreactive level of CLDN11, OCLN, or ZO1 at the BTB, even during stages VIII–IX. Furthermore, different from the expression of the other BTB component proteins, CLDN3 was detected only during stages VI–IX; this result was consistent with a previous study using western blot analysis (Meng et al., 2005). Hence, it was suggested that the barrier function of the BTB is reinforced to some extent by CLDN3 during stages VI–IX. Earlier studies, using the injection of a tracer molecule, illustrated that the distribution of the tracer was limited to the basal compartment during all of the stages of the seminiferous epithelial cycle (Li et al., 2006; Xia et al., 2009; Komljenovic et al., 2009). Taken collectively, it is plausible that the BTB sequesters the contents of adluminal compartment from the systemic circulation even when germ cells migrate

through the BTB.

The intermediate compartment is essential for maintaining the integrity of the BTB

The intermediate cell compartment theory proposes that the intermediate compartment, located between the basal and adluminal compartments, accommodates the passage of germ cells from the basal to the adluminal compartment without compromising the integrity of the BTB (Russell, 1977). However, the existence of the intermediate compartment is controversial, since other studies demonstrated that only one TJ fibril existed per Sertoli cell that sequestered developing germ cells (for review, see Mruk and Cheng 2004). To date, there is no biochemical or molecular evidence to support the existence of the intermediate compartment. The author demonstrated that CLDN11, OCLN, and ZO1 are localized to both of the basal and luminal portions of the preleptotene/leptotene spermatocytes during stages VIII–IX. The presence of two TJ fibrils per Sertoli cell was also confirmed by immunoelectron microscopy. These results indicated that the intermediate compartment temporarily exists during the migration of spermatocytes from the basal to the adluminal compartment. Therefore, the author proposes the putative BTB dynamics model shown in Fig. 1-5. The present study strengthens the notion that new TJ fibrils are formed below the preleptotene/leptotene spermatocytes, followed by the disassembly of the TJ fibrils above these spermatocytes. In this study, two TJ fibrils

per Sertoli cell in the seminiferous tubules were observed at a high-frequency during stages VIII–IX.

Thus, it is plausible that the intermediate compartment exists for a comparatively long period.

CLDN3 has an important role in the migration of spermatocytes through the BTB

The stage-specific localization of CLDN3 at the BTB during stages VI–IX, in contrast to the other TJ molecules, coincided with the emergence of the preleptotene/leptotene spermatocytes. Interestingly, in previous studies of other epithelial cells, CLDN3 recruited endogenous ZO1 to areas of cell-cell contact (Itoh et al., 1999; Ikenouchi et al., 2008). Meng et al. (2005) indicated that the expression of CLDN3 was significantly reduced in *Ar^{invflox(exl-neo)Y};Tg(Amh-Cre)* mice, a model for conditional androgen insensitivity and azoospermia, using microarray and histological analyses. Ether-lipid-deficient glyceronephosphate O-acyltransferase (*Gnpat*)-null mice, showing azoospermia and the arrest of spermatogenesis, also indicated the change of localization patterns and the down-regulation of CLDN3 (Komljenovic et al., 2009). The BTB in both of these mutant mice exhibited an increased permeability to biotin (Meng et al., 2005; Komljenovic et al., 2009). On the basis of these findings, it was suggested that CLDN3 plays an important role in the correct localization of TJ proteins and in establishing new TJ fibrils below the preleptotene/leptotene spermatocytes when these cells migrate from the basal to the adluminal compartment.

Summary

The BTB separates the seminiferous epithelium into the basal and adluminal compartments. During murine spermatogenesis, preleptotene/leptotene spermatocytes migrate from the basal to the adluminal compartment through the BTB during stages VIII–IX. In this chapter, the author focused on the TJ molecules and analyzed their spatiotemporal expression during the murine seminiferous epithelial cycle. Structural analysis revealed that the principal components of the BTB, for example, CLDN3, CLDN11, OCLN, and ZO1, were localized at the basal and luminal sides of the preleptotene/leptotene spermatocytes during the migration stages (VIII–IX). Although the author detected CLDN11, OCLN, and ZO1 throughout adult spermatogenesis, CLDN3 was only detected during stages VI–IX. In conclusion, these findings indicate that the integrity of the BTB is maintained throughout spermatogenesis, and the stage-specific localization of CLDN3 protein plays an important role in regulating BTB integrity and germ cell migration. The author will examine the CLDN3 function during spermatogenesis in the next chapter.

Table and figures

Table 1-1. Primer pairs used for quantitative real-time PCR in this chapter.

Genes (Accession)	Symbol	Primer Sequence (5'-3') F: Forward, R: Reverse	Product Size (bp)
claudin-3 (NM_009902)	<i>Cldn3</i>	F: GCACCCACCAAGATCCTCTA R: TCGTCTGTCACCATCTGGAA	206
claudin-11 (NM_008770)	<i>Cldn11</i>	F: TGGTGGACATCCTCATCCTT R: GCCAGCAGAATAAGGAGCAC	190
occludin (NM_008756)	<i>Ocln</i>	F: CCTACTCCTCCAATGGCAAA R: CTCTTGCCCTTTCCTGCTTT	208
tight junction protein 1 (ZO1) (NM_009386)	<i>Tjp1</i>	F: GCACCATGCCTAAAGCTGTC R: ACTCAACACACCACCATTCG	122
tight junction protein 2 (ZO2) (NM_011597)	<i>Tjp2</i>	F: AATGCGAGGATCGAAATAGC R: TAGCTTCCTCTGGTGTCTG	158
cadherin 1 (: E-cadherin) (NM_009864)	<i>Cdh1</i>	F: ACGTCCATGTGTGTGACTGTG R: AGGAGCAGCAGGATCAGAATC	139
actin, beta (NM_007393)	<i>Actb</i>	F: TGTTACCAACTGGGACGACA R: GGGGTGTTGAAGGTCTCAA	165

ZO1: zonula occludens 1; ZO2: zonula occludens 2.

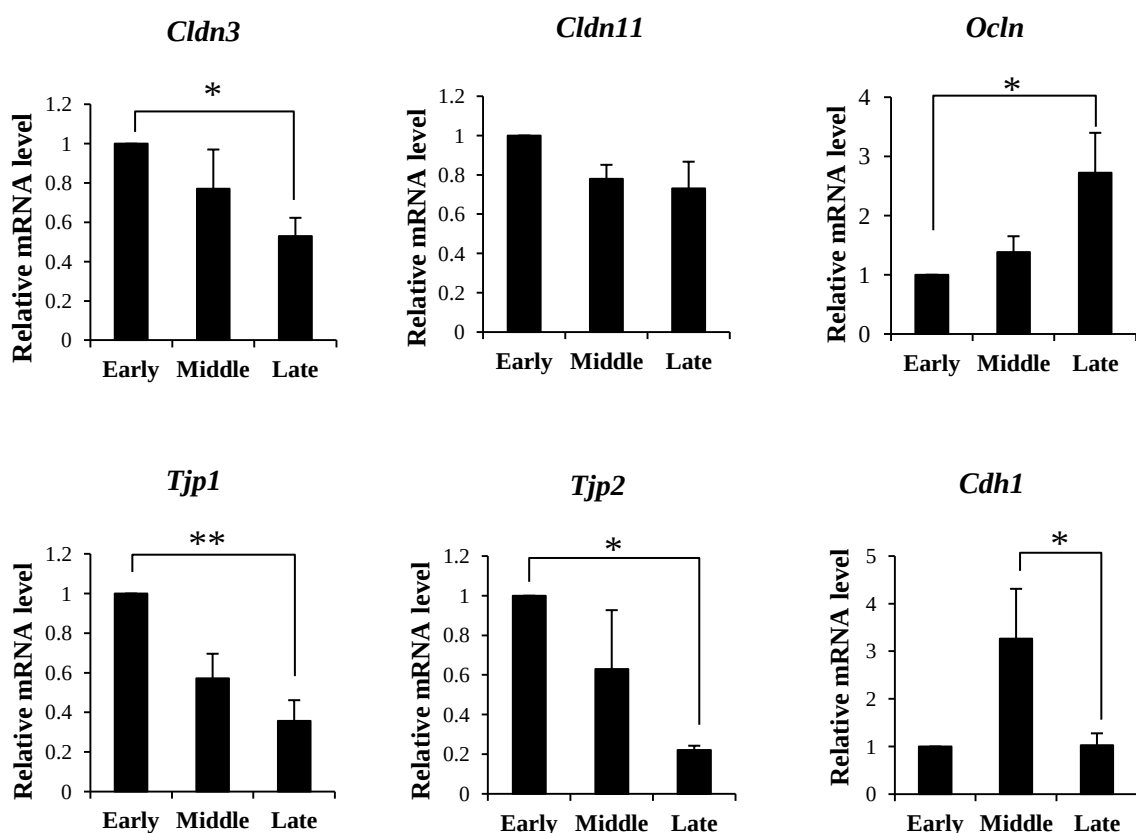


Figure 1-1. Changes in the mRNA levels of *Cldn3*, *Cldn11*, *Ocln*, *Tjp1*, *Tjp2*, and *Cdh1* in staged seminiferous tubules.

The mRNA levels of each gene during the Early stage were arbitrarily set at 1. Early: stages II–VI, Middle: stages VII–VIII, Late: stages IX–I. The number of animals = 5. Results are expressed as the mean $\pm$ SEM and multiple comparisons are performed using the non-parametric Kruskal-Wallis test (Scheffe's method). *: $P < 0.05$, **: $P < 0.01$.

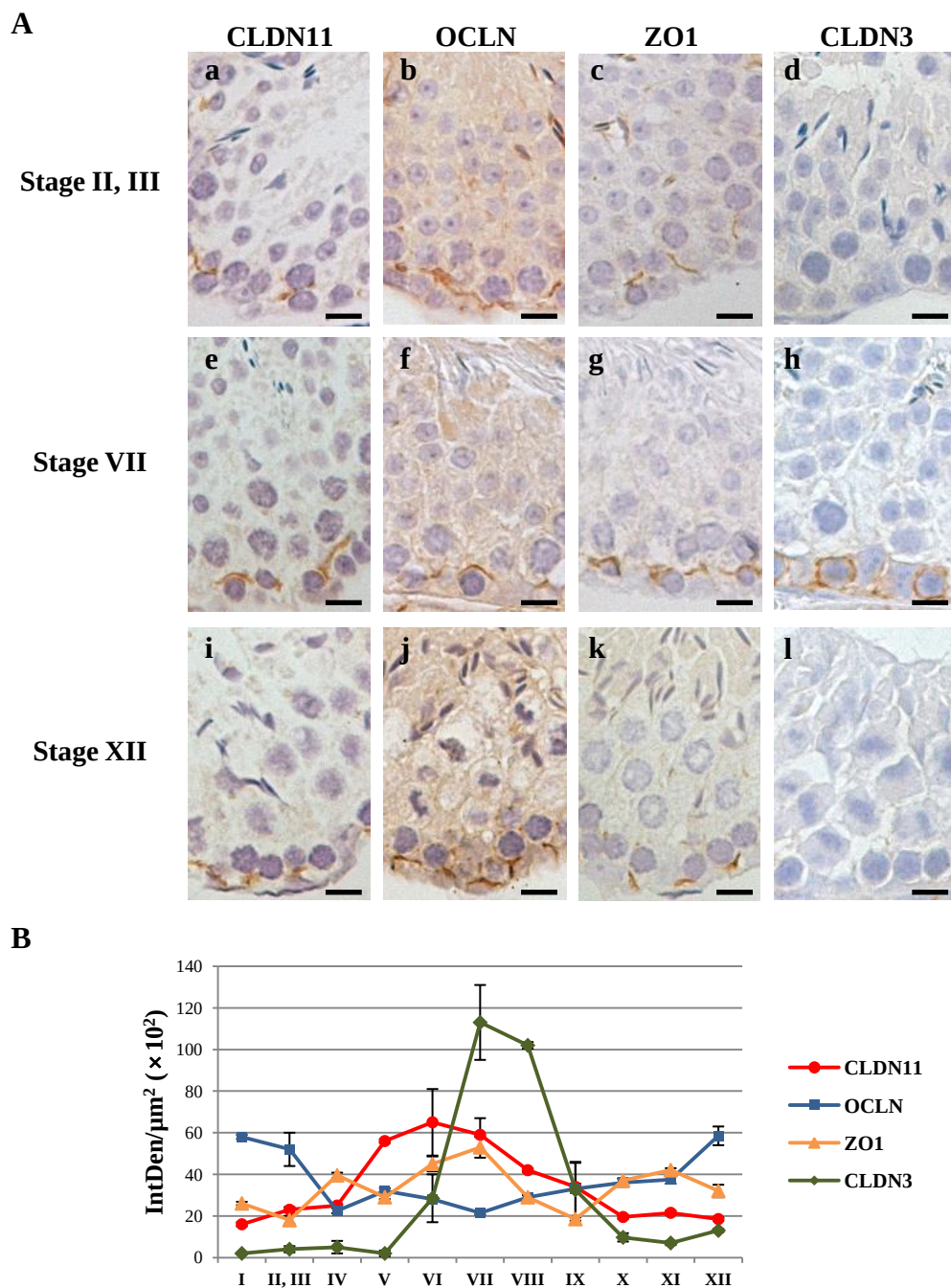


Figure 1-2. Localization and stage specificity of BTB component proteins in adult mouse testes.

A) Immunohistochemistry of CLDN11 (**a, e, i**), OCLN (**b, f, j**), ZO1 (**c, g, k**), and CLDN3 (**d, h, l**) in staged tubules (Stage II, III: **a–d**; Stage VII: **e–h**; Stage XII: **i–l**) determined by PAS-H staining. Bars = 10 μm. **B)** The line graph summarizes the immunoreactive levels of CLDN11 (●), OCLN (■), ZO1 (▲), and CLDN3 (◆) at the basal site of the seminiferous tubules in each stage. Each bar indicates the mean ± SEM (n = 5).

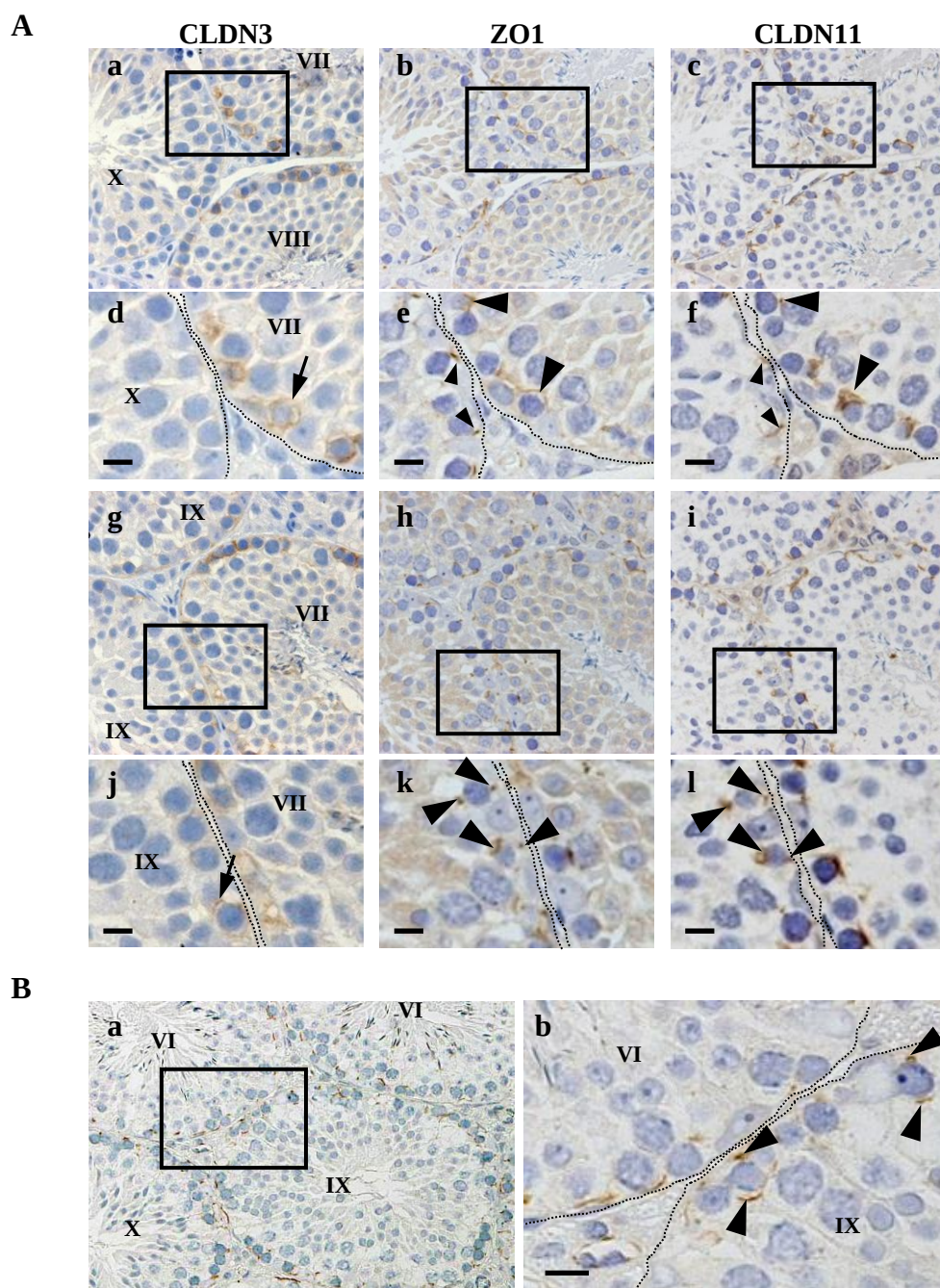


Figure 1-3. Localization of the BTB at stages VII, VIII, IX, and X.

A) (a, b, c) represent serial sections of seminiferous tubules at stages VII, VIII, and X. (d, e, f) represent a magnified view of the boxed area in (a, b, c), respectively. (g, h, i) represent serial sections of seminiferous tubules at stages VII and IX. (j, k, l) represent a magnified view of the boxed area in (g, h, i), respectively. (a, d, g, j): Immunohistochemistry of CLDN3; (b, e, h, k): ZO1; and (c, f, i, l): CLDN11. **B)** Immunohistochemistry of occludin. (b) represents a magnified view of the boxed area in (a). Roman numerals indicate the seminiferous stages determined from the PAS-H-stained serial sections. Dotted lines represent the basal lamina. Bars = 10 μm.

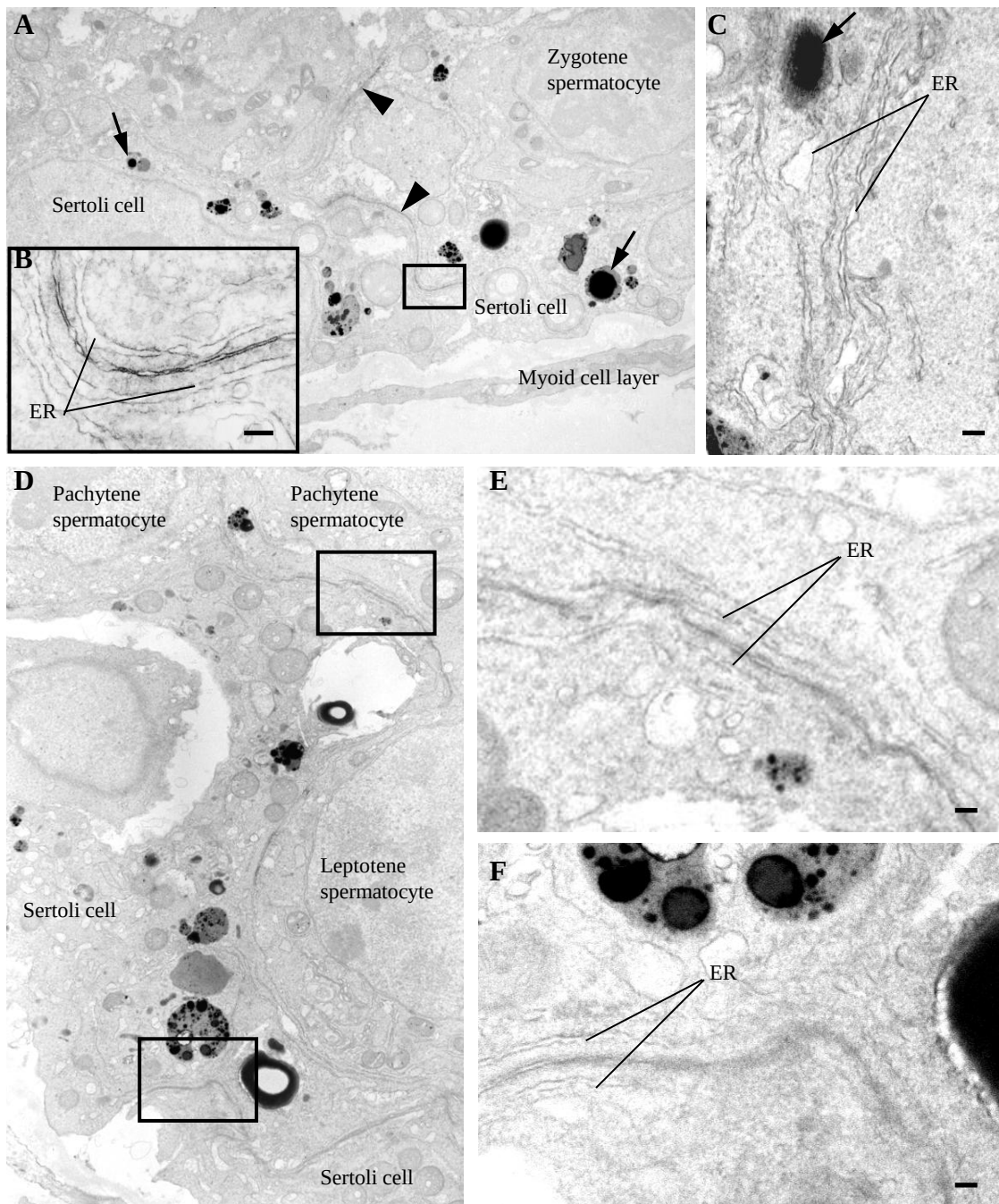


Figure 1-4. Ultrastructural localization of CLDN11 and ZO1 in the adult mouse testis.

A) Immunoelectron microscopy for CLDN11. Immunoreactive staining is localized to the Sertoli cell plasma membrane at the BTB (arrowheads). **B)** A magnified view of the boxed area in **(A)**. The BTB is defined by the presence of the endoplasmic reticulum (ER) sandwiching the plasma membrane of the adjacent Sertoli cells. **C)** A control section of the BTB stained with normal rabbit serum, illustrating that the immunoreactive staining shown in **(A)** and **(B)** are specific for the target proteins. The electron-dense products found in the cytoplasm of the Sertoli cells (arrows) are lipid droplets stained by osmium tetroxide. **D)** The immunoreactive BTB is localized to both of the basal and luminal sides of the leptotene spermatocyte by immunoelectron microscopy for ZO1 (boxed area). **E)** A magnified view of the luminal BTB. **F)** A magnified view of the basal BTB. Bars = 100 nm.

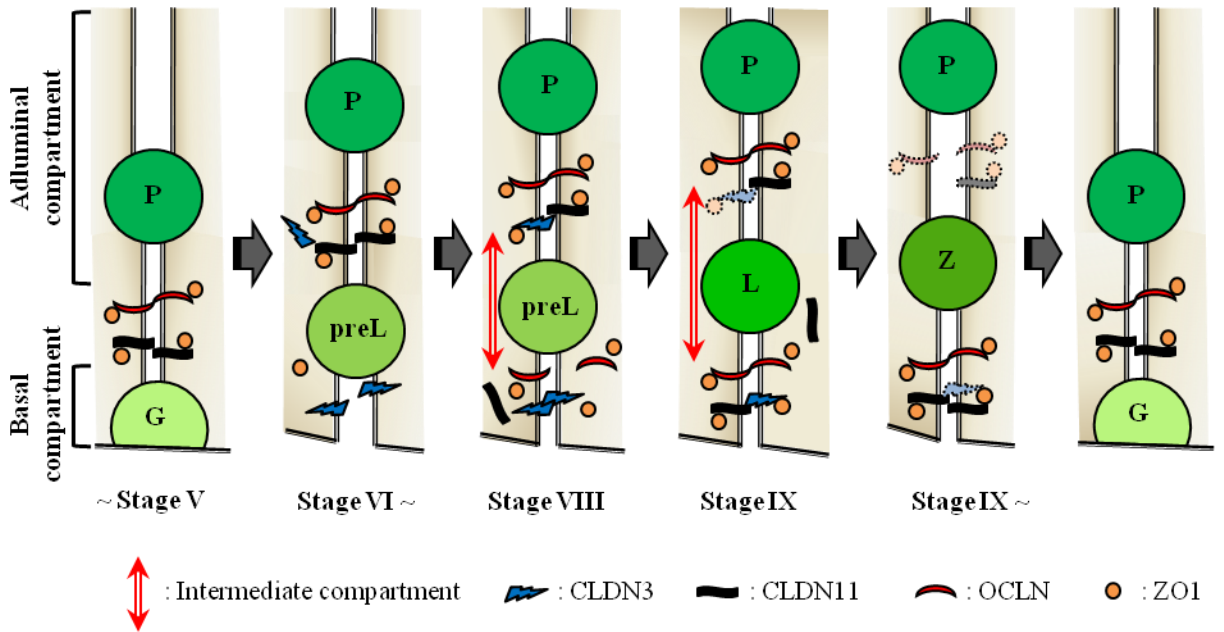


Figure 1-5. Putative BTB dynamic model.

The integrity of the BTB is maintained at all stages. Spermatogonia reside in the basal compartment of the seminiferous tubules. During stages VIII–IX, new TJ fibrils are formed below the preleptotene/leptotene spermatocytes by the recycling or *de novo* synthesis of TJ proteins (architecture of the intermediate compartment). The establishment of new TJ fibrils may be promoted by CLDN3 which is localized at the BTB from stage VI. Thereafter, during stage X, TJ fibrils above the spermatocytes are disassembled. Therefore, during stages X–V, there is only one TJ fibril per Sertoli cell. G, spermatogonia; preL, preleptotene spermatocytes; L, leptotene spermatocytes; P, pachytene spermatocytes; Z, zygotene spermatocytes.

Chapter 2

Stage-specific murine expression of claudin 3 regulates progression of meiosis in early-stage spermatocytes

Introduction

During spermatogenesis, preleptotene/leptotene spermatocytes migrate across the BTB from the basal to the adluminal compartment for further development. In rodents, the germ cell migration across the BTB occurs from late stage VIII to early stage IX (Russell, 1977). As shown in chapter 1, although the TJ proteins including CLDN11, OCLN, and ZO1 localized at the BTB throughout adult spermatogenesis, CLDN3 localized to the basal portion of seminiferous tubules only during stages VI–IX, in accordance with the emergence of the preleptotene/leptotene spermatocytes. Previous studies using epithelial cells showed that CLDN3 recruits endogenous ZO1 to cell-cell contact areas (Itoh et al., 1999; Ikenouchi et al., 2008). On the basis of these findings, temporal CLDN3 expression has been suggested to contribute to the establishment of new TJ fibrils below the preleptotene/leptotene spermatocytes during their migration from the basal to the adluminal compartment (Smith and Braun, 2012).

Although claudins are TJ proteins that regulate cell-cell adhesion in epithelial and endothelial cells (Tsukita et al., 2001), some claudins also localize to non-TJ areas (Gregory et al., 2001; Morrow et al., 2009; Kawai et al., 2011). Interestingly, an experiment on the transplantation of spermatogonial stem cells (SSCs) into testis showed that the TJ proteins including CLDN3 in SSCs regulate the SSC migration from the seminiferous tubular lumen to their niche on the basal lamina through the BTB (Takashima et al.,

2011). Taken collectively, these findings indicate that CLDN3 plays a pivotal role in spermatocyte migration across the BTB during spermatogenesis. However, there is no direct evidence indicates that *Cldn3* ablation causes abnormal spermatogenesis.

In this chapter, to determine the CLDN3 function in spermatogenesis, the author examined the cell types expressing CLDN3 in the mouse testis and evaluated the integrity of spermatogenesis after *Cldn3* knockdown. The results in the present study revealed that not only Sertoli cells that formed the BTB but also the preleptotene/leptotene spermatocytes expressed CLDN3. Moreover, *Cldn3* knockdown testes showed a prolonged premeiotic phase, indicating that temporary expression of CLDN3 might regulate the meiotic progression by promoting spermatocyte migration across the BTB.

Materials and Methods

Animals

Two-month-old C57BL/6N mice (male and female) were purchased from Japan SLC.

ICR.Cg-Tg(Stra8-EGFP)1Ysa/YsaRbrc (*Stra8-EGFP*) mice were developed by Dr. A. Suzuki (Yokohama National University, Yokohama, Japan) and Dr. Y. Saga (National Institute of Genetics, Mishima, Japan), and the breeding pairs were obtained from RIKEN BRC (Tsukuba, Japan). For the construction of the *Stra8-EGFP* transgene, the bacterial artificial chromosome (BAC) clone (RP23-367A8) containing the full length of mouse stimulated by retinoic acid gene 8 (*Stra8*) was used. At the end of *Stra8* open reading frame (orf) in exon 9, the orf of the enhanced green fluorescent protein (*Egfp*) gene combined with β -globin polyA signal site was inserted into RP23-367A8. This BAC clone construct containing this transgene was used for the generation of *Stra8-EGFP* mice. *Stra8* is a gene stimulated by retinoic acid, and it controls the switch from germ cell differentiation to meiosis (Anderson et al., 2008). Because endogenous STRA8 is predominantly expressed in the preleptotene and early leptotene spermatocytes entering meiosis at stages VII and VIII (Mark et al., 2008; Zhou et al., 2008), *Stra8-EGFP* mice produce the fusion protein of STRA8 and EGFP (STRA8-EGFP) at these seminiferous tubule stages under the control of *Stra8* gene expression regulatory mechanisms. In this chapter, 12 weeks was defined as adult

age. For the analyses, mice were obtained by free breeding, maintained under specific pathogen-free conditions, and used according to the Guide for the Care and Use of Laboratory Animals of Hokkaido University, Graduate School of Veterinary Medicine (approved by the Association for Assessment and Accreditation of Laboratory Animal Care International).

In situ hybridization analysis

The testes of adult C57BL/6N mice were fixed by intracardiac perfusion with ice-cold 4% PFA in 0.1 M PB, and then kept in the same fixative overnight at 4 °C. Then, the testes were paraffin-embedded and serial-sectioned for PAS-H staining or *in situ* hybridization. Complementary RNA probes for *Cldn3* were synthesized in the presence of digoxigenin (DIG)-labeled UTP by using a DIG RNA Labeling Kit (Roche Diagnostics, Mannheim, Germany). The primer pairs used for probe synthesis are shown in Table 2-1. Hybridization of probe was performed under stringent conditions as described previously (Kimura et al., 2011). After hybridization, positive signals were detected by visualization of DIG with polyclonal sheep anti-DIG Fab fragments conjugated to alkaline phosphatase (Roche Diagnostics).

Isolation of testicular cells

Testes were isolated from adult *Stra8-EGFP* mice that were euthanized by CO₂ inhalation. Then, the tunica albuginea was removed in ice-cold Dulbecco modified Eagle medium (DMEM)/F12 (Life Technologies). Leydig cells and other interstitial cells were eliminated by incubating the decapsulated testes in collagenase IV (Life Technologies) solution (1 mg/ml in DMEM/F12) for 20 min at 34 °C with shaking at 100 oscillations/min. After being washed 3 times with fresh DMEM/F12, the seminiferous tubules were placed in DMEM/F12 solution containing collagenase IV (1 mg/ml) and trypsin (1 mg/ml; Life Technologies) for 15 min at 34 °C with shaking at 100 oscillations/min. Then, the cell mass was resuspended in 2 ml of trypsin (1 mg/ml) and individual cells were separated by pipetting for 2 min. After being washed with fresh medium, the cell suspension was filtered using a 70-µm cell strainer (BD Biosciences, San Jose, USA), resuspended in ice-cold PBS, and used for subsequent cell sorting and immunostaining.

Cell sorting

EGFP-positive cell sorting was carried out using testicular cell suspension from the *Stra8-EGFP* mice by using MoFlo XDP and Summit software (both from Beckman Coulter Beckman Coulter, Brea, USA). Briefly, the cells were analyzed for forward scatter, side scatter, and EGFP fluorescence with an

argon laser (488 nm, 100 mW). Dead cells were excluded by gating on forward and side scatter. The viable EGFP-positive cells were sorted into ice-cold PBS. Then, total RNA was extracted from purified and unpurified cells with an RNAqueous Kit (Life Technologies).

Immunocytochemical analysis

Testicular cells isolated from *Stra8-EGFP* mice were fixed by 1% PFA for 15 min and rinsed with ice-cold PBS containing 10% fetal bovine serum. Blocking was performed using 2% skim milk/PBS. These cells were incubated with rabbit anti-CLDN3 antibody (1:150; Life Technologies) for 1 h at room temperature. After being washed with PBS, the cells were incubated with Alexa Fluor 546-conjugated donkey anti-rabbit IgG antibody (1:500; Life Technologies) for 30 min at room temperature. For nuclear staining, Hoechst 33342 (1:200; Wako, Osaka, Japan) was used.

Immunohistochemistry and immunofluorescence

Mouse testes fixed by 4% PFA perfusion were used and embedded in paraffin. Then, the 2- μ m testis paraffin sections were deparaffinized and hydrated. For immunohistochemistry, antigen retrieval was performed with buffered citrate (pH 6.0) for 15 min at 105 °C. The samples were treated with methanol

containing 0.3% H₂O₂ to eliminate endogenous peroxidase. The sections were blocked with normal serum and then incubated with rabbit anti-CLDN3 (1:150; Life Technologies), rabbit anti-STRA8 (1:2000; Abcam, Cambridge, UK), goat anti-DMC1 (DMC1 dosage suppressor of mck1 homolog, meiosis-specific homologous recombination; 1:500; Santa Cruz Biotechnology, Santa Cruz, USA), or rat anti-GATA1 (GATA binding protein 1; 1:50; Santa Cruz Biotechnology) at 4 °C overnight. Next, the sections were treated with the appropriate biotin-conjugated secondary antibodies for 30 min at room temperature followed by treatment with the VECTASTAIN EliteABC Reagent (Vector Laboratories) for 30 min at room temperature. The sections were incubated with DAB solution containing 0.006% H₂O₂ until the stain developed, and then they were counterstained with hematoxylin. For histometric analyses, BZ-9000 (Keyence, Osaka, Japan) was used for acquisition of digital images of each immunohistochemical section, and the immunopositive cells were counted by BZ-II Analyzer software (Keyence).

For immunofluorescence analysis, antigen retrieval was performed with Target Retrieval Solution (DakoCytomation, Carpinteria, USA) for 15 min at 105 °C. The sections were incubated with 5% skim milk/PBS and then with rabbit anti-OCN (1:100; Life Technologies) and goat anti-DMC1 (1:500) at 4 °C overnight. After being washed with PBS, the sections were incubated with the appropriate fluorescein isothiocyanate- or tetramethyl rhodamine isothiocyanate-conjugated secondary antibodies for

30 min at room temperature. For nuclear staining, Hoechst 33342 (1:200; Wako) was used.

To evaluate the CLDN3-expressing cells in detail, *Stra8-EGFP* mouse testes fixed by 4 % PFA perfusion were embedded in glycol methacrylate (Technovit 8100, Heraeus Kulzer, Wehrheim, Germany). Then, CLDN3 immunofluorescence was performed using 0.8- μ m semi-thin testis sections. After being photographed under a fluorescence microscope, the sections were stained with 1% toluidine blue (TB) and photographed under a light microscope.

RT-PCR and QPCR

Purified total RNAs were treated with Turbo DNase (Life Technologies) for DNA digestion, and cDNAs were synthesized via RT reaction using ReverTra Ace (Toyobo, Osaka, Japan) and oligo-dT (Life Technologies) or random primers (Promega, Madison, WI, USA). PCR reactions subsequent to RT were performed using the cDNA, Ex Taq DNA Polymerase (Takara Bio, Otsu, Japan), and the gene-specific primers (Table 2-1), and the bands of gel were photographed under a UV lamp. QPCR analysis was performed using the cDNA, the gene-specific primers (Table 2-1), Brilliant III Ultra Fast SYBR Green QPCR Master Mix (Agilent Technologies), and a real-time thermal cycler (MX 3000P, Agilent Technologies). The mRNA expression levels of the target genes were normalized to those of

glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*).

In vivo RNA interference

Cldn3 Stealth RNAi siRNA duplex (MSS273702) was obtained from Life Technologies as annealed and predesigned for *in vivo* siRNA (sense: GAUCACCAUCGUGGCGGGAGUGCUU, antisense: AAGCAGUCCCCGCCACGAUGGUGAUC). Stealth RNAi Negative Control High GC Duplex (Life Technologies) was used as control siRNA. Under anesthesia, the left and right testes of adult male C57BL/6N mice were directly injected with control and *Cldn3* siRNAs (20 μ M, total volume 20 μ l), respectively, by using a 29-gauge needle, as described previously (Lie et al., 2009). To assess the siRNA uptake efficiency, cryosections of testes injected with BLOCK-iT Alexa Fluor red fluorescent oligo (20 μ M; total volume, 20 μ l; Life Technologies) or unlabeled control siRNA were photographed under a fluorescence microscope. Next, to evaluate the knockdown efficiency, mRNA and protein levels of CLDN3 in the testis were evaluated by QPCR and immunohistochemistry 72 h after RNA interference (RNAi) treatment.

BTB integrity assay

The permeability of the BTB was evaluated using Evans blue dye as described previously (Kozler et al., 2003; Alonso et al., 2011). Briefly, Evans blue dye (960.82 Da; Sigma-Aldrich, St Louis, MO, USA) was dissolved in PBS and intraperitoneally injected into adult C57BL/6N mice at 25 mg/kg of body weight after 24 h of RNAi treatment. After 24 h of Evans blue injection, the testes were fixed by 4% PFA perfusion and embedded in paraffin. Then, the testis sections were observed under a fluorescence microscope for the localization of Evans blue dye in seminiferous tubules. Briefly, leakage of Evans blue dye to the adluminal compartment indicates disruption of the BTB.

Incorporation of 5-bromo-2-deoxyuridine into germ cells

5-Bromo-2-deoxyuridine (BrdU; Wako) was dissolved in PBS and injected intraperitoneally into adult C57BL/6N mice at 100 mg/kg of body weight after 24 h of RNAi treatment. The testes were removed from 4% PFA perfusion-fixed animals after 2 or 72 h of BrdU injection. To detect the BrdU-labeled germ cells, the testis paraffin-sections were stained with rat anti-BrdU antibody (1:200; Abcam), biotin-conjugated secondary antibody, and VECTASTAIN EliteABC Reagent. The positive reaction was developed by using DAB solution containing 0.006% H₂O₂.

Statistical analysis

Statistical analysis was performed using nonparametric Mann-Whitney U test ($P < 0.05$). Results are presented as means $\pm$ SEM.

Results

Expression and localization of Cldn3 transcripts in the testis of adult mice

To identify the cell types expressing *Cldn3* mRNA, *in situ* hybridization analysis of *Cldn3* was performed using adult C57BL/6N testes. The seminiferous stages on *in situ* hybridization sections were determined by the morphology of germ cells in PAS-H-stained serial sections. Diffuse *Cldn3*-positive signals were observed in the seminiferous tubules at all stages (Fig. 2-1A), and no positive signal was detected in the sense negative control (Fig. 2-1C). Notably, *Cldn3*-positive cells were predominantly localized to the basal portion of seminiferous tubules during stages VI–IX (Fig. 2-1B, see VIII and IX), and their positive signals tended to be weak at other stages (Fig. 2-1B, see II–III, IV, and X). This stage-dependent localization of testicular *Cldn3* mRNA expression was consistent with the appearance patterns of preleptotene/leptotene spermatocytes (Oakberg, 1956; Russell, 1977).

CLDN3 expression in STRA8-positive spermatocytes

On the basis of the *in situ* hybridization results, the author hypothesized that preleptotene/leptotene spermatocytes as well as Sertoli cells express *Cldn3*. The author used STRA8, which is predominantly expressed at stages VII and VIII (Mark et al., 2008; Zhou et al., 2008), as a marker of preleptotene and

early leptotene spermatocytes in this study. From the testes of adult *Stra8-EGFP* mice, whole testicular cells were dispersed (Fig. 2-2A), and STRA8-EGFP-positive cells were isolated using a cell sorter (Fig. 2-2B). The results of the RT-PCR analyses showed that although the whole testicular cells expressed all the examined TJ component genes, the sorted STRA8-EGFP-positive cells expressed only *Cldn3*, claudin 5 (*Cldn5*), *Cldn11*, and *Tjp1* (encoding ZO1), and not *Ocln* and *Tjp2* (encoding ZO2) (Fig. 2-2C). Immunofluorescence analysis revealed that the STRA8-EGFP-positive cells showed CLDN3-positive signals (Fig. 2D; c, arrows).

CLDN3 localization in the testis of adult mice

In immunohistochemical analysis of adult C57BL/6N testis, CLDN3-positive reactions were observed at the basal portion of the stage IV–IX tubules, linearly surrounding preleptotene/leptotene spermatocytes (Fig. 2-3A; arrowheads). To analyze the intracellular localization of CLDN3 in spermatocytes, the author performed TB staining subsequent to the immunofluorescence analysis of CLDN3 in the testes of adult *Stra8-EGFP* mice (Fig. 2-3B). STRA8-EGFP- or CLDN3-positive reactions were observed at the basal portion of seminiferous tubules (Fig. 2-3B; a and b, respectively). Notably, linear CLDN3-positive signals were observed around the nuclei of STRA8-EGFP-positive spermatocytes

(Fig. 2-3B; c, arrows) and beside the basal lamina of seminiferous tubules (Fig. 2-3B; c, large arrowhead). Furthermore, the duplicated linear patterns of CLDN3-positive signals were observed beside some of the STRA8-EGFP-positive spermatocytes (Fig. 2-3Bc; arrow and small arrowhead beside the rightmost spermatocyte). The comparative observations of TB-stained feature after the immunofluorescence analysis of CLDN3 revealed that the linear pattern of CLDN3-positive signals surrounding the STRA8-EGFP-positive spermatocytes corresponded to their cell membranes (Fig. 2-3B; d, arrows). The CLDN3-positive signals in other portions corresponded to the cytoplasm or cell membrane derived from Sertoli cells (Fig. 2-3B; d, small and large arrowheads).

BTB integrity after *Cldn3* knockdown

BTB integrity was evaluated after *Cldn3* RNAi treatment in adult C57BL/6N testes. The siRNA uptake efficiency was estimated by injection of Alexa Fluor 555-labeled RNA duplex (Fig. 2-4A). Intense red signal was not observed in the seminiferous tubules of unlabeled control siRNA-injected testis (Fig. 2-4A; a), and a weak dotted signal indicated the intrinsic fluorescence in the cytoplasm. In contrast, diffuse intense red signals in the cytoplasm were observed in the cells of both interstitium and seminiferous tubules of labeled RNA-injected testis (Fig. 2-4A; b). As shown in Fig. 2-4B, decreased

Cldn3 mRNA expression (approximately 50 % decrease compared to the control RNAi testis) was observed in the *Cldn3* RNAi testis after 72 h of RNAi treatment by QPCR analysis. Furthermore, the localization of GATA1, a marker of Sertoli cells during stages VII–IX (Yomogida et al., 1994), and that of CLDN3 were examined by immunohistochemistry using serial sections of RNAi-treated testes (Fig. 2-4C). In the control RNAi testis, CLDN3-positive reactions were observed in the GATA1-positive tubules, indicating stages VII–IX (Fig. 2-4C; a and b, asterisks). On the other hand, in the *Cldn3* RNAi testis, these positive reactions disappeared in some of the GATA1-positive tubules (Fig. 2-4C; c and d, asterisk). These results confirmed the knockdown of *Cldn3* in the *Cldn3* RNAi testis.

To elucidate the effect of *Cldn3* knockdown on the expression of other TJ component proteins, the author investigated the mRNA expression levels of *Ocln*, *Cldn5*, *Cldn11*, and *Tjp1* after 72 h of RNAi treatment (Fig. 2-4D). The results of the QPCR analyses showed no significant difference between control and *Cldn3* RNAi testes. Then, BTB integrity was evaluated by the intraperitoneal injection of Evans blue dye after *Cldn3* RNAi treatment (Fig. 2-4E). In both of the control and *Cldn3* RNAi testes, Evans blue dye was observed in the testicular interstitium and the basal compartment of the seminiferous tubules (Fig. 2-4E; a and b), indicating that the BTB prevented leakage of the dye to the lumen and that BTB integrity was maintained even after *Cldn3* knockdown.

Inhibition of the spermatocyte migration across the BTB after *Cldn3* knockdown

The author investigated the spermatocyte migration across the BTB with the double immunofluorescence of OCLN and DMC1, a marker of leptotene and zygotene spermatocytes, after control and *Cldn3* RNAi treatment of adult C57BL/6N mouse testes (Fig. 2-5). In this analysis, the author focused on stage X–XI tubules (50–60 tubule cross-sections in each testis, n = 7) just after the spermatocyte migration across the BTB. In the stage X tubules in the control testis, OCLN-positive signals were observed at the basal side of DMC1-positive spermatocytes (Fig. 2-5C; arrows). However, in half of the stage X tubules in the *Cldn3* RNAi testis (51.92 ± 4.19 %), OCLN-positive signals were observed at the luminal side of stratified or jammed DMC1-positive spermatocytes (Fig. 2-5I; arrows). In the stage XI tubules in both testes, OCLN-positive signals were observed only at the basal side of DMC1-positive spermatocytes (Fig. 2-5F and L; arrows). These results indicated that the spermatocyte migration across the BTB would not be accomplished until after stage X because of *Cldn3* knockdown.

Delayed spermatogenesis after *Cldn3* knockdown

The author investigated the effect of *Cldn3* knockdown on spermatogenesis in adult C57BL/6N

mice after 72 h of RNAi treatment, focusing on the seminiferous stages before and after the passage of spermatocytes through the BTB (Fig. 2-6). In the immunohistochemistry for STRA8, a marker of preleptotene and early leptotene spermatocytes, robust positive germ cells were observed in the stage VII and VIII tubules of the control RNAi testis (Fig. 2-6A; a). However, in the *Cldn3* RNAi testis, these STRA8-positive germ cells were also observed in the stage IX as well as the stage VII and VIII tubules (Fig. 2-6A; b). Furthermore, germ cells positive for DMC1, a marker of leptotene and zygotene spermatocytes, were observed in the stage VIII–XII tubules of the control RNAi testis (Fig. 2-6A; c). In the *Cldn3* RNAi testis, strongly DMC1-positive germ cells were also detected in the stage I–III as well as the stage VIII–XII tubules (Fig. 2-6A; d). Histometric analyses of 200–300 tubule cross-sections in each testis (n = 4) showed that the STRA8-positive germ cells significantly increased in the *Cldn3* RNAi testis compared to the control RNAi testis, but no significant difference was observed in the number of DMC1-positive germ cells between these testes (Fig. 2-6B).

The author hypothesized that the increase of STRA8-positive spermatocytes in stage IX tubules reflected the accumulation of preleptotene/leptotene spermatocytes as a result of *Cldn3* knockdown. Because premeiotic DNA replication occurred in preL spermatocytes (Zhou et al., 2008), the author assessed the accumulation of these cells by detecting DNA replications with the combination of BrdU

and RNAi treatments in adult C57BL/6N mice (n = 3 in each time point) as shown in Fig. 2-7. After 2 h of BrdU treatment, BrdU-labeled spermatocytes were exclusively detected in the stage VII and VIII tubules of the control RNAi testis (Fig. 2-7A). In the *Cldn3* RNAi testis, the BrdU-labeled spermatocytes were detected not only in the stage VII and VIII tubules but also in the stage IX tubules (Fig. 2-7B). Although BrdU-positive cells were also detected at other stages in both of the control and *Cldn3* RNAi testes, these positive cells were morphologically identified as undifferentiated spermatogonia, indicating that the DNA replications were due to mitosis (Fig. 2-7A and B, see I–VI and XI). Furthermore, the author analyzed the cell fate of BrdU-labeled spermatocytes in the testes after 72 h of BrdU treatment (Fig. 2-7C and D). In control RNAi testes, the BrdU-labeled spermatocytes were observed in the stage X, XI, and XII tubules (Fig. 2-7C). In addition to these stages, the stage I tubules also contained BrdU-labeled spermatocytes in the *Cldn3* RNAi testis (Fig. 2-7D). These data strongly indicated a prolonged period of the preleptotene phase.

Discussion

CLDN3 expression in preleptotene/leptotene spermatocytes

The migration of preleptotene/leptotene spermatocytes across the BTB occurs during stages VIII–IX of the seminiferous epithelial cycle in the adult mouse testis (Russell, 1977); however, the mechanisms regulating the germ cell migration across the BTB remain obscure. Results in chapter 1 showed that the CLDN3 protein localized to the BTB only during stages VI–IX, and its expression level peaked at stages VII–VIII, indicating that the timing of CLDN3 localization to the BTB corresponded to that of preleptotene/leptotene spermatocyte migration across the BTB. Therefore, in the present study, the author strongly predicted a crucial role of CLDN3 in the regulation of BTB function.

Several claudins are crucial components of the BTB in Sertoli cells and have a central role in its barrier functions (Pelletier, 2011). However, in the present study, the author detected *Cldn3* mRNA in the basal portion along the circumference of seminiferous tubules in the *in situ* hybridization analysis, and some of the STRA8-expressing preleptotene/leptotene spermatocytes showed CLDN3-positive signals in immunofluorescence analysis. Although germ cells lack TJs (Pelletier, 2011), they express several TJ proteins (Morrow et al., 2009; Takashima et al., 2011). In stage VII, just before the germ cell migration across the BTB, CLDN3 is diffusely distributed in the basal region of tubules surrounding preleptotene

spermatocytes rather than localizing to the BTB (Smith and Braun, 2012). Although CLDN3 expression in germ cells has been suggested previously (Takashima et al., 2011), the sticking structure between germ cells and Sertoli cells makes it difficult to confirm this hypothesis. Results in this chapter clearly demonstrated for the first time that spermatocytes as well as Sertoli cells express CLDN3, depending on the seminiferous epithelial cycles, especially around the period of germ cell migration across the BTB.

Predicted function of CLDN3 in spermatogenesis

In the present study, because linear CLDN3-positive signals corresponded to the cell membrane of spermatocytes, the author considered a role of CLDN3 in spermatocyte migration across the BTB by regulating the cell-cell interactions. In the ovary, a homologous organ to testis, CLDN3 localizes to the surface epithelium. Interestingly, CLDN3 and claudin 4 (CLDN4) are the most highly overexpressed proteins in the epithelial ovarian tumor cells (Hough et al., 2000), and their overexpression promotes metastasis by inducing the migration and invasion of ovarian tumor cells (Agarwal et al., 2005). Furthermore, *in vivo* *Cldn3* RNAi suppressed the growth and metastasis of ovarian tumors (Huang et al., 2009). Moreover, CLDN3 and CLDN4 have been reported to be expressed in medullary thymic epithelial cells (Hamazaki et al., 2007), and CLDN4 expression in CD4/CD8 double-positive thymocytes was

suggested to promote positive selection efficiency (Kawai et al., 2011). These findings indicate that some claudins could modulate cell migration and/or are involved in the determination of cell fate in a TJ-independent manner. Furthermore, SSC transplantation experiments showed that the microinjected donor SSCs in the lumen of seminiferous tubules migrated to the basal compartment through the BTB, colonized in the germline niche of recipient testis, and restarted spermatogenesis (Kanatsu-Shinohara et al., 2008). Interestingly, in this transplantation, *Cldn3* suppression in SSCs caused a reduction in their ability to migrate into germline niches through the BTB (Takashima et al., 2011). Although the direction of cell migration across the BTB differed between the transplanted SSCs and the endogenous spermatocytes, these findings strongly support the notion that CLDN3 regulates germ cell migration across the BTB during spermatogenesis. Importantly, similar to our findings on CLDN3, CLDN5 expression was reported in Sertoli cells, spermatogonia, and preleptotene spermatocytes located in the stage VIII tubules (Morrow et al., 2009). On the basis of these findings, the author proposes that CLDN3 and CLDN5 jointly mediate germ cell migration by regulating the intercellular interaction between Sertoli cells and spermatocytes through homotypic or heterotypic adhesion to other claudin proteins. Further studies are needed to validate this hypothesis.

Inhibition of germ cell migration across the BTB in *Cldn3* knockdown testes

To accomplish the spermatocyte migration across the BTB during stages VIII–IX, new TJ fibrils need to be formed at the basal side of preleptotene/leptotene spermatocytes, followed by the disassembly of the TJ fibrils at the luminal side of these spermatocytes (Russell, 1977; Smith and Braun, 2012). In this study, although the permeability barrier function of the BTB was preserved even after *Cldn3* RNAi treatment, the TJ fibrils were localized to the luminal side of stratified DMC1-positive leptotene or zygotene spermatocytes in stage X tubules. This observation indicates that the spermatocyte migration across the BTB might be partially prevented by the *Cldn3* knockdown and not accomplished until after stage X. However, in the stage XI tubules in the *Cldn3* RNAi testis, the TJ fibrils were detected only at the basal side of zygotene spermatocytes, indicating that the effect of *Cldn3* knockdown was temporary and the spermatocytes could migrate through the BTB by stage XI. Although these results might reflect the temporal efficiency of *Cldn3* knockdown due to the characteristics of siRNA, some complementary mechanisms could also promote spermatocyte migration after *Cldn3* knockdown.

Disordered spermatogenesis in the testes of *Cldn3* knockdown mice

The results of the *Cldn3* RNAi experiments showed an aberrant increase of STRA8-positive

(preleptotene or early leptotene) spermatocytes in stage IX tubules. Furthermore, spermatocytes incorporating BrdU were detected in the stage IX tubules in the *Cldn3* RNAi testis after 2 h of BrdU injection, which was consistent with the results of STRA8 localization after *Cldn3* RNAi treatment. A schematic of disordered spermatogenesis observed in *Cldn3* knockdown mice is shown in Fig. 2-8. After 72 h of BrdU injection, BrdU-labeled spermatocytes were frequently observed in the stage I tubules of *Cldn3* RNAi testes. Spermatogenesis progresses from stage IX to stage I in a span of 72 h (Hogarth and Griswold, 2010). Therefore, these results strongly indicated a prolonged period of the preleptotene phase until at least stage IX in the *Cldn3* knockdown testis. Because the CLDN3-positive signals disappeared in the GATA1-positive tubules, indicating stages VII–IX, the effect of *Cldn3* RNAi might be caused by the *Cldn3* knockdown not only in spermatocytes but also in Sertoli cells. STRA8 is a marker for preleptotene and early leptotene spermatocytes in males because it is required for meiotic initiation in both sexes and its expression is restricted to premeiotic germ cells (Oulad-Abdelghani et al., 1996; Koubova et al., 2006). Furthermore, in males, anti-Müllerian hormone (AMH) is expressed at high levels only in fetal Sertoli cells (Lécureuil et al., 2002). To clarify the direct role of CLDN3 in the maintenance of normal spermatogenesis, generation of conditional knockout mice for preleptotene/leptotene spermatocyte- or Sertoli cell-specific *Cldn3* using *Stra8* or *Amh* gene promoters, respectively, would be useful.

To date, there has been no report that *Cldn3* ablation directly causes abnormal spermatogenesis. Therefore, this is the first study to show that the delay in spermatogenesis is caused by *Cldn3* knockdown in testis. The delayed spermatogenesis in *Cldn3* knockdown testes might be due to the effect of *Cldn3* knockdown on the cell cycle of germ cells. Briefly, it is possible that the persistence of preleptotene spermatocytes through stage IX in *Cldn3* knockdown testes reflected a failure in the cell cycle progression from premeiotic S-phase into G₂ of the leptotene spermatocytes. Indeed, *Cldn11*-null testes contain Sertoli cells that continue to proliferate in adulthood, representing a defect in cell cycle arrest (Mazaud-Guittot et al., 2010). Furthermore, recent reports have found that some signaling factors downstream of TJs regulate cyclin D1 gene expression and protein stability (Farkas et al., 2012). Thus, multiple claudins might regulate cell cycle progression in testes.

In a normal fertile male, the spermatogenic cycle is strictly regulated to produce sperm continuously. Furthermore, to complete the spermatogenesis, germ cells progressively migrate across the entire length of the seminiferous epithelium until they are released into the tubular lumen at stage VIII (Kopera et al., 2010). Results in this chapter strongly suggest that the stage-specific expression of CLDN3 regulates the meiotic progression by promoting germ cell migration from the basal to the adluminal compartment through the BTB. CLDN3 on the surface of germ cells might act like a “key” that disrupts the “gate”

formed by claudin interactions between Sertoli cell membranes by competitive inhibition. Reduction of CLDN3 by RNAi might slow the kinetics of this process, delaying the germ cells entering the intermediate compartment where they receive factors that promote their further differentiation. Alternatively, the presence of CLDN3 on the surface of preleptotene spermatocytes might function as a maturation signal for Sertoli cells to secrete necessary factors to the intermediate compartment for their transition from preleptotene to leptotene spermatocytes. In the absence of CLDN3, the Sertoli cells might delay secretion of these factors until stage IX.

The author proposes that the cyclical expression and localization of CLDN3 during spermatogenesis is mainly regulated by sex hormones. Notably, knockout of a Sertoli cell-specific androgen receptor (*Ar*) causes spermatogenic arrest in meiosis associated with a significant reduction in spermatocytes and *Cldn3* downregulation in testes (Meng et al., 2005; De Gendt et al., 2004). Furthermore, in rodents, the highest AR expression is observed during stages VI–VII, prior to the germ cell migration across the BTB (Zhou et al., 2002). Additionally, in the seasonal breeding adult Djungarian hamster, gonadotropin suppression by short photoperiod treatment caused disordered localization of CLDN3 protein in the seminiferous epithelium, whereas *Cldn3* mRNA level was significantly increased (Tarulli et al., 2008). All these reports indicated the crucial roles of sex hormones in the regulation of CLDN3 expression and localization in the

testis.

In conclusion, the author showed that CLDN3 is expressed in preleptotene/leptotene spermatocytes preparing for migration across the BTB. Furthermore, the current results suggest that temporary expression of CLDN3 during spermatogenesis regulates the progression of meiosis by promoting germ cell migration across the BTB. These findings may provide new insights into the unidentified function of the BTB in spermatogenesis.

Summary

CLDN3 is a protein component of the tight junction strands. TJs between adjacent Sertoli cells form the BTB. During spermatogenesis, seminiferous stage-specific expression of CLDN3 is suggested to regulate the migration of preleptotene/leptotene spermatocytes across the BTB as shown in chapter 1. In this chapter, the author determined the cell types expressing CLDN3 in adult mouse testis and investigated spermatogenesis after testis-specific *in vivo* knockdown of *Cldn3*. The results of *in situ* hybridization revealed that *Cldn3* mRNA was predominantly expressed in germ cells near the basal lamina of seminiferous stage VI–IX tubules. Furthermore, CLDN3 protein was localized to not only the BTB but also the cell membrane of STRA8-expressing preleptotene/leptotene spermatocytes in the testis of adult *ICR.Cg-Tg(Stra8-EGFP)1Ysa/YsaRbrc* mice. Although *Cldn3* knockdown did not affect BTB integrity, it caused a partial delay in spermatocyte migration across the BTB. Moreover, *Cldn3* knockdown resulted in a prolonged period of the preleptotene phase during spermatogenesis. These data indicate that the seminiferous stage-specific expression and localization of CLDN3 during spermatogenesis regulate the progression of meiosis by promoting germ cell migration across the BTB.

Table and figures

Table 2-1. Primer pairs used in this chapter.

Gene name (Accession)	Symbol	Primer Sequence (5'-3') F: Forward, R: Reverse	Product Size (bp)	Application
occludin (NM_008756)	<i>Ocln</i>	F: CCTACTCCTCCAATGGCAAA R: CTCTTGCCCTTTCCTGCTTT	208	RT-PCR and QPCR
claudin 3 (NM_009902)	<i>Cldn3</i>	F: CCAGTCTCCAAAGCCACAG R: TTCCTAGGCCCGGTAGTCAG	1083	in situ hybridization
claudin 3 (NM_009902)	<i>Cldn3</i>	F: GCACCCACCAAGATCCTCTA R: TCGTCTGTCACCATCTGGAA	206	RT-PCR and QPCR
claudin 5 (NM_013805)	<i>Cldn5</i>	F: TGCTGCCTTAATGTCCAGTG R: CTTCCAGGAGGAAGGCAAC	221	RT-PCR and QPCR
claudin 11 (NM_008770)	<i>Cldn11</i>	F: TGGTGGACATCCTCATCCTT R: GCCAGCAGAATAAGGAGCAC	190	RT-PCR and QPCR
tight junction protein 1 (ZO1) (NM_009386)	<i>Tjp1</i>	F: GCACCATGCCTAAAGCTGTC R: ACTCAACACACCACCATTGC	122	RT-PCR and QPCR
tight junction protein 2 (ZO2) (NM_011597)	<i>Tjp2</i>	F: AATGCGAGGATCGAAATAGC R: TAGCTTCCTCTGGTGTCTCTG	158	QPCR
glyceraldehyde-3-phosphate dehydrogenase (NM_008084)	<i>Gapdh</i>	F: TGTGTCCGTCGTGGATCTGA R: TTGCTGTTGAAGTCGCAGGAG	150	RT-PCR and QPCR

ZO1, zonula occludens 1; ZO2, zonula occludens 2.

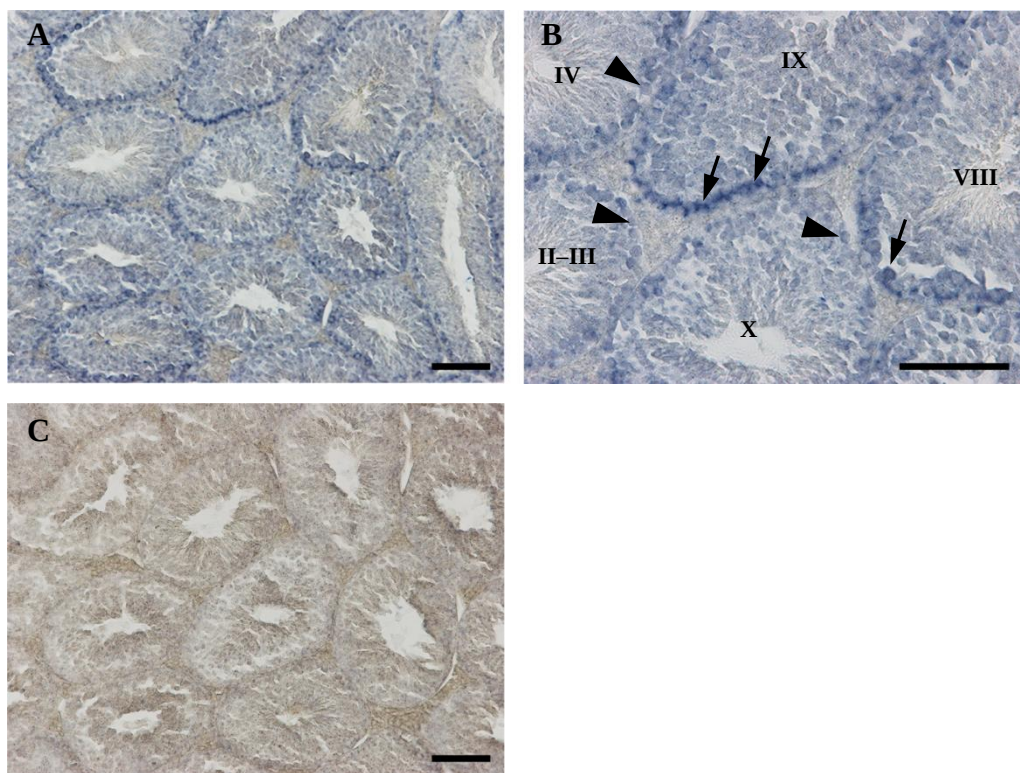


Figure 2-1. Localization of *Cldn3* transcripts in the adult mouse testis.

In situ hybridization for *Cldn3* was performed using testis sections from adult C57BL/6N mice. **A** and **B**) Testis sections with *Cldn3* antisense probe. Diffuse navy blue positive signals for transcripts are observed in the seminiferous tubules at all stages (**A**). These positive signals are predominantly observed in the cells near the basal lamina of the stage VI–IX tubules (**B**; arrows), but are reduced at other stages (**B**; arrowheads). **C**) Negative control section with the corresponding sense probe. No significant positive signal is detected. Roman numerals indicate the seminiferous stages determined from the PAS-H-stained serial sections. Bars = 50 μ m.

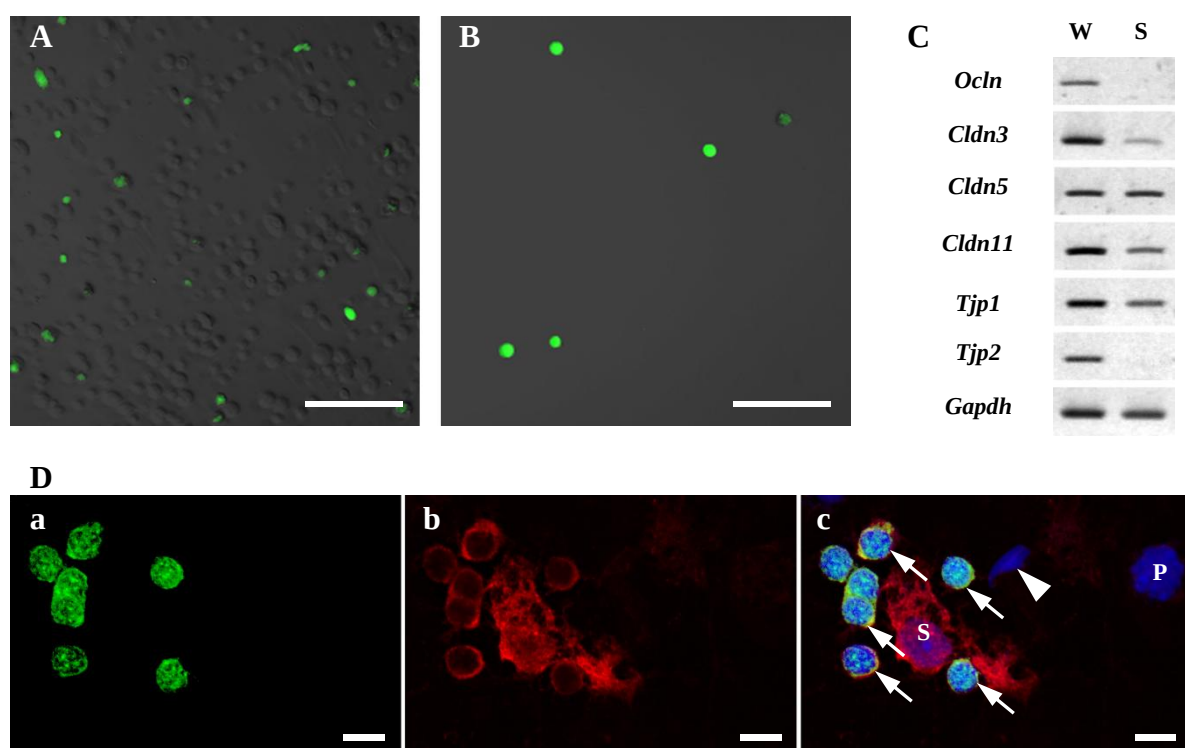


Figure 2-2. CLDN3 expression in adult mouse spermatocytes.

Enzymatically isolated testicular cells from adult *Stra8-EGFP* mice were analyzed by cell sorting for EGFP signals. **A)** Testicular cells before cell sorting for EGFP signals. Some STRA8-EGFP-expressing cells are observed in the testicular cells (green). **B)** Testicular cells after cell sorting for EGFP signals. Only STRA8-EGFP-expressing cells are observed (green). Bars = 100 μ m. **C)** RT-PCR results showing the expression of genes of TJ components obtained from testicular cells before and after cell sorting for EGFP signals. In whole testicular cells (W), all examined genes were detected. In the sorted STRA8-EGFP-expressing cells (S), the expression of *Cldn3*, *Cldn5*, *Cldn11*, and *Tjp1* (ZO1), but not of *Ocln* and *Tjp2* (ZO2), was detected. **D)** CLDN3 immunofluorescence in the testicular cells isolated from *Stra8-EGFP* mice. Each panel shows the STRA8-EGFP-expressing testicular cells (**a**; green), CLDN3-positive cells in the same samples from panel D-a (**b**; red), and merged signals of **a** and **b** (**c**; yellow) with Hoechst 33342 nuclear staining. STRA8-EGFP-expressing cells show CLDN3-positive reactions (**c**; arrows). S, Sertoli cell; P, pachytene spermatocyte; arrowhead; spermatid. Bars = 10 μ m.

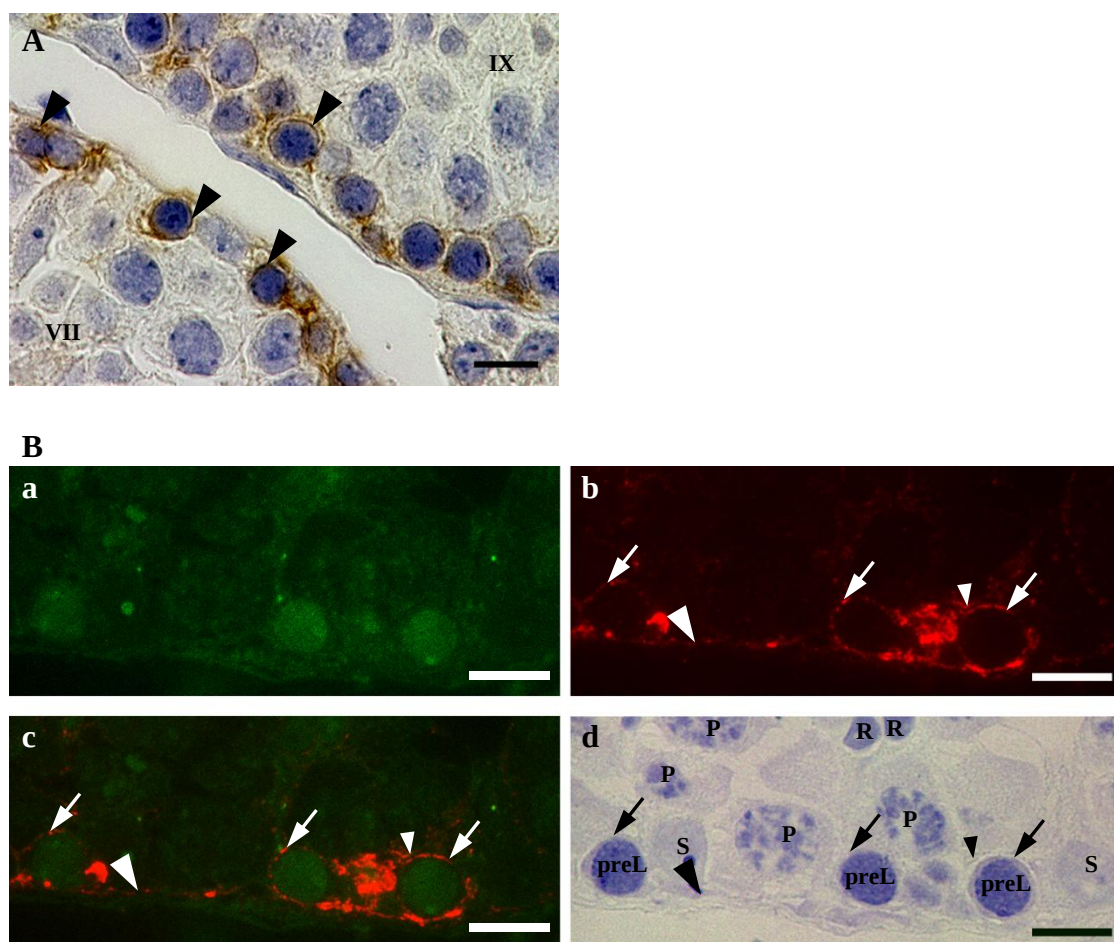


Figure 2-3. Intracellular localization of CLDN3 protein in the adult mouse testis.

A) Immunohistochemistry for CLDN3 protein in C57BL/6N mice. CLDN3-positive reactions localize in the basal part of the stage VII and IX tubules, linearly surrounding preleptotene/leptotene spermatocytes (**A**; arrowheads). **B)** Immunofluorescence for CLDN3 in the *Stra8-EGFP* mice. The STRA8-EGFP-expressing spermatocytes are observed at the basal portion of seminiferous tubules (**a**; green). In the same section in **a**, linear CLDN3-positive signals (**b** and **c**; arrows) are observed around the nuclei of STRA8-EGFP-expressing spermatocytes and beside the basal lamina of seminiferous tubules (large arrowhead). In the same TB-stained section in **a–c**, the CLDN3-positive reactions correspond to the cell membrane of STRA8-EGFP-positive spermatocytes (**d**; arrows) and Sertoli cells (**d**; small and large arrowheads). Roman numerals indicate the seminiferous stages determined from the PAS-H-stained serial sections. S, Sertoli cell; preL, preleptotene; L, leptotene; P, pachytene spermatocytes; R, round spermatids. Bars = 10 μ m.

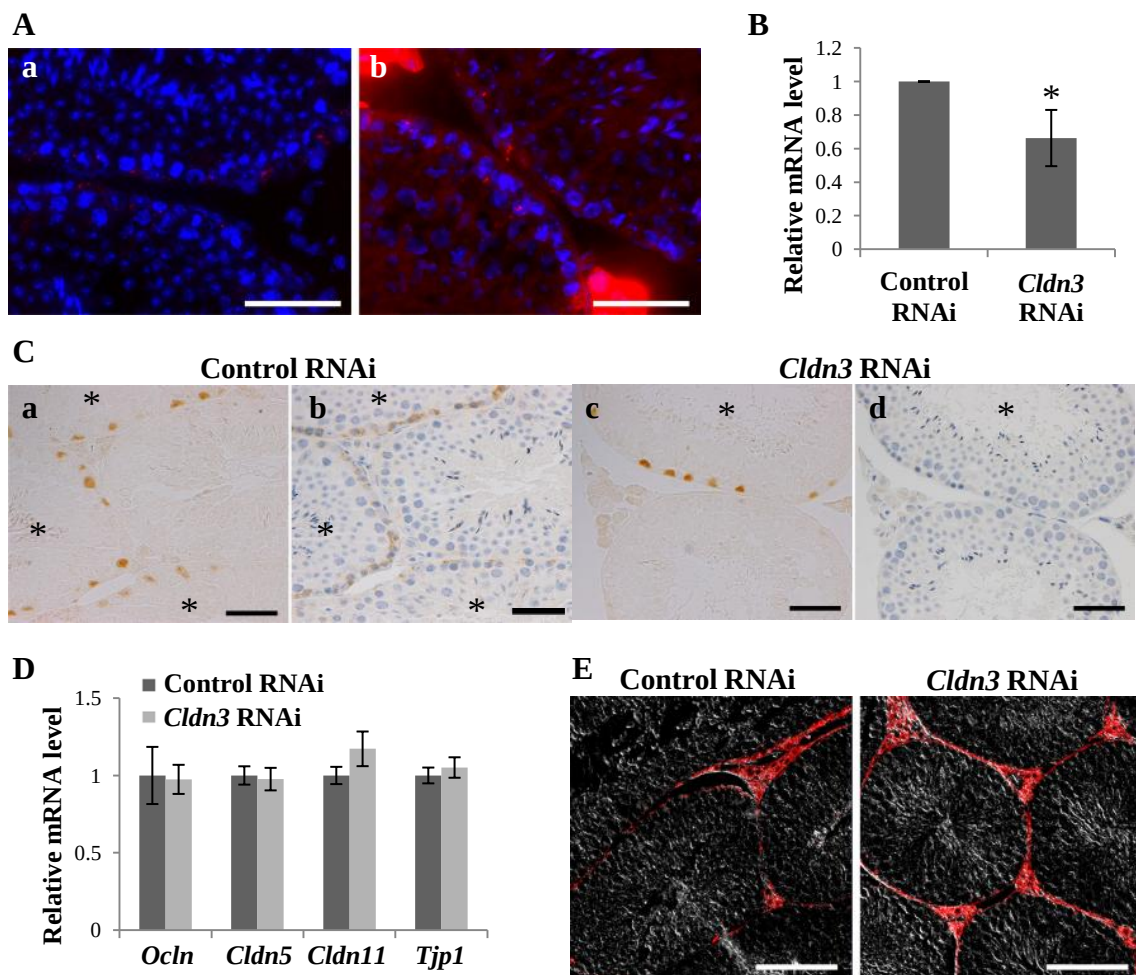


Figure 2-4. BTB integrity after *Cldn3* knockdown.

Cldn3 knockdown was performed by siRNA injection into adult C57BL/6N testes. **A)** The siRNA uptake estimated by injection of Alexa Fluor 555-labeled RNA duplex. No red signal expected for the intrinsic fluorescence in the unlabeled control siRNA injected testis is observed (**a**), whereas intense red fluorescence signals are observed in the seminiferous tubules of the labeled RNA duplex-injected testis (**b**). **B)** *Cldn3* mRNA expression in the testis analyzed by QPCR 72 h after RNAi treatment. Values are presented as the mean $\pm$ SEM (n = 4). * $P < 0.05$, Mann-Whitney *U*-test. **C)** Localization of GATA1 and CLDN3 in the testis 72 h after RNAi treatment. In control RNAi testes, positive immunoreactions for GATA1 (**a**) and CLDN3 (**b**) are observed in the same tubules (asterisks) on serial sections. The CLDN3-positive reaction disappears in some of the GATA1-positive tubules (**c**; asterisk) of *Cldn3* RNAi testes (**d**; asterisk). **D)** QPCR analyses of mRNA expression levels of *Ocln*, *Cldn5*, *Cldn11*, and *Tjp1* 72 h after RNAi treatment. Values are presented as the mean $\pm$ SEM (n = 4). **E)** BTB integrity evaluated by the intraperitoneal injection of Evans blue dye 48 h after RNAi treatment. In both control (**a**) and *Cldn3* (**b**) RNAi testes, Evans blue dye (red) is sequestered in the testicular interstitium or the basal compartment of seminiferous tubules. Bars = 50 μ m (**C**) and 100 μ m (**E**).

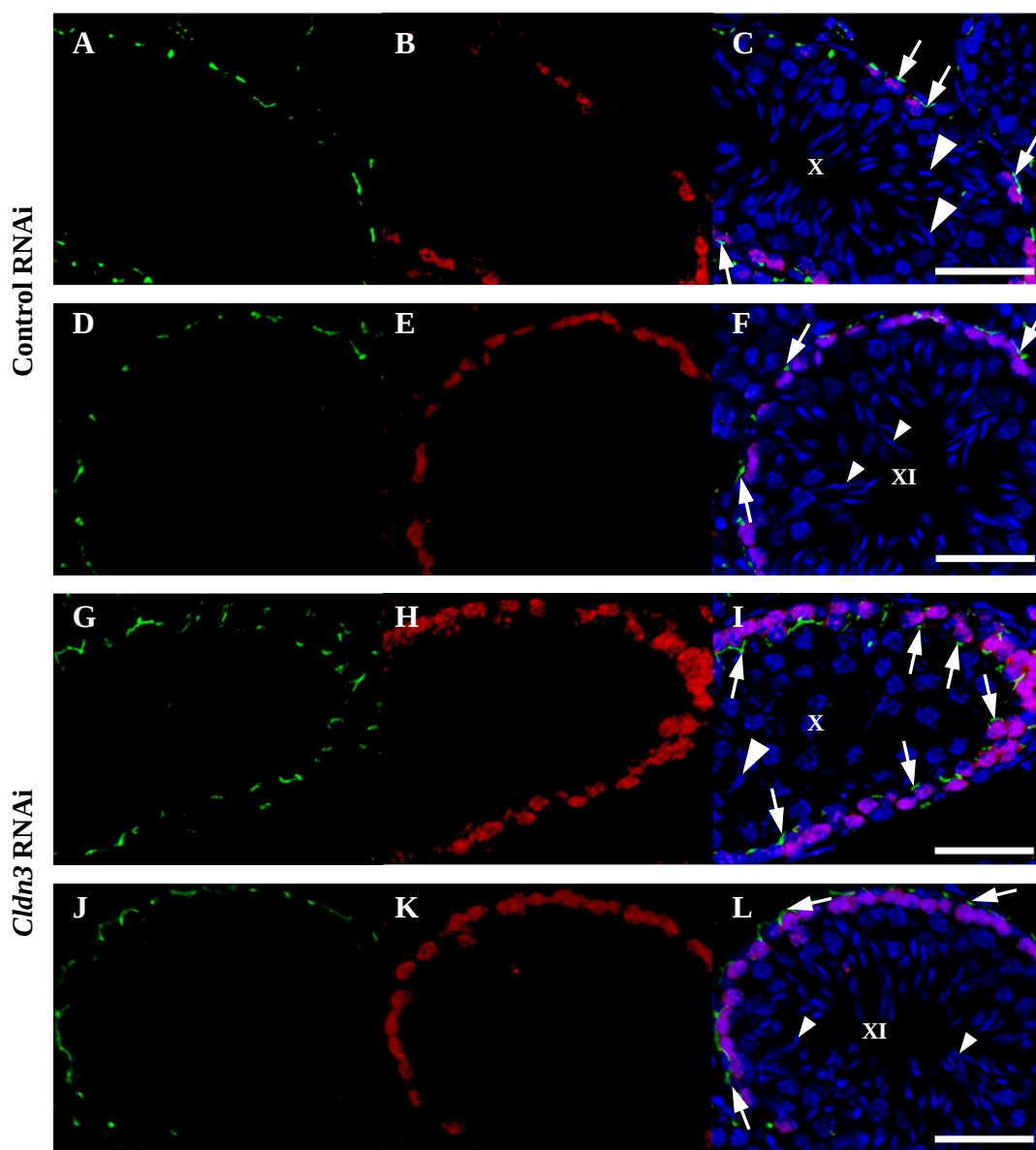


Figure 2-5. Spermatocyte migration across the BTB after *Cldn3* knockdown.

Using the testes of 72 h RNAi-treated C57BL/6N mice, the author performed immunofluorescence for OCLN and DMC1 subsequently stained with Hoechst 33342. **A–F)** Control RNAi testes. In the stage X–XI tubules, OCLN-positive signals (**A** and **D**; green) are observed at the basal side (**C** and **F**; arrows) of the DMC1-positive cells (**B**, **E** and **C**, **F**; red and purple). **G–L)** *Cldn3* RNAi testes. In the stage X tubules, OCLN-positive signals (**G**; green) are observed at the luminal side (**I**; arrows) of stratified DMC1-positive spermatocytes (**H** and **I**; red and purple). However, in the stage XI tubules, OCLN-positive signals (**J**; green) are observed at the basal side (**L**, arrows) of the DMC1-positive cells (**K** and **L**; red and purple). Roman numerals indicate the seminiferous stages determined from the presence of step 10 (**C** and **F**; large arrowheads) and step 11 spermatids (**F** and **L**; small arrowheads). Bars = 50 μ m.

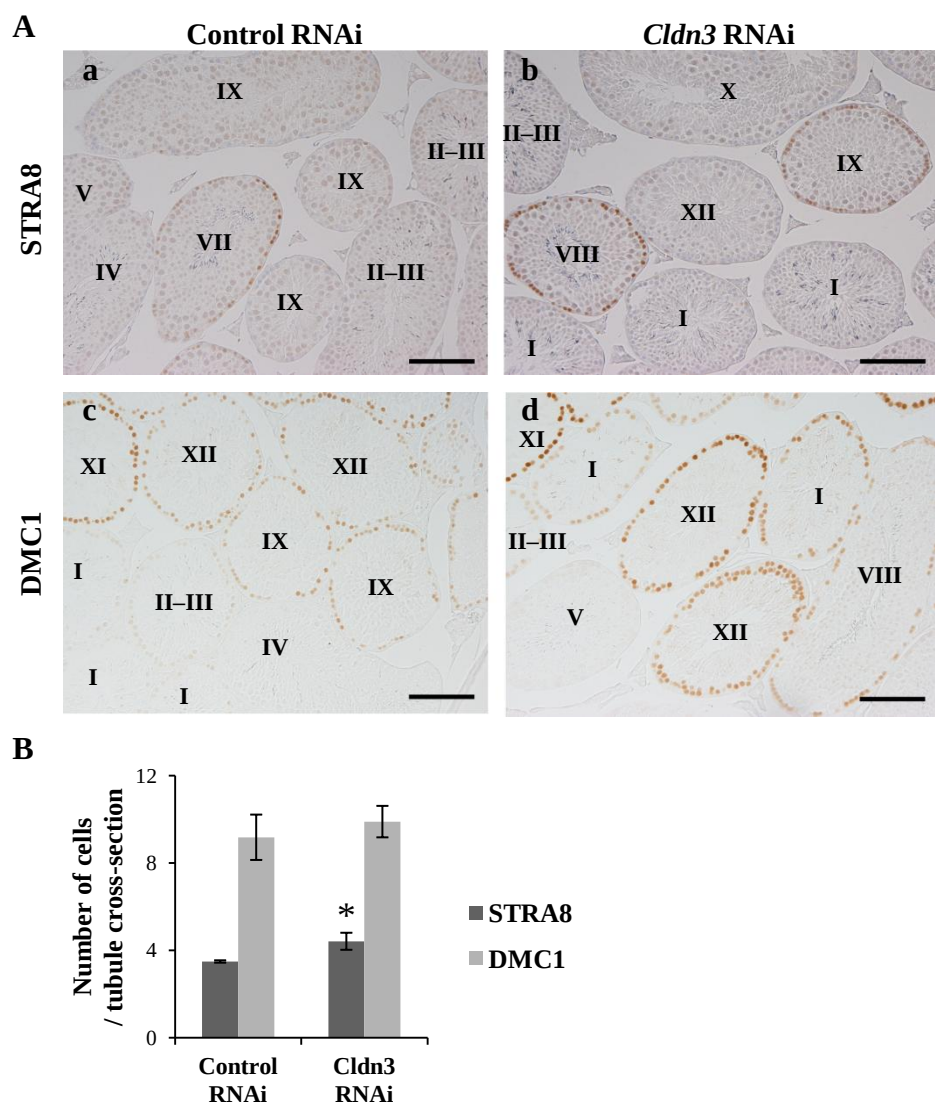


Figure 2-6. Integrity of spermatogenesis after *Cldn3* knockdown.

After 72 h of RNAi treatment of adult C57BL/6N testes, the integrity of spermatogenesis was examined by the detection of stage-specific STRA8- and DMC1-positive cells. **A)** Immunohistochemistry for STRA8 and DMC1 was performed in the testes 72 h after RNAi treatment. Cells positive for STRA8, a marker for preleptotene and early leptotene spermatocytes, are observed in the stage VII tubules of control RNAi testes (**a**). In *Cldn3* RNAi testes, STRA8-positive cells are observed in the stage VIII and IX tubules (**b**). Cells positive for DMC1, a marker for leptotene and zygotene spermatocytes, are observed in the stage X, XI, and XII tubules of control RNAi testes (**c**). In *Cldn3* RNAi testes, DMC1-positive cells are observed in the stage I–III as well as the stage XI and XII tubules (**d**). Roman numerals indicate the seminiferous stages determined from the PAS-H-stained serial sections. Bars = 50 μ m. **B)** Number of immunoreactive spermatocytes for STRA8 and DMC1 in RNAi-treated testes. Values are presented as means $\pm$ SEM ($n = 4$) among 200–300 tubule cross-sections in each testis. * $P < 0.05$, Mann-Whitney U test.

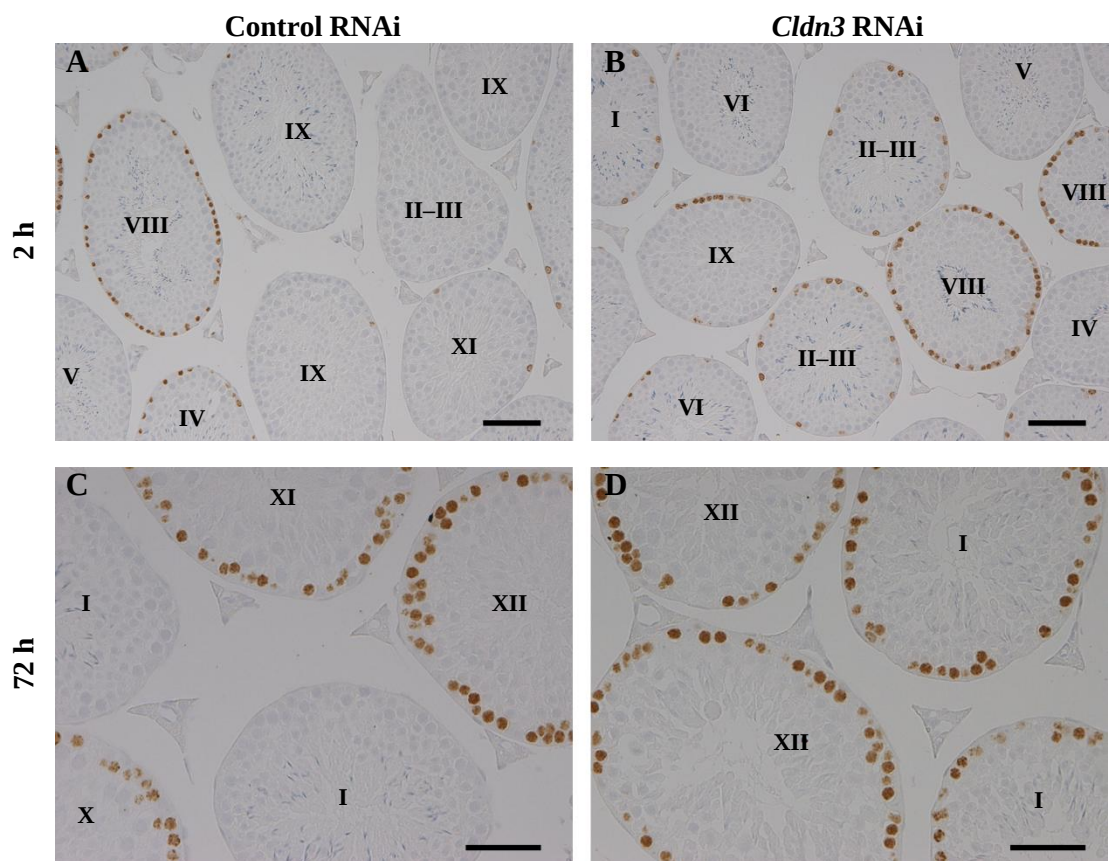


Figure 2-7. Progression of meiosis after *Cldn3* knockdown.

Peritoneal BrdU injections were administered after 24 h of RNAi treatment of adult C57BL/6N testes. **A and B)** Detection of BrdU-labeled cells after 2 h of BrdU treatment. The BrdU-labeled spermatocytes are mainly detected in the stage VIII tubules of the control RNAi testis (**A**), while they are detected even in the stage IX as well as stage VIII tubules of the *Cldn3* RNAi testis (**B**). **C and D)** Detection of BrdU-labeled cells after 72 h of BrdU treatment. In control RNAi testes, the BrdU-labeled spermatocytes are observed in the stage X, XI, and XII tubules (**C**). In addition to these stages, the stage I tubules also contain BrdU-labeled spermatocytes in the *Cldn3* RNAi testis (**D**). Roman numerals indicate the seminiferous stages determined from the PAS-H-stained serial sections. Bars = 50 μ m.

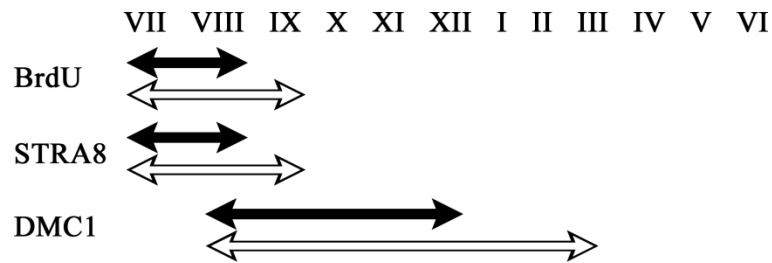


Figure 2-8. Illustration of disordered spermatogenesis after *Cldn3* knockdown.

The stage-dependent existence of BrdU-labeled cells after 2 h of BrdU treatment and of STRA8- or DMC1-positive spermatocytes in control and *Cldn3* RNAi testes are indicated by black and white arrows, respectively. Roman numerals indicate the seminiferous stages.

Chapter 3

Vitamin A deprivation affects the progression of the spermatogenic wave and initial formation of the blood-testis barrier, resulting in irreversible testicular degeneration in mice

Introduction

The first wave of spermatogenesis, meiosis initiation, occurs after birth and continues cyclically throughout adulthood (Fig. 3-1A). In mice, the spermatogenic cycle is divided into distinct stages (I–XII) with a stage-specific set of germ cells in the seminiferous tubules (Oakberg, 1956). Four cycles of 8.62 days each (for a total of 34.48 days) are required to progress from spermatogonia to spermatozoa (Hogarth and Griswold, 2010).

Male germ cell differentiation relies largely on Sertoli cells for structural and nutritional support. Sertoli cells maintain the integrity of spermatogenesis by forming the BTB (Cheng and Mruk, 2002; Pelletier, 2011). In adult animals, the BTB divides the seminiferous epithelium into the basal and adluminal compartments, thereby establishing a suitable milieu for germ cell development. In several species, the BTB is initially established during the prepubertal period when the majority of germ cells derived from the first spermatogenic wave reach the early-pachytene stage (Sun and Gondos, 1986; Morales et al., 2007; Willems et al., 2010). In rats, BTB assembly does not occur synchronously along the length of the seminiferous tubules, despite the emergence of pachytene spermatocytes (Morales et al., 2007). Hence, initial assembly of the BTB may be regulated by cooperation between the action of pachytene spermatocytes derived from the first spermatogenic wave and other unknown factors. After BTB assembly, preleptotene/leptotene spermatocytes derived from the second wave onward need to

migrate across the BTB from the basal to the adluminal compartment for further development during spermatogenesis (Fig. 3-1A). In rodents, germ cell migration across the BTB occurs from late-stage VIII to early-stage IX (Russell, 1977).

Interestingly, as shown in chapter 1, to accommodate such germ cell migration, the expression and localization of TJ proteins that constitute the BTB are altered in a seminiferous epithelium cycle-dependent manner (Hasegawa and Saga, 2012). Furthermore, although the expression levels of TJ transcripts and proteins in the testis increase with age in the prepubertal period and decrease with sexual maturation, these proteins consistently localize to the BTB throughout the reproductive period (Hellani et al., 2000; Moroi et al., 1998; Yan et al., 2008). Recently, it has been suggested that maintenance of BTB integrity is essential for normal spermatogenesis (Gow et al., 1999; Saitou et al., 2000; Mok et al., 2012; Fink et al., 2009).

In mammals, retinoic acid (RA), the active derivative of vitamin A, has been shown to be an essential inducer of meiosis in both sexes (Baillet and Mandon-Pepin, 2012). Indeed, testes of rats and mice fed a vitamin A-deficient (VAD) diet show spermatogenic arrest at spermatogonia (van Pelt and de Rooij, 1990; van Pelt et al., 1995). RA administration and dietary retinoid replenishment in rodents fed the VAD diet result in the re-initiation of spermatogenesis, starting from the remaining spermatogonia, and synchronized spermatogenesis (Ismail et al., 1990). RA acts through binding to

nuclear RA receptors (RARs) and retinoid X receptors (RXRs) in various testicular cell types and is thought to exert its effects mainly via the action of RARs in Sertoli cells and early germ cells (Hogarth and Griswold, 2010).

A previous study reported that the RAR-dependent effects of RA in Sertoli cells contribute to prepubertal testis development (Vernet et al., 2006). Moreover, as RA signaling in the testis is closely associated with the periodic cycle functions of Sertoli cells (Vernet et al., 2006; Sugimoto et al., 2011), it is possible that vitamin A affects the regulation of BTB integrity by alteration of the seminiferous epithelial cycle. Indeed, in rats, vitamin A deprivation after weaning has been reported to induce disruption of the BTB (Huang et al., 1988; Morales and Cavicchia, 2002). However, some reports have indicated that vitamin A deficiency has no effect on BTB preservation (Ismail and Morales, 1992). To clarify the importance of vitamin A in the maintenance of spermatogenesis during testes development, it is essential to assess spermatogenesis progression with changes in BTB integrity and the vitamin A pathway.

The study in this chapter assessed the impact of vitamin A deprivation on the murine seminiferous epithelium from the prepubertal period to adulthood, with a focus on spermatogenic progression and BTB assembly. The results showed that feeding the VAD diet to the parental generation (Fig. 3-1B) induced critical defects in spermatogenesis progression and altered the BTB

integrity in adult testes compared with that in the prepubertal testes of the progeny. On the basis of the results in this chapter, the author propose that BTB integrity is regulated by vitamin A metabolism with control of meiosis and is required for persistent differentiation of spermatocytes rather than the initiation of meiosis.

Materials and methods

Animals

Two-month-old male and female C57BL/6N mice were purchased from Japan SLC. VAD diet feeding and RA replacements were performed as described in a previous study (van Pelt and de Rooij, 1990). Briefly, mice were fed a VAD diet (D13110GC; Research Diets, New Brunswick, USA). After 4–12 weeks of feeding this diet, the animals were used as breeding pairs. After weaning, male pups received the VAD diet until 90 days postpartum (dpp). Testes of mice fed the VAD diet and those fed a normal vitamin A-sufficient (VAS) diet (Labo MR Standard, NOSAN, Yokohama, Japan) were weighed, and epididymides and testes were harvested at 5–90 dpp. At 91 dpp, when the body weight was slightly decreased, all-trans-RA (Nacalai Tesque, Kyoto, Japan, 0.5 mg/head) was intraperitoneally injected into mice fed the VAD diet, and the VAD diet was changed to the VAS diet. All-trans-RA was dissolved in a mixture of 25 µl ethanol and 75 µl sesame oil (MP Biomedicals, Santa Ana, USA). Testes were weighed and collected after 2–44 days of RA replacement (Fig. 3-1B). For analyses, mice were obtained by free breeding, maintained under specific pathogen-free conditions and used according to the Guide for the Care and Use of Laboratory Animals of Hokkaido University, Graduate School of Veterinary Medicine (approved by the Association for Assessment and Accreditation of Laboratory Animal Care International).

Immunohistochemistry

For immunohistochemical analyses, testes were removed and immersion-fixed in 4% PFA in 0.1 M PB. Following fixation, testes were dehydrated in ethanol and embedded in paraffin. Sections were then deparaffinized and hydrated. Antigen retrieval was performed for 15 min at 105 °C with Target Retrieval Solution (DakoCytomation) for OCLN and CLDN11 or with buffered citrate (pH 6.0) for the following proteins: STRA8, DMC1, and synaptonemal complex protein 3 (SCP3, also known as SYCP3). Samples were treated with methanol containing 0.3% H₂O₂ to eliminate endogenous peroxidase. After blocking with normal serum, sections were incubated with rabbit anti-OCLN (1:100; Life Technologies), rabbit anti-CLDN11 (1:100; Life Technologies), rabbit anti-STRA8 (1:2000; Abcam), goat anti-DMC1 (1:500; Santa Cruz Biotechnology), rabbit anti-SCP3 (1:800; Novus Biologicals, Littleton, USA), or rabbit anti-single stranded DNA (ssDNA; 1:200; IBL, Fujioka, Japan) at 4 °C overnight. Next, the sections were treated with biotin-conjugated goat anti-rabbit IgG antibodies (SABPO kit, Nichirei) or with biotin-conjugated donkey anti-goat IgG antibodies (1:100; Santa Cruz Biotechnology) for 30 min at room temperature, followed by treatment with streptavidin-biotin complex (SABPO kit) for 30 min at room temperature. The sections were incubated with DAB solution containing 0.006% H₂O₂ until the stain developed and were then counterstained

with hematoxylin. For histometric analyses, BZ-9000 (Keyence) was used for acquisition of digital images of each IHC section, and the immunopositive cells were counted with the BZ-II Analyzer software (Keyence).

Histopathology

Paraffin sections were prepared from testes and epididymides fixed in 4% PFA fixed as described above for PAS-H or von Kossa staining. For von Kossa staining, sections were incubated with 5% silver nitrate solution under sunlight for 60 min. The slides were then washed in water, differentiated with 5% sodium thiosulfate pentahydrate for 3 min, rinsed, and counterstained for 5 min with nuclear fast red.

RT-PCR and QPCR

Total RNA was extracted from whole testes harvested from mice fed the VAD diet at 5–90 dpp and at 16–44 days after RA replacement using TRIzol reagent (Life Technologies). Purified total RNA was treated with Turbo DNase (Life Technologies) for DNA digestion, and cDNAs were synthesized via RT reactions using ReverTra Ace (Toyobo) and oligo-dT primers (Life Technologies). QPCR analysis subsequent to RT was performed using the prepared cDNA, gene-specific primers (Table 3-1),

Brilliant III Ultra Fast SYBR Green QPCR Master Mix (Agilent Technologies) and a real-time thermal cycler (MX 3000P; Agilent Technologies). Levels of mRNA expression of the target genes were normalized to those of *Actb*.

Statistical analyses

Results are expressed as the means $\pm$ SEM, and statistical analyses were performed using PASW Statistics for Windows, Version 18.0 (IBM SPSS, Chicago, USA). The Mann-Whitney *U* test was used for comparison between 2 groups. One-way analysis of variance (ANOVA) followed by Dunnett's test was used to compare changes between treatment groups and corresponding untreated groups. *P*-values less than 0.05 were considered statistically significant.

Results

Changes in the weight of the testes in mice fed the VAD diet

The changes in the weight of testes collected from mice fed the VAD diet and control mice fed the VAS diet are shown in Fig. 3-2A. From 5–50 dpp, the weight of testes of the VAD diet group increased similarly to that of the control group, indicating that prepubertal testis development in mice fed the VAD diet proceeded normally until approximately 50 dpp. However, the weight of the testes collected from mice fed the VAD diet gradually decreased from 60 dpp onwards. At 70–90 dpp, just before RA administration, the weight of the testes collected from mice fed the VAD diet decreased significantly compared with that of control mice.

Effects of VAD diet feeding on RA signaling in the mouse testes

To assess the functional abnormalities of RA signaling in mice fed the VAD diet, mRNA levels of RA signaling molecules were analyzed in the testes of prepubertal mice (0–20 dpp) and sexually mature mice (90 dpp). Aldehyde dehydrogenase family 1, subfamily A2 (*Aldh1a2*) and cytochrome P450 family 26, subfamily a, polypeptide 1 (*Cyp26a1*) were examined as RA signaling molecules (Fig. 3-2B and C). *Aldh1a2* mRNA levels were lower in mice fed the VAD diet than in control mice, and statistically significant differences were detected at 15 and 90 dpp (Fig. 3-2B). Furthermore,

significant decreases in *Cyp26a1* mRNA expression in the testes of mice fed the VAD diet were detected at 5, 10, and 90 dpp, with the most dramatic decrease observed at 90 dpp (Fig. 3-2C).

Histological abnormalities of the testes and epididymides in mice fed the VAD diet

Although the seminiferous epithelium showed no significant histological differences between control mice and mice fed the VAD diet during the prepubertal period (at 10 dpp as shown in Fig. 3-3A and B), the seminiferous epithelium of mice fed the VAD diet showed severe histological abnormalities characterized by epithelial vacuolization observed from 70 dpp (Fig. 3-3D; arrows). This vacuolization was barely detectable in testes from control mice throughout the observation period (Fig. 3-3C). By 80 dpp, progressive atrophy with marked depletion of germ cells was observed in all seminiferous tubules of mice fed the VAD diet (Fig. 3-3E). Moreover, from 80 dpp onwards, von Kossa staining revealed that dystrophic calcification occurred in the severely degenerated tubules of mice fed the VAD diet (Fig. 3-3F). Intratubular calcification was observed in $6.78\% \pm 3.62\%$ of seminiferous tubules of mice fed the VAD diet ($n = 3$) at 80 dpp, and this increased to $19.34\% \pm 3.60\%$ ($n = 4$) at 90 dpp. In the epididymides of mice fed the VAD diet, no significant histological changes were observed, and the epididymides contained spermatozoa, as expected, until 70 dpp (Fig. 3-3G). However, severe squamous metaplasia was frequently observed in the corpus epididymis from

80 dpp onwards (Fig. 3-3H).

Induction of germ cell apoptosis in the testes of mice fed the VAD diet

To elucidate whether germ cell apoptosis was induced concurrently with testicular degeneration in mice fed the VAD diet, immunohistochemical analysis for ssDNA, an apoptotic cell marker, was performed (Fig. 3-4). In prepubertal testes (5–20 dpp), a number of ssDNA-positive cells were observed in both control mice and mice fed the VAD diet (Fig. 3-4A). From 50–90 dpp, testes from control mice continually showed few ssDNA-positive cells in the seminiferous tubules (Fig. 3-4A and B). In contrast, testes from mice fed the VAD diet contained numerous ssDNA-positive cells from 50–70 dpp, especially in the luminal parts of seminiferous tubules. These apoptotic cells seemed to be mainly pachytene spermatocytes and round spermatids, reflecting the apoptotic induction in germ cells (Fig. 3-4C). In a histometric analysis, mice fed the VAD diet showed a higher number of ssDNA-positive cells as compared with control mice from 50–70 dpp, and a statistically significant difference was observed at 60 dpp (Fig. 3-4A).

Effects of the VAD diet on the progression of meiosis

To elucidate the effects of vitamin A insufficiency on the seminiferous epithelium, the

progression of meiosis was examined from the prepubertal period to adulthood (Fig. 3-5). For immunohistochemical analysis, STRA8, DMC1 and SYCP3 were used as markers of preleptotene and early leptotene spermatocytes, leptotene and zygotene spermatocytes, and leptotene to metaphase I spermatocytes, respectively (Fig. 3-1A) (Mark et al., 2008; Zhou et al., 2008; Hermo et al., 2010; Hamer et al., 2006; La Salle et al., 2008; Oka et al., 2010). Fig. 3-5A–C show the time course of immunopositive cell numbers in mouse testes. STRA8-positive cells were detected from 10 dpp in the testes of mice fed the VAD diet and control mice (Fig. 3-5A). However, the number of STRA8-positive cells in mice fed the VAD diet tended to be lower than that in control mice throughout the observation period (Fig. 3-5A and D–G), and significant differences were observed at 15, 70, 80, and 90 dpp (Fig. 3-5A). However, DMC1- and SYCP3-positive cells were detected at similar levels from 15–50 dpp in the testes of mice fed the VAD diet and control mice (Fig. 3-5B and C, Fig. 3-5H vs. 3-5I and Fig. 3-5L vs. 3-5M). The numbers of DMC1- or SYCP3-positive cells were significantly decreased in mice fed the VAD diet from 70 and 60 dpp onwards, respectively (Fig. 3-5B and C, Fig. 3-5J vs. K and Fig. 3-5N vs. O). After 80 dpp, it was difficult to detect immunopositive spermatocytes for all examined markers in the testes of mice fed the VAD diet, reflecting spermatogenic arrest (Fig. 3-5A–C).

Assembly and disruption of the BTB in the testes of mice fed the VAD diet

To assess the effects of the VAD diet on BTB assembly, the mRNA expression of BTB component TJ genes, including *Ocln*, *Cldn3*, *Cldn11*, and *Tjp1* (encoding ZO1), was evaluated in the testes in the prepubertal period (5–20 dpp) and at a sexually mature age (90 dpp; Fig. 3-6). Consistent with previous reports (Russell, 1977; Hasegawa and Saga, 2012; Moroi et al., 1998), mRNA expression of the above genes in control mice tended to increase during the prepubertal period (5–20 dpp) and then decreased by sexual maturation (90 dpp; Fig. 3-6A–D). In mice fed the VAD diet, the mRNA expression of these genes tended to be lower than that in control mice from 5–20 dpp (Fig. 3-6A–D). Significant differences in the expression of *Ocln* mRNA at 5 dpp, *Cldn11* mRNA at 15 dpp and *Tjp1* mRNA at 5 and 15 dpp were observed between mice fed the VAD diet and control mice (Fig. 3-6A, C and D). At 90 dpp, the mRNA expression levels of *Ocln* and *Cldn11* were significantly higher in mice fed the VAD diet than in control mice (Fig. 3-6A and C). No significant changes were observed in *Cldn3* expression between mice fed the VAD diet and control mice throughout the observation period (Fig. 3-6B).

To evaluate BTB integrity, OCLN and CLDN11 localization was compared between testes from control mice and mice fed the VAD diet from the prepubertal period to adulthood (Fig. 3-6E–N). Consistent with a previous study (Hellani et al., 2000), OCLN was weakly and diffusely distributed

from the apical to basal regions of Sertoli cells in control mice at 10 dpp (Fig. 3-6E) and began to accumulate in the basal part of the seminiferous tubules from 15 dpp (Fig. 3-6F). In mice fed the VAD diet, OCLN showed a weak and diffuse pattern in seminiferous tubules, even at 15 dpp (Fig. 3-6J), whereas OCLN seemed to localize to the basal portion of the seminiferous tubules by 20 dpp (data not shown). During 10–20 dpp, a similar localization pattern to OCLN was observed in CLDN11 (data not shown). In control mice at 60 dpp, OCLN localized to the basal side of leptotene or zygotene spermatocytes in stage X tubules (Fig. 3-6G, arrows), indicating normal migration of spermatocytes across the BTB. However, in stage X tubules of mice fed the VAD diet, OCLN localized to the luminal side as well as the basal side of leptotene or zygotene spermatocytes at 60 dpp (Fig. 3-6K, arrowheads). In mice fed the VAD diet, OCLN positivity disappeared with severe epithelial vacuolization at the basal regions of tubules at 70 dpp (Fig. 3-6L), indicating BTB disruption. Furthermore, in testes from mice fed the VAD diet at 90 dpp, Sertoli cell detachment from the basement membrane was observed in $25.58\% \pm 3.06\%$ tubules ($n = 3$), and OCLN- and CLDN11-positive staining was diffusely observed in abnormal tubules at 90 dpp (Fig. 3-6H vs. M, Fig. 3-6I vs. N).

Re-initiation of spermatogenesis and reassembly of the BTB after RA replenishment in mice fed the

VAD diet

As shown in Fig. 3-7, re-initiation of spermatogenesis was evaluated with the assembly of the BTB after RA administration and dietary vitamin A replenishment in adult mice fed the VAD diet (see also Fig. 3-1B). From 91 dpp, day 0 after RA replenishment, weight of the testes in mice fed the VAD diet gradually increased throughout the observation period (Fig. 3-7A). After RA replenishment, the number of seminiferous tubules containing the germ cells increased with the increase of the diameter (Fig. 3-7B vs. C). Furthermore, at day 35 after RA replenishment, spermatogenesis progressed to step 16 spermatids, germ cells just prior to spermiation (Fig. 3-7C; arrows). These observations reflected the re-initiation of spermatogenesis after RA replenishment, which is consistent with a previous report (van Pelt et al., 1995). Despite the re-initiation of spermatogenesis, intratubular calcification was detected, even after RA replenishment, throughout the observation period (Fig. 3-7B vs. C), and the incidence rate of von Kossa-positive seminiferous tubules did not change significantly before ($19.34\% \pm 3.60\%$ at 90 dpp, $n = 4$) or after RA replenishment ($14.33\% \pm 1.59\%$ at day 34–36, $n = 12$). The levels of mRNA expression of *Ocln* and *Cldn11* were significantly decreased in a time-dependent manner after RA replenishment (Fig. 3-7D and F), but no significant changes were observed in the levels of *Cldn3* and *Tjp1* mRNAs (Fig. 3-7E and G). In immunohistochemical analysis, although OCLN and CLDN11 positivity was unclear and scattered in the seminiferous epithelium until day 9

after RA replenishment (Fig. 3-7 H, I, L and M), OCLN and CLDN11 localized to the proper basal portions of the seminiferous tubules with the emergence of pachytene and preleptotene/leptotene spermatocytes (Fig. 3-7J and N; arrows and arrowheads, respectively) from approximately day 16 after RA replenishment. Subsequently, stage-specific changes in OCLN and CLDN11 localization were typically observed in RA-replenished testes (Fig. 3-7K and O).

Discussion

Efficiency of the VAD diet feeding strategy

In the male mouse, meiosis is initiated after birth and continues throughout the reproductive period. Increasing evidence has indicated that vitamin A is required for the maintenance of spermatogenesis (Hogarth and Griswold, 2010). During prepubertal testis development, neonates receive vitamin A from the maternal milk. Vitamin A is stored primarily in the liver and, to a lesser extent, in other tissues. To induce vitamin A insufficiency in the prepubertal period itself, the author fed parental mice the VAD diet from at least 4 weeks prior to mating (Fig. 3-1B) as previously described (van Pelt AM and de Rooij, 1990). Mice fed the VAD diet showed reduced expression of RA signaling molecular transcripts (i.e., *Aldh1a2* and *Cyp26a1*) compared with those in control mice fed the VAS diet during the prepubertal period (5–20 dpp). Furthermore, dramatic decreases in the expression levels of these genes were observed at adulthood (90 dpp) in mice fed the VAD diet. RA synthesis from vitamin A is typically controlled by tissue-specific activity of retinaldehyde dehydrogenase (RALDH). In the testes, *Aldh1a2* (encoding RALDH2) is expressed in Sertoli and germ cells (Sugimoto et al., 2011). RA is then oxidized into its inactive forms by CYP26, and *Cyp26a1* is expressed by Sertoli cells and transcribed by the activation of RA signaling (Sugimoto et al., 2011). Taken together, these findings suggest that functional abnormality of RA signaling is

induced beginning in the prepubertal period and that the effects of VAD were critical at 90 dpp in the present VAD diet feeding strategy.

Seminiferous epithelial disruption with germ cell apoptosis in testes of mice fed the VAD diet

In mice fed the VAD diet, histological changes characterized by vacuolization of the seminiferous epithelium were observed from 70 dpp onwards. This observation corresponded to a significant reduction in weight of the testes in mice fed the VAD diet. Vacuolization of the seminiferous epithelium is a well-known response of Sertoli cells to various kinds of damages, and often occurs before extensive germ cell degeneration (Carette et al., 2010; Eid et al., 2012; Kyrölähti et al., 2011; Li et al., 2011; Russell et al., 1990). RA has been suggested to mainly exert its effects via the action of RARs in Sertoli cells and germ cells (Hogarth and Griswold, 2010); therefore, the author speculates that the VAD condition would directly cause vacuolization in the seminiferous epithelium. However, no vacuolization was observed during the prepubertal period in the testes of mice fed the VAD diet. Furthermore, massive apoptotic induction of germ cells was observed from 60 dpp onwards, and spermatogenesis was almost completely halted at the spermatogonial stages by 80 dpp. This germ cell death would likely contribute to the dramatic decrease in weight of the testes in adult mice fed the VAD diet. Although the efficiency of VAD may cause differences between the prepubertal period and

adulthood, these results reflected differences in the effects of RA during testicular development.

Unexpectedly, the author noted calcification in severely degenerated seminiferous tubules of mice fed the VAD diet from 80 dpp onwards. In clinical reports in humans, testicular calcification has been detected in association with several pathological conditions, including infertility, cryptorchidism, testicular neoplasms, Klinefelter's syndrome, varicocele, and torsion of the testis (Dagash and Mackinnon, 2007; Ganem et al., 1999; Kim et al., 2003; Miller et al., 2007), and in normal testes (von Eckardstein et al., 2001). However, the etiology of testicular calcification remains to be determined. Recently, intratesticular calcification has been reported to occur in an age-dependent manner in mutant mice showing Sertoli cell dysfunction with progressive germ cell degeneration (Kyrölähti et al., 2011; Boyer et al., 2008; O'Shaughnessy et al., 2009). In the present study, adult mice fed the VAD diet showed Sertoli cell detachment from the basement membrane of seminiferous tubules. Therefore, these data suggested that the VAD condition induced abnormal polarity and dysfunction of Sertoli cells. Intratubular calcification was detectable even after RA replenishment, indicating that the resumption of spermatogenesis could no longer occur in specific parts of the tubules. Indeed, in the rat testes, prolonged deprivation of vitamin A beyond 10–11 weeks has been shown to result in permanent failure of spermatogenesis, despite RA replenishment (Ismail et al., 1990). Taken together, these data suggest that the onset of calcification in the seminiferous epithelium could reflect the irreversible

damage caused by long-term vitamin A deprivation.

Delayed spermatogenesis and BTB assembly in testes from prepubertal mice fed the VAD diet

At 10 dpp, STRA8-positive (preleptotene or early-leptotene) spermatocytes derived from the first wave were detected at similar levels in testes from control mice and mice fed the VAD diet. However, in mice fed the VAD diet, the number of STRA8-positive cells tended to be lower than that in control mice from 15 dpp onwards, and a significant difference was observed at 15 dpp. *Stra8* is an RA-stimulated gene controlling the transition from the preleptotene to leptotene spermatocyte (Anderson et al., 2008). Therefore, the functional abnormality of RA signaling in mice fed the VAD diet might affect the STRA8 expression in some of the preleptotene or leptotene spermatocytes. However, the author confirmed that mice fed the VAD diet showed STRA8 expression in all of the preleptotene and early leptotene spermatocytes distinguishable by the typical chromatin pattern of these cells using immunofluorescence analysis (data not shown). As shown in Fig. 3-1A, normal murine seminiferous tubules at 15 dpp contained pachytene spermatocytes accompanying preleptotene/early-leptotene spermatocytes derived from the first and second waves, respectively (Goetz et al., 1984; Chung et al., 2004). Therefore, the reduced number of STRA8-positive cells from 15 dpp may reflect suppressed meiosis initiation or onset of spermatogonial differentiation from the

second wave onwards. Despite the difference in the number of STRA8-positive cells, mice fed the VAD diet showed no differences in the numbers of DMC1-positive cells (leptotene and zygotene spermatocytes) or SYCP3-positive cells (leptotene to metaphase I spermatocytes) up to 50 dpp. Furthermore, up to 70 dpp, no abnormal changes were observed in the epididymides of mice fed the VAD diet, and these epididymides contained spermatozoa, as under normal conditions. Consistent with our results, *RAR α* -deficient mice, which exhibit progressive breakdown of the spermatogenic process, showed a normal number of pachytene spermatocytes at 4 weeks of age regardless of delay in the onset of the second wave (Chung et al., 2004). Therefore, the imbalance between the numbers of STRA8-, DMC1-, and SYCP3-positive cells in mice fed the VAD diet up to 50 dpp might reflect the abnormal progression of spermatogenesis. Importantly, mRNA expression levels of BTB components, including *Ocln*, *Cldn11* and *Tjp1*, were significantly reduced in mice fed the VAD diet compared with those in control mice at 5–15 dpp. Furthermore, although the BTB was formed by 15 dpp in normal mice, as previously described (Willems et al., 2010; Moroi et al., 1998), the initial assembly of the BTB, estimated by OCLN and CLDN11 positivity in the basal portion of seminiferous tubules, was barely detectable at 15 dpp and delayed until 20 dpp in testes from mice fed the VAD diet. Because OCLN and CLDN11 are principal components of the BTB in mouse testes (Pelletier, 2011), loss of proper localization of these TJ proteins indicates defective BTB fence function. These findings suggest

that the VAD condition in prepubertal mice delayed both the appearance of STRA8-positive spermatocytes (preleptotene or early-leptotene) from the second wave and the initial formation of the BTB, although meiosis of the first wave and subsequent spermatogenesis from zygotene spermatocytes proceeded until 50 dpp.

After re-initiation of spermatogenesis by RA replenishment in spermatogenic-arrested testes of mice fed the VAD diet, it took 35 days for spermatogonia to differentiate into step 16 spermatids. Furthermore, at day 16 after RA replenishment, almost all tubules, except for calcified tubules, contained germ cells that had progressed to pachytene and preleptotene/leptotene spermatocytes. Because the differentiation from A1 spermatogonia to spermatozoa requires approximately 35 days during normal spermatogenesis (Hogarth and Griswold, 2010), spermatogenesis in mice fed the VAD diet proceeded normally soon after RA replenishment. Although *Ocln* expression in the testis was reported to be activated by RA signaling (Hasegawa and Saga, 2012), RA did not immediately rescue OCLN and CLDN11 localization to the basal portions of seminiferous tubules in mice fed the VAD diet until day 16 after RA replenishment. Interestingly, at day 16, almost all tubules contained pachytene and preleptotene/leptotene spermatocytes. As mentioned above, at 15 dpp, the majority of seminiferous tubules contained pachytene spermatocytes from the first wave together with preleptotene/leptotene spermatocytes from the second wave (Goetz et al., 1984; Chung et al., 2004). In

the seminiferous epithelium, Sertoli and germ cells coordinately establish an intimate and elaborate cellular network for cell-cell communications and regulate each other's functions via bidirectional trafficking (Cheng and Mruk, 2002). Taken together, these findings and the author's results from the VAD diet study strongly suggest that the presence of spermatocyte sets derived from both of the first and second wave, especially pachytene spermatocytes from the first wave and preleptotene/leptotene spermatocytes from the second wave, are required for the initial establishment of the BTB. Furthermore, RA signals may play a crucial role in regulating these processes during the prepubertal period.

Failure of spermatogenesis and disrupted BTB integrity in mice fed the VAD diet

In mice fed the VAD diet, a dramatic induction of germ cell apoptosis occurred from 60 dpp onwards, and spermatogenesis was almost completely halted at the spermatogonial stages by 80 dpp. The mice fed the VAD diet at 60 dpp showed aberrant OCLN localization to the luminal side of leptotene or zygotene spermatocytes in stage X tubules, indicating disruption of BTB integrity. Recently, stage-specific expression of OCLN was found to be regulated by RA signaling, and knockdown of *Ocln* was shown to induce apoptosis in germ cells in stage IX–XII tubules (Hasegawa and Saga, 2012). On the basis of these findings, the VAD diet was expected to induce apoptosis in

germ cells in adulthood by altering the stage-specific localization of TJ proteins. Interestingly, zygotene or pachytene spermatocytes are thought to mainly be affected by incomplete BTB assembly (Morales et al., 2007). Indeed, in testes from mice fed the VAD diet, although the number of SYCP3-positive cells (leptotene to metaphase I spermatocytes) decreased from 60 dpp onwards, STRA8- (preleptotene and early-leptotene spermatocytes) or DMC1-positive cells (leptotene and zygotene spermatocytes) decreased from 70 dpp onwards. These results indicated that germ cell apoptosis was primarily induced from the pachytene spermatocyte stage onwards, consistent with the observations in Fig. 3-4 and subsequently during the former stages of spermatocytes due to BTB disruption resulting from vitamin A deprivation.

In mice fed the VAD diet, mRNA expression levels of *Ocln* and *Cldn11* significantly increased at 90 dpp and gradually decreased after re-initiation of spermatogenesis by RA replenishment. These changes may be attributable simply to alterations in testicular cellularity (i.e., the ratio of somatic cells to germ cells). However, because postmeiotic germ cells, especially spermatids, have been reported to inhibit CLDN11 expression in rat testes (Florin et al., 2005), higher expression of TJ genes at 90 dpp in mice fed the VAD diet may also reflect the loss of postmeiotic germ cells.

Despite the delayed and incomplete BTB formation, spermatogenesis in mice fed the VAD diet successfully proceeded up to 50 dpp but was arrested at a later period. The delayed and incomplete

BTB formation and age-dependent testicular atrophy observed in the present study were consistent with results reported in mice with selective ablation of the androgen receptor in Sertoli cells (Willems et al., 2010; O'Shaughnessy et al., 2009; De Gendt et al., 2004). These findings suggested that functional correlations between RA and sex hormones and an altered balance of these functions are critical for inducing seminiferous epithelial damage in adulthood. It is noteworthy that, similar to results in this chapter, *Ocln*-null mice showed typical testicular development with a normal set of germ cells at 6 weeks of age and later developed testicular atrophy and became sterile (Saitou et al., 2000). Furthermore, spermatogenesis of *Cldn11*-null mice progressed to round spermatids at 28 dpp (Mazaud-Guittot et al., 2010), after which the mice became sterile due to BTB disruption (Gow et al., 1999). Interestingly, in a seasonal breeder, the mink, the onset of spermatogenesis is not dependent on formation of the impermeable BTB (Pelletier, 1986). Therefore, these reports and the author's VAD study from the prepubertal period to adulthood indicate that assembly of the BTB may not be essential for the initiation and progression of germ cell differentiation in the prepubertal period but may be required for the maintenance of spermatogenesis from the prepubertal period to adulthood.

Summary

The BTB is essential for maintaining homeostasis in the seminiferous epithelium. Although many studies have reported that vitamin A is required for the maintenance of spermatogenesis, the relationships between the BTB, spermatogenesis and vitamin A have not been elucidated. In this chapter, the author analyzed BTB assembly and spermatogenesis in the testes of mice fed the VAD diet from the prepubertal period to adulthood. During the prepubertal period, no changes were observed in the initiation and progression of the first spermatogenic wave in mice fed the VAD diet. However, the numbers of preleptotene/leptotene spermatocytes derived from the second spermatogenic wave onwards were decreased, and initial BTB formation was also delayed, as evidenced by the decreased expression of mRNAs encoding BTB components and VA signaling molecules. From 60 days postpartum, mice fed the VAD diet exhibited apoptosis of germ cells, arrest of meiosis, disruption of the BTB, and dramatically decreased testis size. Furthermore, vacuolization and calcification were observed in the seminiferous epithelium of adult mice fed the VAD diet. Re-initiation of spermatogenesis by VA replenishment in adult mice fed the VAD diet rescued BTB assembly after when the second spermatogenic wave initiated from the arrested spermatogonia reached the preleptotene/leptotene spermatocytes. These results suggested that BTB integrity was regulated by VA metabolism with meiotic progression and that the impermeable BTB was required for persistent

spermatogenesis rather than meiotic initiation. In conclusion, consumption of the VAD diet led to critical defects in spermatogenesis progression and altered the dynamics of BTB assembly.

Table and figures

Table 3-1. Primer pairs used in this chapter.

Gene name (Accession)	Symbol	Primer sequence (5'-3') F: Forward, R: Reverse	Product size (bp)
aldehyde dehydrogenase family 1, subfamily A2 (NM_009022)	<i>Aldh1a2</i>	F: TCTGTTGGACAAGCTTGCAG R: CCAGCCTGCATAATACCTCAG	148
cytochrome P450, family 26, subfamily a, polypeptide 1 (NM_007811)	<i>Cyp26a1</i>	F: ATTGAGCACTCGTGGGAGAG R: CTTCTCGAACTTTCTGGAGGAC	163
occludin (NM_008756)	<i>Ocln</i>	F: CCTACTCCTCCAATGGCAAA R: CTCTTGCCCTTTCTGCTTT	208
claudin 3 (NM_009902)	<i>Cldn3</i>	F: GCACCCACCAAGATCCTCTA R: TCGTCTGTCACCATCTGGAA	206
claudin 11 (NM_008770)	<i>Cldn11</i>	F: TGGTGGACATCCTCATCCTT R: GCCAGCAGAATAAGGAGCAC	190
tight junction protein 1 (ZO1) (NM_009386)	<i>Tjp1</i>	F: GCACCATGCCTAAAGCTGTC R: ACTCAACACACCACCATTGC	122
actin, beta (NM_007393)	<i>Actb</i>	F: ACTGCTCTGGCTCCTAGCAC R: CAGCTCAGTAACAGTCCGCC	196

ZO1, zonula occludens 1.

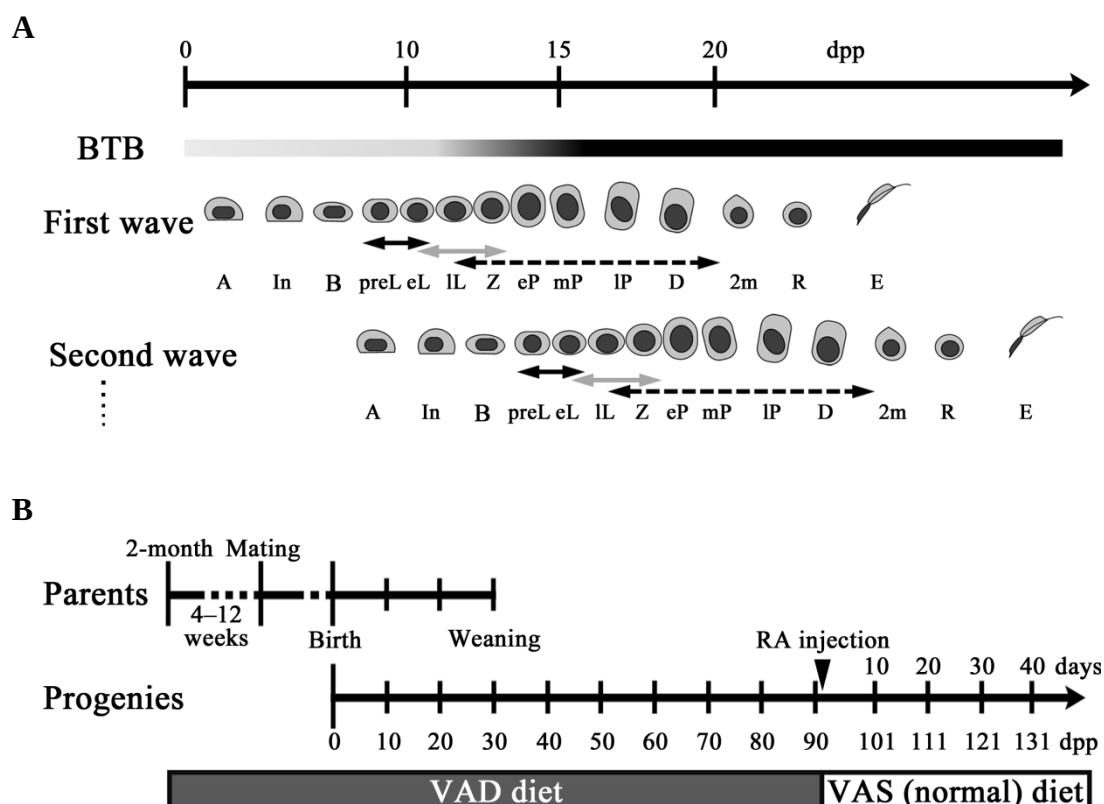


Figure 3-1. Illustration of spermatogenic progression and BTB assembly during the prepubertal period and VAD diet feeding schedule.

A) Time-course progression of spermatogenic waves with BTB assembly in prepubertal mice. The degree of BTB assembly and impermeability are indicated by the darkness of the shading at each time point. A, type A spermatogonia; In, intermediate spermatogonia; B, type B spermatogonia; preL, preleptotene spermatocytes; eL, early leptotene spermatocytes; IL, late leptotene spermatocytes; eP, early pachytene spermatocytes; mP, mid pachytene spermatocytes; lP, late pachytene spermatocytes; Z, zygotene spermatocytes; D, diplotene spermatocytes; 2m, secondary spermatocytes; R, round spermatids; E, elongating spermatids. Solid black arrows, gray arrows, and black dashed arrows represent STRA8-, DMC1-, and SYCP3-positive spermatocytes, respectively. **B)** VAD diet feeding schedule used in the present study. Parental mice were fed the VAD diet from 4–12 weeks prior to mating, and male pups received the same diet until 90 dpp. At 91 dpp, mice fed the VAD diet were injected with RA and were then placed on a normal VAS diet.

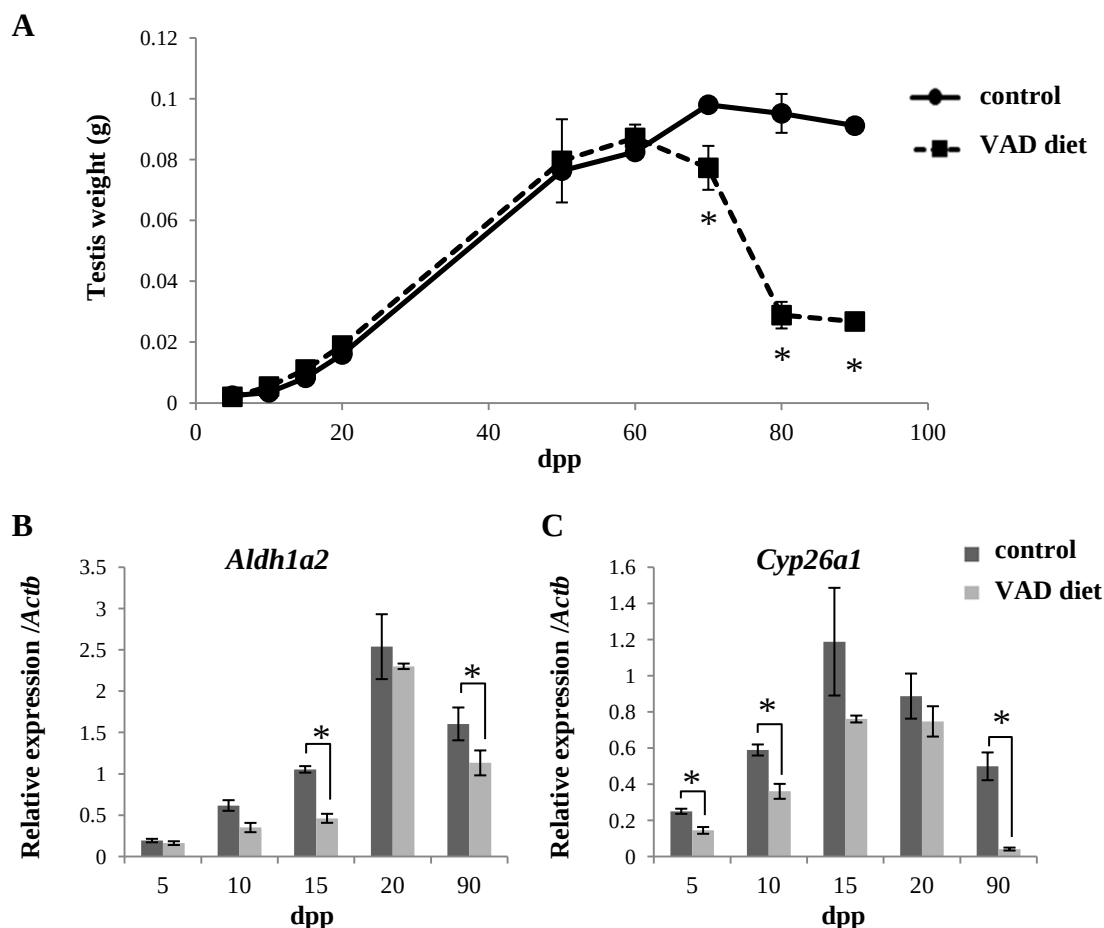


Figure 3-2. Testes weight and the expression of RA signaling molecule transcripts in the testes of mice fed the VAD diet.

A) Changes in the testes weight of mice fed the VAD diet and control mice fed the VAS diet. Values are presented as means $\pm$ SEM. * $P < 0.05$, Mann-Whitney U test. **B–C)** QPCR analyses of *Aldh1a2* (**B**) and *Cyp26a1* mRNAs (**C**) during the prepubertal period (5–20 dpp) and adulthood (90 dpp) in mice fed the VAD diet and control mice fed the VAS diet. Values are presented as means $\pm$ SEM. * $P < 0.05$, Mann-Whitney U test.

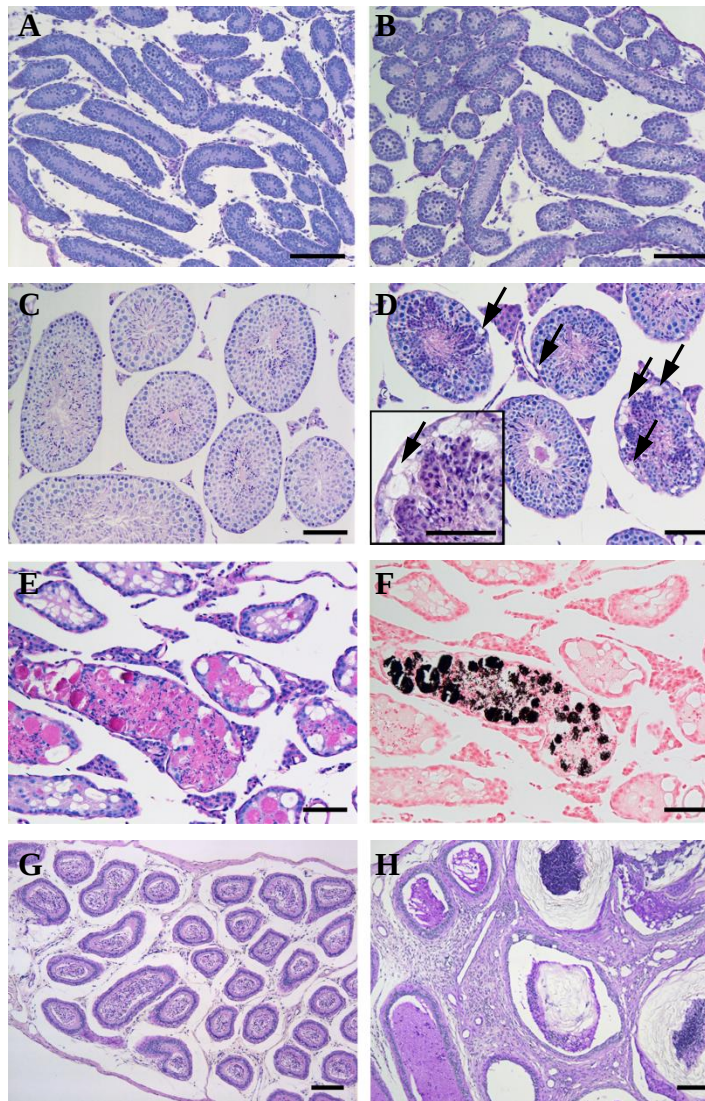


Figure 3-3. Histopathological abnormalities in the seminiferous epithelium and epididymides of mice fed the VAD diet.

A–F) Histological features of seminiferous tubules in mice fed the control VAS diet and those fed the VAD diet. Panels **A–E** and **F** show PAS-H and von Kossa staining, respectively. At 10 dpp, no differences were observed between the testes of control mice (**A**) and mice fed the VAD diet (**B**). In testes from control mice at 90 dpp, no histopathological abnormalities were detected (**C**). Some tubules in the testes of mice fed the VAD diet showed epithelial vacuolization at 70 dpp (**D**; arrows). The insert box in panel **D** highlights the epithelial vacuolization at the basal regions of a tubule (arrow). Progressive atrophy of all tubules with marked depletion of germ cells was observed at 80 dpp in testes from mice fed the VAD diet (**E**). In serial sections of panel **E**, von Kossa-positive calcification was detected in severely degenerated tubules (**F**). **G–H)** PAS-H-stained epididymides of mice fed the VAD diet. No significant abnormalities were detected at 70 dpp, and spermatozoa were observed in the lumen of ducts (**G**). Severe squamous metaplasia in the corpus epididymis was observed at 80 dpp (**H**). Bars = 100 μ m.

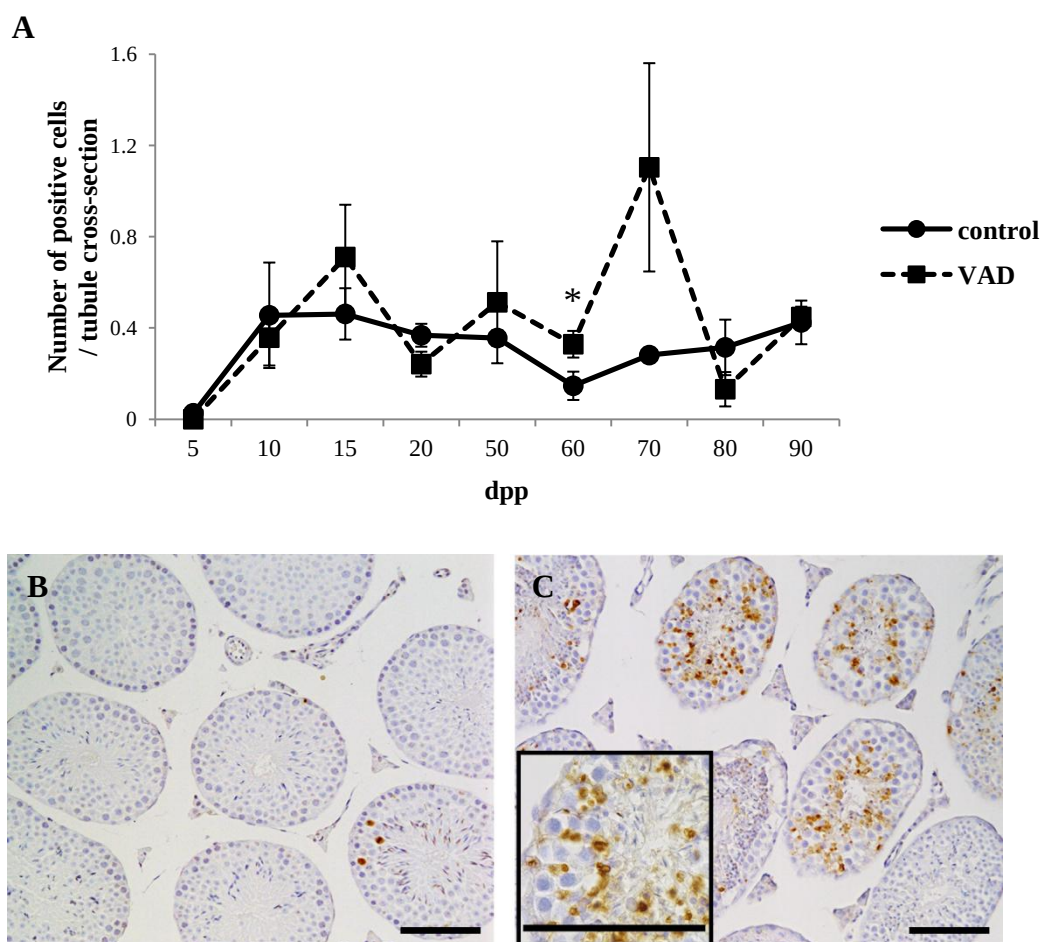


Figure 3-4. Germ cell apoptosis in the testes of mice fed the VAD diet.

A) Total numbers of ssDNA-positive cells in the testes of control mice fed the VAS diet and mice fed the VAD diet. ssDNA-positive cells were counted in 200–500 tubules per testis of control mice and mice fed the VAD diet from 5–90 dpp using immunohistochemical sections. Values are presented as means $\pm$ SEM. * $P < 0.05$, Mann-Whitney U test. **B–C)** Appearance of ssDNA-positive cells in the seminiferous epithelium. ssDNA-positive cells were only infrequently detected in testes from control mice at 70 dpp (**B**). Testes from mice fed the VAD diet contained numerous ssDNA-positive cells at 70 dpp (**C**). The insert box in panel **C** highlights the ssDNA-positive cells in the luminal area of the seminiferous tubules. Bars = 100 μ m.

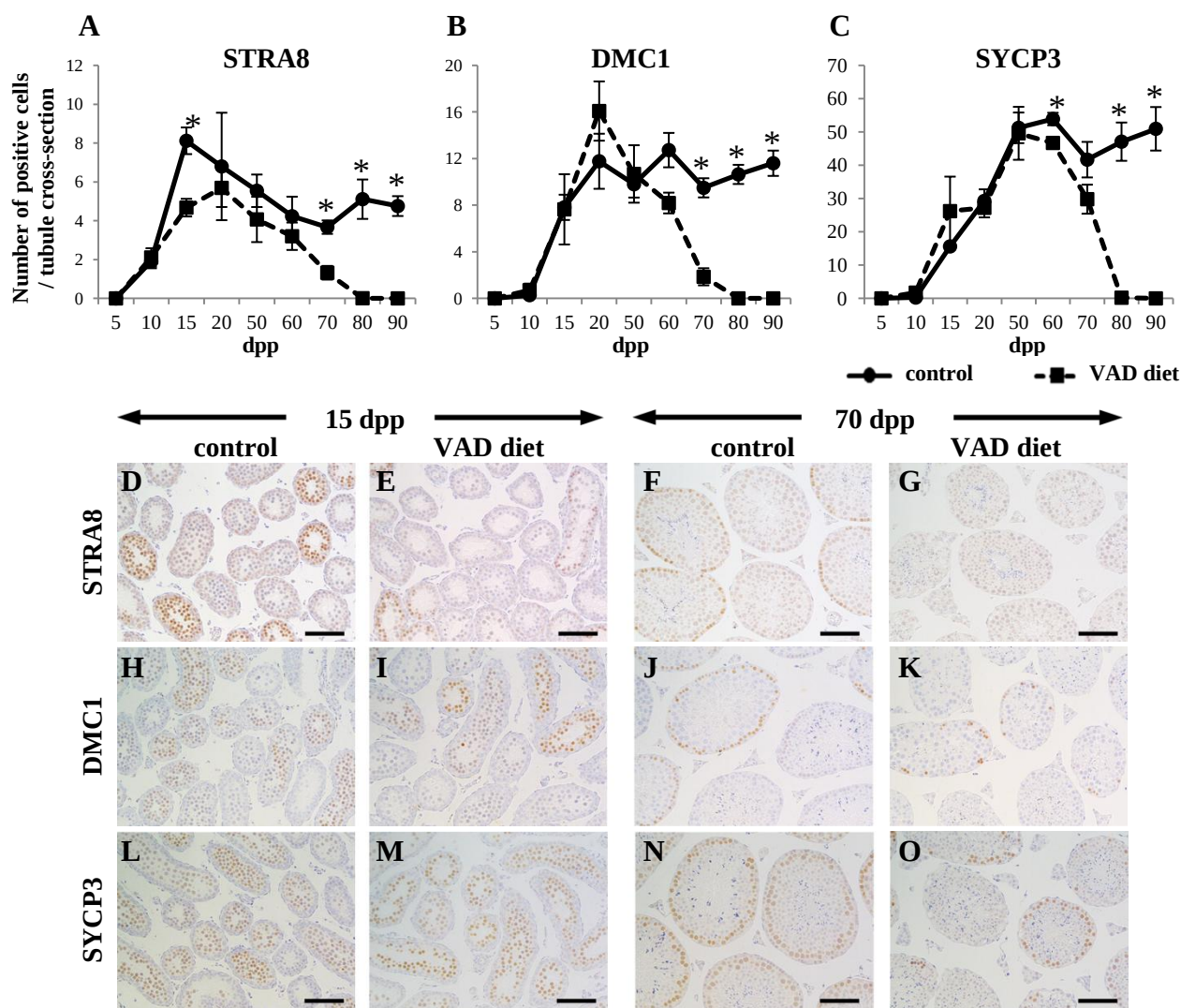


Figure 3-5. Meiotic progression in the testes of mice fed the VAD diet.

A–C) The numbers of STRA8-positive cells (**A**), DMC1-positive cells (**B**) and SYCP3-positive cells (**C**) in the testes of control mice fed the VAS diet and mice fed the VAD diet. Positive cells were counted in 200–500 tubules per testis. Values are presented as means $\pm$ SEM. * $P < 0.05$, Mann-Whitney U test. **D–G)** The appearance of STRA8-positive cells in the seminiferous tubules. Cells positive for STRA8, a marker for preleptotene and early leptotene spermatocytes, were observed at 15 (**D**) and 70 dpp (**F**) in control testes. In testes from mice fed the VAD diet, although STRA8-positive cells were observed at 15 dpp (**E**), they were barely detectable at 70 dpp (**G**). **H–K)** The appearance of DMC1-positive cells in the seminiferous tubules. Cells positive for DMC1, a marker for leptotene and zygotene spermatocytes, were observed at similar levels at 15 dpp in control testes (**H**) and testes from mice fed the VAD diet (**I**). In contrast, at 70 dpp, DMC1-positive cells were decreased in mice fed the VAD diet (**K**) compared with control mice (**J**). **L–O)** The appearance of SYCP3-positive cells in the seminiferous tubules. Cells positive for SYCP3, a marker for leptotene to metaphase I spermatocytes, were detected in almost all tubules in both the control mice (**L**) and mice fed the VAD diet (**M**) at 15 dpp and in control mice only at 70 dpp (**N**). SYCP3-positive cells were not detectable in some tubules of testes from mice fed the VAD diet at 70 dpp (**O**). Bars = 50 μ m.

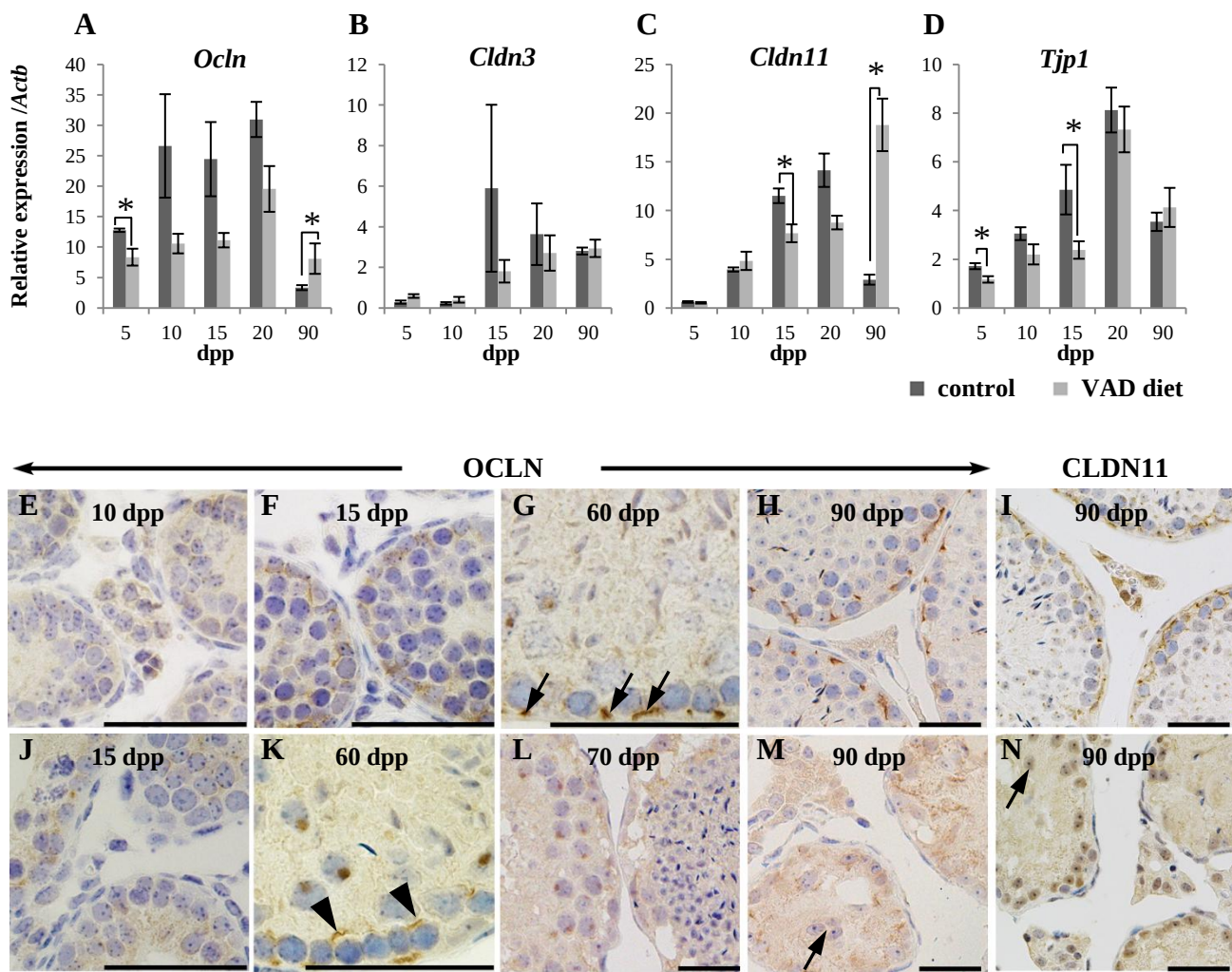


Figure 3-6. BTB integrity in the testes of mice fed the VAD diet.

A–D) mRNA levels of *Ocln* (**A**), *Cldn3* (**B**), *Cldn11* (**C**), and *Tjp1* (**D**) in the testes of control mice fed the VAS diet and mice fed the VAD diet during the prepubertal period (5–20 dpp) and adulthood (90 dpp). Values are presented as means $\pm$ SEM. * $P < 0.05$, Mann-Whitney U test. **E–N)** BTB localization in control mice and mice fed the VAD diet. Positive reactions were detected by immunohistochemistry for OCLN (**E–H** and **J–M**) and CLDN11 (**I** and **N**). In testes from control mice, specific OCLN localization was barely detectable at 10 dpp (**E**), while OCLN was accumulated in the basal part of the seminiferous tubules at 15 dpp (**F**). Few immunoreactions for OCLN were observed at 15 dpp in testes from mice fed the VAD diet (**J**). In stage X tubules of control mice at 60 dpp, OCLN localized only to the basal side of leptotene or zygotene spermatocytes (**G**; arrows). In stage X tubules of mice fed the VAD diet at 60 dpp, OCLN localized to the luminal side of leptotene or zygotene spermatocytes (**K**; arrowheads). At 70 dpp in testes from mice fed the VAD diet, immunopositive OCLN signals decreased, and epithelial vacuolization was observed (**L**). At 90 dpp in testes from control mice, Sertoli cell nuclei were aligned along the basement membrane of tubules with OCLN (**H**) and CLDN11 (**I**) localization. In contrast, testes from mice fed the VAD diet showed diffuse distribution of OCLN (**M**) and CLDN11 (**N**) with detached Sertoli cells at 90 dpp (**M** and **N**; arrows). Bars = 50 μ m.

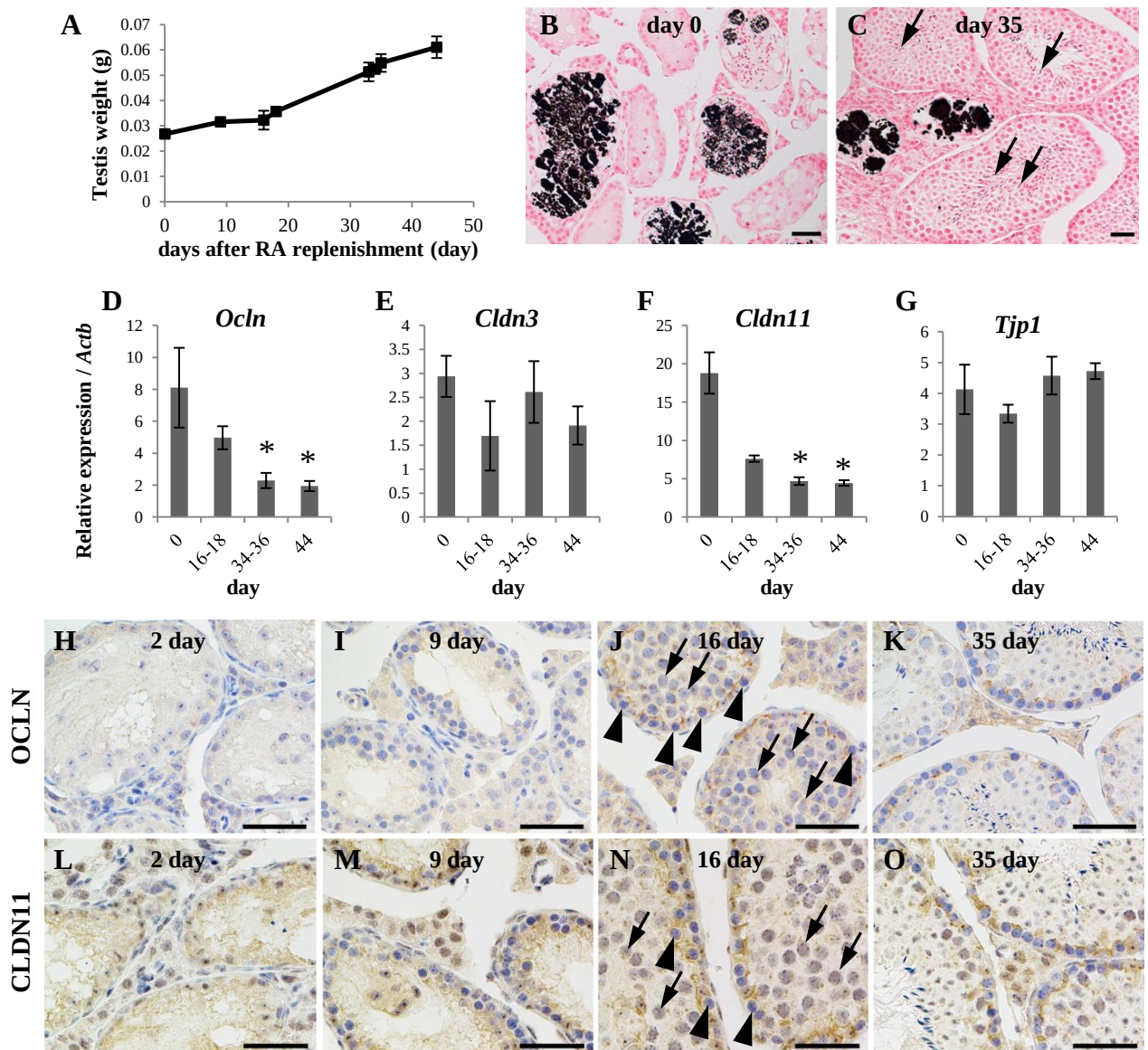


Figure 3-7. Effects of RA replenishment on the testes of mice fed the VAD diet.

A) Changes in testes weight after RA replenishment in mice fed the VAD diet. Values are presented as means $\pm$ SEM. **B–C)** Testes of mice fed the VAD diet after RA replenishment. Intratubular calcifications were observed by von Kossa staining on day 0 (**B**) and day 35 (**C**). At day 35, the majority of tubules contained step 16 spermatids (**C**; arrows). Bars = 50 μ m. **(D–G)** mRNA levels of *Ocln* (**D**), *Cldn3* (**E**), *Cldn11* (**F**), and *Tjp1* (**G**) in the testes of mice fed the VAD diet after RA replenishment. Values are presented as means $\pm$ SEM. *Significant difference vs. day 0 ($P < 0.05$, one-way ANOVA followed by Dunnett's test). **H–O)** BTB organization in the testes of mice fed the VAD diet after RA replenishment. An OCLN-positive signal was barely detectable at days 2 (**H**) and 9 (**I**) after RA replenishment but was clearly localized to the basal part of seminiferous tubules at day 16 (**J**), with the presence of pachytene spermatocytes (**J**; arrows) and preleptotene/leptotene spermatocytes (**J**; arrowheads). Similarly, CLDN11-positive signal was diffused at day 2 (**L**) and day 9 (**M**) after RA replenishment, but was clearly localized to the basal part of seminiferous tubules at day 16 (**N**), with the presence of pachytene spermatocytes (**N**; arrows) and preleptotene/leptotene spermatocytes (**N**; arrowheads). Normal localization of OCLN and CLDN11 in the basal part of the tubules was also observed at day 35 (**K** and **O**). Bars = 50 μ m.

Conclusion

Male germ cells, sperm, are produced in a cyclic and complicated process called spermatogenesis, which occurs in the seminiferous tubules in the testes. The differentiation of germ cells is supported, nurtured, and supervised by somatic Sertoli cells, which line the seminiferous tubules. During testis development in prepubertal period, Sertoli cells form the blood-testis barrier (BTB) which divides the seminiferous epithelium into the basal and adluminal compartments. The BTB restricts the entry of molecules in the interstitial space into the adluminal compartment, thereby BTB has been considered to play an important role in maintaining homeostasis of seminiferous epithelium. However, to accomplish germ cell differentiation, preleptotene/leptotene spermatocytes residing in basal compartment need to migrate across the BTB into adluminal compartment. Because the precise function of the BTB in spermatogenesis remains obscure, in this thesis, the author attempted to reveal the relationships between dynamics of BTB component proteins and meiosis progression during murine spermatogenesis.

At first, the author proved the dynamics of the BTB components during adult spermatogenesis focusing on spermatocytes migration across the BTB. Structural analysis revealed that the BTB component tight junction (TJ) proteins localized at the BTB throughout adult spermatogenesis. Although the TJ proteins including CLDN11, OCLN, and ZO1 were at the adluminal side of germ

cells before their BTB migration, new TJ fibrils composed of these molecules are formed at the basal side of the preleptotene/leptotene spermatocytes preparing for migration across the BTB. Therefore, the intermediate compartment, a microenvironment sandwiched between TJ proteins from the apico-basal direction, is formed temporarily, and it is considered that the old TJ fibrils at the adluminal side are degraded to accomplish the passage of germ cells. Remarkably, unlike other BTB-constructing TJ proteins, CLDN3 localized to the basal portion of seminiferous tubules only around migration stages, in accordance with the emergence of the preleptotene/leptotene spermatocytes. These results indicated that the barrier function of the BTB is strictly maintained even in spermatocyte migration across the BTB, and the stage-specific localization of CLDN3 protein plays an important role in regulation of germ cell migration.

The author next examined the cell types expressing CLDN3 in the mouse testis and evaluated the integrity of spermatogenesis after *Cldn3* knockdown in order to verify the CLDN3 function in spermatogenesis. Astonishingly, besides Sertoli cells that form BTB, preleptotene/leptotene spermatocytes lacking TJ structure expressed the mRNA and protein of CLDN3. *Cldn3* knockdown caused a partial delay in spermatocyte migration across the BTB, resulting in a prolonged period of the preleptotene phase during spermatogenesis. These data strongly indicated that Sertoli cells as well as preleptotene/leptotene spermatocytes participated in the regulation of BTB by expressing CLDN3, and

that CLDN3 regulates the progression of spermatogenesis by promoting germ cell migration across the BTB.

Finally, the author attempted to validate the relationship between the BTB assembly and spermatogenic progression using vitamin A deficient mice showing spermatogenic arrest. Although vitamin A deficiency induced no changes in the initiation and progression of the first spermatogenic wave during prepubertal period, the numbers of preleptotene/leptotene spermatocytes derived from the second spermatogenic wave onwards were decreased in coincidence with the delay in the BTB assembly. Furthermore, BTB disruption in vitamin A deficient mice preceded testicular degeneration and complete spermatogenic arrest during adult age. From these results the author proposes that BTB integrity is regulated by vitamin A metabolism with control of meiosis and is required for persistent differentiation of germ cells rather than the initiation of spermatogenesis.

The present study suggests that Sertoli and germ cells coordinately establish an intimate and elaborate cellular network, morphologically observed as BTB, regulating their reciprocal functions during the germ cell development. Therefore, the author concluded that the BTB structure acts as “Gatekeeper” to regulate persistent spermatogenesis, and its functional and structural disruptions with altered dynamics of component TJ proteins could lead animals to male infertility.

References

1. Agarwal, R., D'Souza, T. and Morin, P.J. 2005. Claudin-3 and claudin-4 expression in ovarian epithelial cells enhances invasion and is associated with increased matrix metalloproteinase-2 activity. *Cancer Res.* 65: 7378–7385.
2. Alonso, A., Reinze, E., Fatar, M., Hennerici, M.G. and Meairs, S. 2011. Clearance of albumin following ultrasound-induced blood-brain barrier opening is mediated by glial but not neuronal cells. *Brain Res.* 1411: 9–16.
3. Anderson, E.L., Baltus, A.E., Roepers-Gajadien, H.L., Hassold, T.J., de Rooij, D.G., van Pelt, A.M. and Page, D.C. 2008. Stra8 and its inducer, retinoic acid, regulate meiotic initiation in both spermatogenesis and oogenesis in mice. *Proc. Natl. Acad. Sci. U. S. A.* 105: 14976–14980.
4. Baillet, A. and Mandon-Pepin, B. 2012. Mammalian ovary differentiation - a focus on female meiosis. *Mol. Cell. Endocrinol.* 356: 13–23.
5. Boyer, A., Hermo, L., Paquet, M., Robaire, B. and Boerboom, D. 2008. Seminiferous tubule degeneration and infertility in mice with sustained activation of WNT/CTNNB1 signaling in Sertoli cells. *Biol. Reprod.* 79: 475–485.

6. Carette, D., Weider, K., Gilleron, J., Giese, S., Dompierre, J., Bergmann, M., Brehm, R., Denizot, J.P., Segretain, D. and Pointis, G. 2010. Major involvement of connexin 43 in seminiferous epithelial junction dynamics and male fertility. *Dev. Biol.* 346: 54–67.
7. Cheng, Y.M., Lee, N.P., Mruk, D.D., Lee, W.M. and Cheng, C.Y. 2003. Fer kinase/Fer T and adherens junction dynamics in the testis: An in vitro and in vivo study. *Biol. Reprod.* 69:656–672.
8. Cheng, C. and Mruk, D. 2002. Cell junction dynamics in the testis: Sertoli-germ cell interactions and male contraceptive development. *Physiol. Rev.* 82: 825–874.
9. Chung, S.S., Sung, W., Wang, X. and Wolgemuth, D.J. 2004. Retinoic acid receptor alpha is required for synchronization of spermatogenic cycles and its absence results in progressive breakdown of the spermatogenic process. *Dev. Dyn.* 230: 754–766.
10. Dagash, H. and Mackinnon, E.A. 2007. Testicular microlithiasis: what does it mean clinically? *BJU Int.* 99: 157–160.
11. De Gendt, K., Swinnen, J.V., Saunders, P.T., Schoonjans, L., Dewerchin, M., Devos, A., Tan, K., Atanassova, N., Claessens, F., Lécureuil, C., Heyns, W., Carmeliet, P., Guillou, F., Sharpe, R.M. and Verhoeven, G. 2004. A Sertoli cell-selective knockout of the androgen receptor causes spermatogenic arrest in meiosis. *Proc. Natl. Acad. Sci. U. S. A.* 101: 1327–1332.

12. Eid, N., Ito, Y. and Otsuki, Y. 2012. Enhanced mitophagy in Sertoli cells of ethanol-treated rats: morphological evidence and clinical relevance. *J. Mol. Histol.* 43: 71–80.
13. Farkas, A.E., Capaldo, C.T. and Nusrat, A. 2012. Regulation of epithelial proliferation by tight junction proteins. *Ann. N. Y. Acad. Sci.* 1258: 115–124.
14. Fink, C., Weigel, R., Fink, L., Wilhelm, J., Kliesch, S., Zeiler, M., Bergmann, M. and Brehm, R. 2009. Claudin-11 is over-expressed and dislocated from the blood-testis barrier in Sertoli cells associated with testicular intraepithelial neoplasia in men. *Histochem. Cell. Biol.* 131: 755–764.
15. Florin, A., Maire, M., Bozec, A., Hellani, A., Chater, S., Bars, R., Chuzel, F. and Benahmed, M. 2005. Androgens and postmeiotic germ cells regulate claudin-11 expression in rat Sertoli cells. *Endocrinology* 146: 1532–1540.
16. Ganem, J., Workman, K. and Shaban, S. 1999. Testicular microlithiasis is associated with testicular pathology. *Urology* 4295: 209–213.
17. Goetz, P., Chandley, A.C. and Speed, R.M. 1984. Morphological and temporal sequence of meiotic prophase development at puberty in the male mouse. *J. Cell Sci.* 65: 249–263.
18. Gow, A., Southwood, C.M., Li, J.S., Pariali, M., Riordan, G.P., Brodie, S.E., Danias, J., Bronstein, J.M., Kachar, B. and Lazzarini, R.A. 1999. CNS myelin and Sertoli cell tight junction strands are absent in Osp/Claudin-11 null mice. *Cell* 99: 649–659.

19. Gregory, M., Dufresne, J., Hermo, L. and Cyr, D. 2001. Claudin-1 is not restricted to tight junctions in the rat epididymis. *Endocrinology* 142: 854–863.
20. Hamazaki, Y., Fujita, H., Kobayashi, T., Choi, Y., Scott, H.S., Matsumoto, M. and Minato, N. 2007. Medullary thymic epithelial cells expressing Aire represent a unique lineage derived from cells expressing claudin. *Nat. Immunol.* 8: 304–311.
21. Hamer, G., Gell, K., Kouznetsova, A., Novak, I., Benavente, R. and Höög, C. 2006. Characterization of a novel meiosis-specific protein within the central element of the synaptonemal complex. *J. Cell Sci.* 119: 4025–4032.
22. Hasegawa, K. and Saga, Y. 2012. Retinoic acid signaling in Sertoli cells regulates organization of the blood-testis barrier through cyclical changes in gene expression. *Development* 139: 4347–4355.
23. Hellani, A., Ji, J., Mauduit, C., Deschildre, C., Tabone, E. and Benahmed, M. 2000. Developmental and hormonal regulation of the expression of oligodendrocyte-specific protein/claudin-11 in mouse testis. *Endocrinology* 141: 3012–3019.
24. Hermo, L., Pelletier, R.M., Cyr, D.G. and Smith, C.E. 2010. Surfing the wave, cycle, life history, and genes/proteins expressed by testicular germ cells. Part 1: background to spermatogenesis, spermatogonia, and spermatocytes. *Microsc. Res. Tech.* 73: 241–278.

25. Hentrich, A., Wolter, M., Szardening-Kirchner, C., Lüers, G.H., Bergmann, M., Kliesch, S. and Konrad, L. 2011. Reduced numbers of Sertoli, germ, and spermatogonial stem cells in impaired spermatogenesis. *Mod. Pathol.* 24: 1380–1389.
26. Hogarth, C.A. and Griswold, M.D. 2010. The key role of vitamin A in spermatogenesis. *J. Clin. Invest.* 120: 956–962.
27. Hough, C.D., Sherman-Baust, C.A., Pizer, E.S., Montz, F.J., Im, D.D., Rosenshein, N.B., Cho, K.R., Riggins, G.J. and Morin, P.J. 2000. Large-scale serial analysis of gene expression reveals genes differentially expressed in ovarian cancer. *Cancer Res.* 60: 6281–6287.
28. Huang, H.F., Yang, C.S., Meyerhofer, M., Gould, S. and Boccabella, A.V. 1988. Disruption of sustentacular (Sertoli) cell tight junctions and regression of spermatogenesis in vitamin A deficient rats. *Acta. Anat. (Basel)* 3: 10–15.
29. Huang, Y.H., Bao, Y., Peng, W., Goldberg, M., Love, K., Bumcrot, D.A., Cole, G., Langer, R., Anderson, D.G. and Sawicki, J.A. 2009. Claudin-3 gene silencing with siRNA suppresses ovarian tumor growth and metastasis. *Proc. Natl. Acad. Sci. U. S. A.* 106: 3426–3430.
30. Ikenouchi, J., Sasaki, H., Tsukita, S., Furuse, M. and Tsukita, S. 2008. Loss of occludin affects tricellular localization of tricellulin. *Mol. Biol. Cell* 19: 4687–4693.
31. Ismail, N. and Morales, C.R. 1992. Effects of vitamin A deficiency on the inter-Sertoli cell tight

- junctions and on the germ cell population. *Microsc. Res. Tech.* 20: 43–49.
32. Ismail, N., Morales, C. and Clermont, Y. 1990. Role of spermatogonia in the stage-synchronization of the seminiferous epithelium in vitamin A-deficient rats. *Am. J. Anat.* 188: 57–63.
33. Itoh, M., Furuse, M., Morita, K., Kubota, K., Saitou, M. and Tsukita, S. 1999. Direct binding of three tight junction-associated MAGUKs, ZO-1, ZO-2, and ZO-3, with the COOH terminal of claudins. *J. Cell Biol.* 147: 1351–1363.
34. Itoh, M., Nagafuchi, A., Moroi, S. and Tsukita, S. 1997. Involvement of ZO-1 in cadherin-based cell adhesion through its direct binding to α catenin and actin filaments. *J. Cell Biol.* 138: 181–192.
35. Kaitu'u-Lino, T., Sluka, P., Foo, C.F. and Stanton, P.G. 2007. Claudin-11 expression and localization is regulated by androgens in rat Sertoli cells in vitro. *Reproduction* 133:1169–1179.
36. Kanatsu-Shinohara, M., Takehashi, M., Takashima, S., Lee, J., Morimoto, H., Chuma, S., Raducanu, A., Nakatsuji, M., Fässler, R. and Shinohara, T. 2008. Homing of mouse spermatogonial stem cells to germline niche depends on β 1-integrin. *Cell Stem Cell* 3: 533–542.
37. Kawai, Y., Hamazaki, Y., Fujita, H., Fujita, A., Sato, T., Furuse, M., Fujimoto, T., Jetten, A.M., Agata, Y. and Minato, N. 2011. Claudin-4 induction by E-protein activity in later stages of CD4/8

- double-positive thymocytes to increase positive selection efficiency. *Proc. Natl. Acad. Sci. U. S. A.* 108: 4075–4080.
38. Kim, B., Winter, T.C. and Ryu, J. 2003. Testicular microlithiasis: clinical significance and review of the literature. *Eur. Radiol.* 13: 2567–2576.
 39. Kimura, J., Ichii, O., Otsuka, S., Kanazawa, T., Namiki, Y., Hashimoto, Y. and Kon, Y. 2011. Quantitative and qualitative urinary cellular patterns correlate with progression of murine glomerulonephritis. *PloS One* 6: e16472.
 40. Komljenovic, D., Sandhoff, R., Teigler, A., Heid, H., Just, W.W. and Gorgas, K. 2009. Disruption of blood-testis barrier dynamics in ether-lipid-deficient mice. *Cell Tissue Res.* 337: 281–299.
 41. Kopera, I.A., Bilinska, B., Cheng, C.Y. and Mruk, D.D. 2010. Sertoli-germ cell junctions in the testis: a review of recent data. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 365: 1593–1605.
 42. Kotaja, N., Kimmins, S., Brancorsini, S., Hentsch, D., Vonesch, J.L., Davidson, I., Parvinen, M. and Sassone-Corsi, P. 2004. Preparation, isolation and characterization of stage-specific spermatogenic cells for cellular and molecular analysis. *Nat. Methods* 1:249–254.
 43. 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.

44. Kozler, P. and Pokorný, J. 2003. Altered blood-brain barrier permeability and its effect on the distribution of Evans blue and sodium fluorescein in the rat brain applied by intracarotid injection. *Physiol. Res.* 52: 607–614.
45. Kuriyama, G., Takekoshi, S., Tojo, K., Nakai, Y., Kuhar, M.J. and Osamura, R.Y. 2004. Cocaine- and amphetamine-regulated transcript peptide in the rat anterior pituitary gland is localized in gonadotrophs and suppresses prolactin secretion. *Endocrinology* 145: 2542–2550.
46. Kyrölähti, A., Euler, R., Bielinska, M., Schoeller, E.L., Moley, K.H., Toppari, J., Heikinheimo, M. and Wilson, D.B. 2011. GATA4 regulates Sertoli cell function and fertility in adult male mice. *Mol. Cell. Endocrinol.* 333: 85–95.
47. La Salle, S., Sun, F., Zhang, X.D., Matunis, M.J. and Handel, M.A. 2008. Developmental control of sumoylation pathway proteins in mouse male germ cells. *Dev. Biol.* 321: 227–237.
48. Lécureuil, C., Fontaine, I., Crepieux, P. and Guillou, F. 2002. Sertoli and granulosa cell-specific Cre recombinase activity in transgenic mice. *Genesis* 33: 114–118.
49. Lee, N.P. and Cheng, C.Y. 2004. Ectoplasmic specialization, a testis-specific cell-cell actin-based adherens junction type: is this a potential target for male contraceptive development? *Hum. Reprod. Update* 10: 349–369.

50. Lie, P.P., Mruk, D.D., Lee, W.M. and Cheng, C.Y. 2009. Epidermal growth factor receptor pathway substrate 8 (Eps8) is a novel regulator of cell adhesion and the blood-testis barrier integrity in the seminiferous epithelium. *FASEB J.* 23: 2555–2567.
51. Li, M.W., Xia, W., Mruk, D.D., Wang, C.Q., Yan, H.H., Siu, M.K., Lui, W.Y., Lee, W.M. and Cheng, C.Y. 2006. Tumor necrosis factor α reversibly disrupts the blood-testis barrier and impairs Sertoli-germ cell adhesion in the seminiferous epithelium of adult rat testes. *J. Endocrinol.* 190: 313–329.
52. Li, W., Wu, Z., Zhao, J., Guo, S., Li, Z., Feng, X., Ma, L., Zhang, J.S., Liu, X.P. and Zhang, Y.Q. 2011. Transient protection from heat-stress induced apoptotic stimulation by metastasis-associated protein 1 in pachytene spermatocytes. *PLoS One* 6: e26013.
53. Mark, M., Jacobs, H., Oulad-Abdelghani, M., Dennefeld, C., Féret, B., Vernet, N., Codreanu, C.A., Chambon, P. and Ghyselinck, B. 2008. STRA8-deficient spermatocytes initiate, but fail to complete, meiosis and undergo premature chromosome condensation. *J. Cell Sci.* 121: 3233–3242.
54. Mazaud-Guittot, S., Meugnier, E., Pesenti, S., Wu, X., Vidal, H., Gow, A. and Le Magueresse-Battistoni, B. 2010. Claudin 11 deficiency in mice results in loss of the Sertoli cell epithelial phenotype in the testis. *Biol. Reprod.* 82: 202–213.

55. Meng, J., Greenlee, A.R., Taub, C.J. and Braun, R.E. 2011. Sertoli cell-specific deletion of the androgen receptor compromises testicular immune privilege in mice. *Biol. Reprod.* 85:254–260.
56. Meng, J., Holdcraft, R.W., Shima, J.E., Griswold, M.D. and Braun, R.E. 2005. Androgens regulate the permeability of the blood-testis barrier. *Proc. Natl. Acad. Sci. U. S. A.* 102: 16696–16700.
57. Miller, F.N., Rosairo, S., Clarke, J.L., Sriprasad, S., Muir, G.H. and Sidhu, P.S. 2007. Testicular calcification and microlithiasis: association with primary intra-testicular malignancy in 3,477 patients. *Eur. Radiol.* 17: 363–369.
58. Mok, K.W., Mruk, D.D., Lee, W.M. and Cheng, C.Y. 2012. Spermatogonial stem cells alone are not sufficient to re-initiate spermatogenesis in the rat testis following adjuvin-induced infertility. *Int. J. Androl.* 35: 86–101.
59. Morales, A. and Cavicchia, J.C. 2002. Spermatogenesis and blood-testis barrier in rats after long-term vitamin A deprivation. *Tissue Cell* 34: 349–355.
60. Morales, A., Mohamed, F. and Cavicchia, J.C. 2007. Apoptosis and blood-testis barrier during the first spermatogenic wave in the pubertal rat. *Anat. Rec. (Hoboken)* 290: 206–214.
61. Morrow, C.M., Tyagi, G., Simon, L., Carnes, K., Murphy, K.M., Cooke, P.S., Hofmann, M.C. and Hess, R.A. 2009. Claudin 5 expression in mouse seminiferous epithelium is dependent upon

- the transcription factor ets variant 5 and contributes to blood-testis barrier function. *Biol. Reprod.* 81: 871–879.
62. Moroi, S., Saitou, M., Fujimoto, K., Sakakibara, A., Furuse, M., Yoshida, O. and Tsukita, S. 1998. Occludin is concentrated at tight junctions of mouse/rat but not human/guinea pig Sertoli cells in testes. *Am. J. Physiol.* 274: C1708–C1717.
63. Mruk, D.D. and Cheng, C.Y. 2004. Sertoli-Sertoli and Sertoli-germ cell interactions and their significance in germ cell movement in the seminiferous epithelium during spermatogenesis. *Endocr. Rev.* 25: 747–806.
64. Oakberg, EF. 1956. A description of spermiogenesis in the mouse and its use in analysis of the cycle of the seminiferous epithelium and germ cell renewal. *Am. J. Anat.* 99: 391–413.
65. Oka, A., Mita, A., Takada, Y., Koseki, H. and Shiroishi, T. 2010. Reproductive isolation in hybrid mice due to spermatogenesis defects at three meiotic stages. *Genetics* 186: 339–351.
66. O'Shaughnessy, P.J., Monteiro, A., Verhoeven, G., De Gendt, K. and Abel, M.H. 2009. Occurrence of testicular microlithiasis in androgen insensitive hypogonadal mice. *Reprod. Biol. Endocrinol.* 7: 88.
67. Oulad-Abdelghani, M., Bouillet, P., Décimo, D., Gansmuller, A., Heyberger, S., Dollé, P., Bronner, S., Lutz, Y. and Chambon, P. 1996. Characterization of a premeiotic germ cell-specific

- cytoplasmic protein encoded by *Stra8*, a novel retinoic acid-responsive gene. *J. Cell Biol.* 135: 469–477.
68. Pelletier, R.M. 1986. Cyclic formation and decay of the blood-testis barrier in the mink (*Mustela vison*), a seasonal breeder. *Am. J. Anat.* 175: 91–117.
 69. Pelletier, R.M. 2011. The blood-testis barrier: the junctional permeability, the proteins and the lipids. *Prog. Histochem. Cytochem.* 46: 49–127.
 70. Russell, L. 1977. Movement of spermatocytes from the basal to the adluminal compartment of the rat testis. *Am. J. Anat.* 148: 313–328.
 71. Russell, L.D., Ettlin, R.A., Sinha Hikim, A.P. and Clegg, E.D. 1990. Histological and histopathological evaluation of the testis. Clearwater, FL: Cache. River Press.
 72. Saitou, M., Furuse, M., Sasaki, H., Schulzke, J.D., Fromm, M., Takano, H., Noda, T. and Tsukita, S. 2000. Complex phenotype of mice lacking occludin, a component of tight junction strands. *Mol. Biol. Cell* 11: 4131–4142.
 73. Smith, B.E. and Braun, R.E. 2012. Germ cell migration across Sertoli cell tight junctions. *Science* 338: 798–802.

74. Sugimoto, R., Nabeshima, Y. and Yoshida, S. 2011. Retinoic acid metabolism links the periodical differentiation of germ cells with the cycle of Sertoli cells in mouse seminiferous epithelium. *Mech. Dev.* 128: 610–624.
75. Sun, E. and Gondos, B. 1986. Formation of the blood-testis barrier in the rabbit. *Cell Tissue Res.* 243: 575–578.
76. Takashima, S., Kanatsu-Shinohara, M., Tanaka, T., Takehashi, M., Morimoto, H. and Shinohara, T. 2011. Rac mediates mouse spermatogonial stem cell homing to germline niches by regulating transmigration through the blood-testis barrier. *Cell Stem Cell* 9: 463–475.
77. Tarulli, G.A., Meachem, S.J., Schlatt, S. and Stanton, P.G. 2008. Regulation of testicular tight junctions by gonadotrophins in the adult Djungarian hamster in vivo. *Reproduction* 135: 867–877.
78. Tsukita, S., Furuse, M. and Itoh, M. 2001. Multifunctional strands in tight junctions. *Nat. Rev. Mol. Cell Biol.* 2: 285–293.
79. van Pelt, A.M. and de Rooij, D.G. 1990. Synchronization of the seminiferous epithelium after vitamin A replacement in vitamin A-deficient mice. *Biol. Reprod.* 3: 363–367.

80. van Pelt, A.M., van Dissel-Emiliani, F.M., Gaemers, I.C., van der Burg, M.J., Tanke, H.J. and de Rooij, D.G. 1995. Characteristics of A spermatogonia and preleptotene spermatocytes in the vitamin A-deficient rat testis. *Biol. Reprod.* 53: 570–578.
81. Vernet, N., Dennefeld, C., Guillou, F., Chambon, P., Ghyselinck, N.B. and Mark, M. 2006. Prepubertal testis development relies on retinoic acid but not rexinoid receptors in Sertoli cells. *EMBO J.* 25: 5816–5825.
82. von Eckardstein, S., Tsakmakidis, G., Kamischke, A., Rolf, C. and Nieschlag, E. 2001. Sonographic testicular microlithiasis as an indicator of premalignant conditions in normal and infertile men. *J. Androl.* 22: 818–824.
83. Willems, A., Batlouni, S.R., Esnal, A., Swinnen, J.V., Saunders, P.T., Sharpe, R.M., França, L.R., Gendt, K. and Verhoeven, G. 2010. Selective ablation of the androgen receptor in mouse Sertoli cells affects Sertoli cell maturation, barrier formation and cytoskeletal development. *PLoS One* 5: e14168.
84. Xia, W., Mruk, D.D., Lee, W.M. and Cheng C.Y. 2005. Cytokines and junction restructuring during spermatogenesis – a lesson to learn from the testis. *Cytokine Growth Factor Rev.* 16: 469–493.
85. Xia, W., Wong, E.W, Mruk, D.D. and Cheng, C.Y. 2009. TGF- β 3 and TNF- α perturb blood-testis

- barrier (BTB) dynamics by accelerating the clathrin-mediated endocytosis of integral membrane proteins: A new concept of BTB regulation during spermatogenesis. *Dev. Biol.* 327: 48–61.
86. Yan, H.H., Mruk, D.D., Lee, W.M. and Cheng, C.Y. 2008. Blood-testis barrier dynamics are regulated by testosterone and cytokines via their differential effects on the kinetics of protein endocytosis and recycling in Sertoli cells. *FASEB J.* 22: 1945–1959.
 87. Yomogida, K., Ohtani, H., Harigae, H., Ito, E., Nishimune, Y., Engel, J.D. and Yamamoto, M. 1994. Developmental stage- and spermatogenic cycle-specific expression of transcription factor GATA-1 in mouse Sertoli cells. *Development* 120: 1759–1766.
 88. Zhou, Q., Nie, R., Li, Y., Friel, P., Mitchell, D., Hess, R.A., Small, C. and Griswold, M.D. 2008. Expression of stimulated by retinoic acid gene 8 (Stra8) in spermatogenic cells induced by retinoic acid: an in vivo study in vitamin A-sufficient postnatal murine testes. *Biol. Reprod.* 79: 35–42.
 89. Zhou, Q., Nie, R., Prins, G.S., Saunders, P.T., Katzenellenbogen, B.S. and Hess, R.A. 2002. Localization of androgen and estrogen receptors in adult male mouse reproductive tract. *J. Androl.* 23: 870–881.

Acknowledgements

I thank Dr. Atsushi Suzuki and Dr. Yumiko Saga for kindly providing the breeding pairs of *ICR.Cg-Tg(Strab-EGFP)1Ysa/YsaRbrc* mice (RIKEN BRC, Tsukuba, Japan). I would like to extend thanks and my appreciation to those who have encouraged me during years of graduate studies. First and foremost, I would like to express my gratitude towards my supervisor Dr. Yasuhiro Kon for his support, advice, and guidance throughout the duration of this project. I would like thank Dr. Kazuhiro Kimura, Dr. Mayumi Ishizuka, Dr. Masashi Nagano, and Dr. Nobuya Sasaki for time taken to provide advice, critical comments and assistance in completing this thesis. I would also like to thank Dr. Osamu Ichii and Dr. Saori Otsuka for their encouragement, support, advice, and great times. Finally, I deeply appreciated to the animals supporting this study.

Conclusion in Japanese

マウス血液精巣関門の新たな機能の解明

—精子発生の調節を担うゲートキーパーとしての役割—

北海道大学大学院獣医学研究科

比較形態機能学講座 解剖学教室

千原 正尚

雄の配偶子である精子は、精巣内に存在する曲精細管で産生される。曲精細管の上皮（精上皮）には、将来精子となる様々な分化段階の精細胞（精祖細胞、精母細胞、および精子細胞）とそれらの分化・成熟を支持するセルトリ細胞が存在する。マウスは生後数日中に精細胞分化の **First wave** を開始し、以降、規則的な精細胞分化の波が精上皮周期を形成していく。**First wave** 由来精母細胞がパキテン期に達する生後 15 日頃、密着結合帯である血液精巣関門 (**BTB**) が隣接するセルトリ細胞間に形成され、精上皮は基底区画と傍腔区画とに分けられる。精細胞は分化に伴い、次第に精細管の基底側から管腔側へと移動するが、その過程において、プレレプトテン期／レプトテン期精母細胞は **BTB** を通過する。通説として、**BTB** は傍腔区画内を

体循環から隔離し、減数分裂後の精細胞の抗原を自己の免疫システムから隔離する免疫学的障壁として機能すると考えられてきた。しかしながら、BTB を破壊したマウス精巣において、自己免疫反応の誘導は証明されておらず、BTB が精子発生に果たす役割とその制御機構については不明である。本研究では、BTB 構成蛋白の動態と精細胞分化との関連を検証し、BTB の機能解明を試みた。

はじめに、成熟マウスの精子形成過程における BTB 構成蛋白の動態を解析した。密着結合蛋白である CLDN11、OCLN、および ZO1 は、精上皮周期の全ステージにおいて精祖細胞と精母細胞の間に線状に観察され、精細胞の BTB 通過が起こる前後のステージではプレレプトテン期／レプトテン期精母細胞の基底側と管腔側の両方に観察された。本結果は、精細胞の BTB 通過時には、まず移動する精細胞の基底側に新しい BTB が形成され、その後管腔側にあ
る古い BTB が分解されることを強く示唆した。このため、BTB による傍腔区画隔離能は精子形成過程を通して厳密に維持されていることが明らかとなった。一方、CLDN3 は精細胞の BTB 通過が起こる前後のステージ特異的に BTB 近傍に局在したことから、BTB 構成蛋白では特に CLDN3 が精細胞の BTB 通過に重要な役割を有することが考えられた。

続いて、成熟マウス精巣における CLDN3 の発現部位を精査した結果、CLDN3 は BTB を構築するセルトリ細胞のみならず、密着結合帯を形成しないプレレプトテン期／レプトテン期精母細胞の細胞膜にも局在した。さらに、*in vivo* RNAi 法によって *Cldn3* 発現を抑制した精巣

において、精細胞の BTB 通過の抑制に加え、プレレプトテン期精母細胞の存在期間の延長が観察された。これらの結果より、CLDN3 は精細胞の BTB 通過を制御するとともに、精細胞分化の進行を調節している可能性が示された。

最後に、「生体におけるビタミン A 欠乏は可逆的に精子形成を停止させる」ことに着目し、ビタミン A 欠乏食給餌 (VAD) マウスを用いて BTB の構築と精細胞分化の関連を解析した。VAD マウスの性成熟過程を観察した結果、First wave の精細胞分化の開始と進行に異常は認められなかったが、Second wave 以降の精細胞分化におけるプレレプトテン期／レプトテン期精母細胞数の減少ならびに BTB の初期形成の遅延が認められた。さらに、性成熟後の VAD マウスでは、顕著な精巣変性と精子形成の停止に先行して BTB の崩壊が観察された。ビタミン A 再補填による精子形成再開後、プレレプトテン期／レプトテン期およびパキテン期精母細胞の出現と一致して BTB の再形成が認められたことから、BTB の初期形成には特定の分化段階に達した精細胞とセルトリ細胞の協調作用が必要であり、また、BTB は精細胞分化の開始ではなく、持続的な精子形成を維持する為に必須の構造であると考えられた。

上述の結果は、精細胞とセルトリ細胞は相互に密接に関わり合いながら BTB の局在を制御し、精細管内を精子形成に適した微小環境に維持していることを示す。本研究により、著者は、BTB は免疫学的障壁というよりはむしろ、精細胞の体系的な分化調節を担うゲートキーパーとしての役割を有する構造であり、その破綻は雄性不妊症の一因となることを提唱する。